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Translational Research and Onco-Omics Applications in the Era of Cancer Personal Genomics

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Preface

This book attempts to address the complexity field of translational cancer research and presents concise chapters written by internationally respected experts on various important aspects of translational cancer genomics, offering a comprehensive overview of the onco-omics applications in the new era of cancer personal genomics research field.

The last 15 years of the twenty-first century were characterized by the notion that tumor cells display characteristic molecular alterations that have significantly changed our understanding of cancer-driving pathways.

Our hope is that this book can stimulate innovative translational research collaborations by providing insights into how onco-omics applications using cutting-edge technologies can be integrated into the clinical practices.

Mexico City, Mexico

Erika Ruiz-Garcia
Horacio Astudillo-de la Vega

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We would like to thank all our coauthors who worked diligently on their contributions to the book. All of them believed in our original proposal and provided invaluable feedback regarding chapters in the book; without their support, this book would not have been possible.

The inspiration of this book came from the work of daily job in our institutions, from research laboratories, and from our cancer patients.

Erika and I are very fortunate to have each other because among us we find the encouragement, trust, and love working together, in the same way that we complement each other in our personal lives.

Finally, we would like to acknowledge our family (our parents, Alma Leticia, Rolanda, and Esteban Horacio; our siblings, Dulce María, Bithiah, Esteban, Emmanuel, Rolando, and Alexander; and our lovely pets, Doré, Dominó, Oreo, Mochaccino, and Nano) for their company, love, and support to write this book, which has been a great job in our professional and personal life.

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1 Pharmaco-Geno-Proteo-Metabolomics and Translational Research in Cancer

Edith A. Fernández-Figueroa, Saul Lino-Silva, Jorge E. Peña-Velasco, and Claudia Rangel-Escareño

Abstract

The diagnosis, prognosis and treatment of cancer has had a great improvement due to the "omics" technologies such as genomics, proteomics, epigenomics, pharmacogenomics, and metabolomics. The technological progress of these technologies has allowed precision medicine to become a clinical reality. The study of different biomolecules such as DNA, RNA and proteins has helped to detect alterations in genes, changes in gene expression profiles and loss or gain of protein function, which allows us to make associations and better understand the cancer biology. Data obtained from different "omics" technologies gives a complementary spectrum of information that helps us to understand and unveil new information for a better diagnosis, prognosis, prediction of new molecular targets of anticancer therapies, etc. This chapter presents a general landscape of the interaction between the Pharmaco-Geno-Proteo-Metabolomic and translational medicine research in cancer.

Keywords

Oncogenomic · Transcriptomic · Proteomic · Metabolomic · Interactome · Proteogenomics · Pharmacogenomics · Biomarker · Datases · NGS

1.1 "Omics"

Clinical diagnosis plays an important role in health care in developed and developing countries [1]. The need to improve diagnostic methods, prognoses and therapeutic targets has set the standard for focusing on precision medicine, whose main objectives consider molecular and biological characteristics of patients, and thereby identify the genetic markers that have an impact in the response to the treatment of certain diseases [2, 3]. The availability of this knowledge is possible thanks to the access to diverse genomic platforms, to the development of massive sequencing and the methods of analysis of large databases that have been generated over time and that finally, allow us to know specific molecular

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markers of a particular disease [2]. Precision medicine encompasses two different approaches, stratified and personalized medicine, which consist in testing new drug therapies in groups of patients with specific molecular alterations and determining each patient's particular response to the treatment in order to obtain conclusions at a population level [1, 2]. Biomarkers used for early detection of cancer need be adequately sensitive, as well as disease targeted and with properly selected antigens to monitor the response to the treatment [4].

Recent advancements in technologies used to expand the knowledge of human genome, epigenome, metabolome, transcriptome and proteome at the population level provide complementary approaches to understand certain biological mechanisms involved in disease. All the data gathered from these approaches is required to understand the synergistic interactions (Fig. 1.1) [5]. The sample collection, sample preparation, technical procedures, data analysis and validation are aspects of great importance for the use of "omic" techniques [4]. Special care must be taken for the planification of sample collection when using different technologies as they would need distinct samples from a single patient.

1.2 Genomics

Genomic changes that occur in DNA sequences, such as single nucleotide variations (SNVs), small insertions and deletions (INDELs) and structural variants (SVs) can result in the development of cancer [6]. The study of the structure and function of DNA is studied by "genomics" and through it

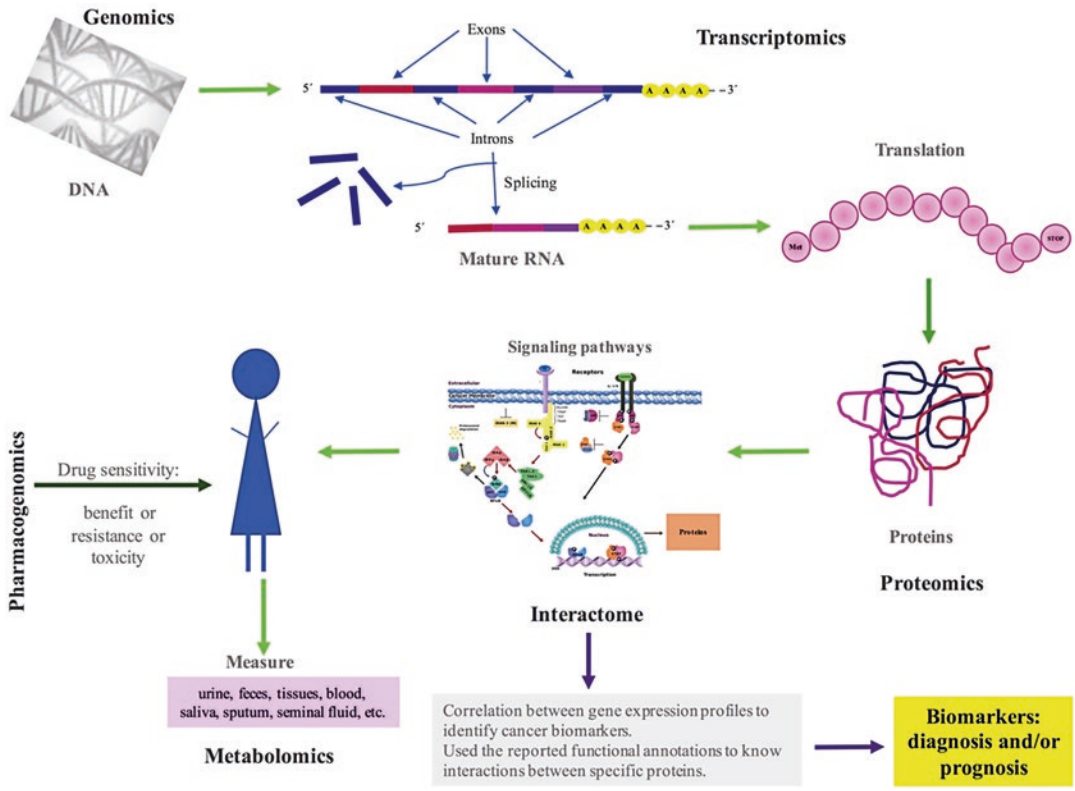

Fig. 1.1 The "omics" technologies. The technological progress of these technologies has allowed precision medicine to become a clinical reality. "Omics" sciences provide complementary approaches to understand certain biological mechanisms involved in diseases

several gene defects that help to understand the pathogenesis of germline diseases had been discovered, examples of this are BRCA1/2 gene mutations in breast and ovarian cancer and absence of mismatch-repair enzyme in colorectal cancer [7]. Single or multi-gene disorders can be identified using sequencing technologies such as Sanger sequencing or next-generation sequencing (NGS) respectively. Given the quantity of data generated with those techniques it has been possible to identify genomic signatures that define a specific tumor. On the other hand, the study of gene expression using transcriptome techniques is very informative when we wish analyze differential expression between cases and controls or in subsets tumors [7].

Association of data between Genome-wide association studies (GWAS) and transcriptome profiles using network building algorithms have allowed researchers to determine that genes such as EGFR, TERT and HIF are the center of the complex and robust gene network in non-small lung cancer. The latter being involved in cancer development and progression as well as cell proliferation, apoptosis, metabolism invasion, metastasis, etc. [5].

1.3 Proteomics

Another platform that has helped to understand the pathogenesis of cancer is proteomic. For a long time, immunohistochemistry has been used to characterize tumors, but the applications are limited. The above, has created the need to use new technologies to analyze the expression of proteins in cancer cells and function alterations due to phosphorylation and other post-translational modifications [7]. The expression of mRNAs cannot completely reflect the amount of a protein; however, translational control of proteins permits faster changes at this level, the efficiency of this mechanism is determinant for the proteins functions [8].

The different proteomic techniques have made the investigation of changes of protein expression profiles possible. For example, mass spectrometry (MS)-based quantitative proteomic methods are commonly used nowadays. Besides the identification of proteins, MS/MS technology, is able to identify gene fusion proteins and propose them as disease biomarkers or drug targets [6].

Secretome is the compendium of secreted proteins released from cell, tissue or organisms and it has been analyzed in cancer. It has become important due to the need to discover diagnosis/prognosis cancer biomarkers [8]. Analysis of protein patterns provides a more comprehensive view of gene expression and regulation of biological mechanisms in cancer and it may provide novel specific therapeutic targets for cancer treatment and diagnosis/prognosis biomarkers.

1.4 Proteogenomics and Interactome

The integration and correlation of proteomic data (high resolution-mass spectrometry) with genomic and transcriptomic data defines the proteogenomic concept, which identifies novel proteins and discovers new events in genome annotation [6, 9]. The integration between these technologies promotes the discovery of biomarkers and therapeutic targets with potential diagnosis/prognosis capabilities, and new proteins that can be associated with biological processes in cancer biology in order to understand the intracellular processes [10]. Association between proteomic and genomic data allows the study of protein coding regions, alternative splicing, frame-shift translation, signal peptides, etc. [6]. Proteomic and transcriptome data can be used to identify novel splice variants, gene fusion events, protein coding evidence, etc. [10].

Several research initiatives such as TCGA (The Cancer Genome Atlas), ICGC (The International Cancer Genome Consortium), CPTAC (The Clinical Proteomic Tumor Analysis Consortium), and others, have studied cancer at the molecular level. Currently, they provide access to public databases that include a large cohort of human tumor genomes, molecular alterations in children's tumors and proteomics data from the tumors [10]. The analysis of proteogenomic data provides insight into the relation-

ship between genomic variation and the observed cancer phenotypes. For example, 162 single amino acid variants have been identified in colorectal cancer [10, 11] and 11 unique fusion peptides in non-small cell lung cancer [6].

On the other hand, interactome is defined such the physical interaction between proteins, the association of them is different in cells, tissues or organs. Depending of the presence and interactions between proteins in cells and tissues can be defined protein-protein interaction maps. The human interactome has more than 27,000 interactions between approximately 9600 proteins [12]. Cancer has a dynamic biology that turn it to a very complex disease, many molecules and associated pathways, the interactome, and using network analysis and gene ontology enrichment had help to know genes and proteins involved in metastasis from breast, prostate cancer [13].

1.5 Metabolomics

Metabolites, studied by metabolomics, is an analytic tool used to follow changes in biofluids or tissues which vary according to the physiology, developmental or pathological state of the cell, tissue or organism, taken this idea, the samples to analyze metabolites are diverse and include: urine, feces, tissues, blood, saliva, sputum, seminal fluid, etc. However, the presence of these molecules depends not only on the disease per se, but also, on factors to which each individual is exposed such as diet, xenobiotic exposure, collection technique, etc. [4, 14, 15]. Metabolomics allows us to measure the metabolites profile which in turn affects the observed phenotype [14]. Metabolites can be characterized using several types of analyses using LS-MS (Liquid chromatography-Mass spectrometry) and NMR (Nuclear magnetic resonance) [4, 14].

The use of metabolomics has allowed us to make associations between pathogenesis and progression of neoplastic disorders with changes in the lipidoma [7]. The major approaches to discover and characterize those metabolites, which are differentially regulated in various conditions, are the use of software packages to reveal the chromatography profiles and validation and the quantity of metabolites, the use of those methodologies could be interesting to test in cancer patients in order to contribute in a better tumor classification and/or response to treatment [4, 16]. In the future, the use of metabolomics and lipidomic biomarkers could help to eliminate false negative or false positive results that still generate other studies such as CT scans, used for the diagnostic of pancreatic or lung cancer [4].

1.6 Pharmacogenomics

Germline genomic variants that generate drug sensitivity (benefit, resistance or toxicity) are studied by pharmacogenomics [7]. The efficacy and toxicity of a drug treatment is heavily influenced by the genomic variation of each person [17]. Pharmacogenomics aims to study the variation in the drug response of the patients due to these genomic variations and, in the light of this knowledge, develop a personalized and targeted therapy in order to optimize the efficacy of a drug treatment and reduce its toxicity [18]. In order to do so, an association between a certain genotype and a drug induced phenotype needs to be determined by analyzing the pharmacodynamic and pharmacokinetic effects of the drug. These effects refer to the intensity of the response a drug elicits in the organism, the pharmacodynamic effects, and the rates at which a drug is absorbed, distributed, metabolized and excreted, termed the pharmacokinetic effects [19].

NGS technologies, such as whole genome sequencing (WGS) and whole exome sequencing (WES), have provided the opportunity to study the effects that genetic variants have over the response to drug therapy at a large scale [19] and with higher precision than microarray technologies [20] which is why approaches employing NGS are being gradually adopted for pharmacogenomics' research [19]. The reach of this research framework is not limited to a specific disease [21]; however, it has had a significant impact in cancer therapeutics [17, 21, 22] where

the complexity of the disease has forced researchers to study it from a multiple-gene perspective instead of a single-gene approach.

Variants obtained from cancer genomics research are categorized into somatic variants, pertaining to the tumor, and germline variants, which belong to the patient's normal tissue. Although somatic variations are acquired and, therefore, play an important role in the development of cancer; germline variations are also of great importance for pharmacogenomics research since they will influence the pharmacokinetics and pharmacodynamics of the drug therapy independently of the development of cancer [17, 23].

1.7 Translation into Clinical Practice

The results obtained from the analysis of these "omics" platforms need to be integrated in order to define potential biomarkers for disease detection, therapy targets or drug response, necessary for the translation of these findings into the clinical practice. Genomic, transcriptomic and proteomic platforms provide genetic information; however, patients' drug response is not only dependent on these factors but also on environmental factors; integration of this information is also required to classify the susceptibility to a particular disease or response to treatments of subpopulations of patients [24].

Although drug response is affected by many factors, due to advancements in sequencing technologies, the focus of precision medicine has been shifted towards the field of genomics specially to study the impact of genetic variations in pharmacokinetics and pharmacodynamics responses towards drugs. Drug metabolizing enzymes like the ones in the Cytochrome P450's family have been shown to harbor single nucleotide polymorphisms (SNPs) that affect the drug metabolism [25, 26]. Sequencing of a patient's genome and exome is also useful so as to determine the particular mutational landscape which could provide evidence of their eligibility for targeted therapies. In 2011, the sequencing of the whole genome, targeted exome and transcriptome provided a rationale for clinical trial with CDK inhibitors for a colorectal cancer patient with amplification and overexpression of CDK8, point mutations in NRAS, TP53, AURKA, FAS and MYH11. A second patient with malignant melanoma exhibited point mutations in HRAS and a structural rearrangement affecting CDKN2C who could potentially qualify for a clinical trial with a combination of PI3K and MEK inhibitors [27].

Clinical significance of SNVs and SVs is of major importance for the translation of the results from WGS and WES to clinical practice. For this purpose, annotation of these variants is required. Some of the most widely used tools for this purpose are ANNOVAR [28], VEP [29] and SnpEff [30] which provide useful information for the filtering and prioritization of the SNVs found in a variant analysis. Several Genome Wide Association Studies (GWAS) have provided associations between SNVs and a great amount of diseases and responses to drug treatments which have been stored in publicly available databases such as the Human Gene Mutation Database (HGMD) [31], the Online Mendelian Inheritance in Man (OMIM) [32], PharmGKB [33] and DrugBank [34].

Figure 1.2 summarizes the workflow for the use of "omic" technologies, since samples are obtained to found a potential biomarker.

1.8 Conclusions

The characterization of new mutation and driver genes to development new target therapeutic strategies require the "omic" validation techniques that could help to further improve cancer patients' survival and avoid invasive procedures that lot of current methods generate. On the other hand, the combined results of those omics approaches to investigate gene expression and regulation of different proteins in cancer may provide novel information for new therapeutic targets for cancer treatment and/or biomarkers for diagnosis/prognosis.

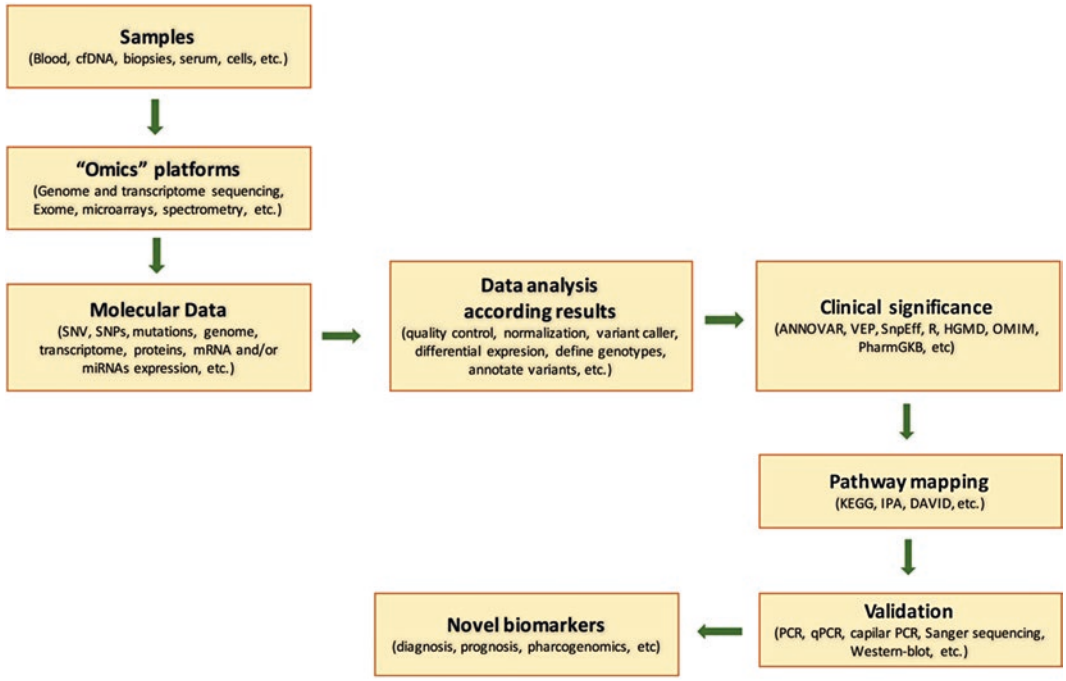

Fig. 1.2 Summary workflow for the use of "omic" technologies

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2 Next Generation Sequencing (NGS): A Revolutionary Technology in Pharmacogenomics and Personalized Medicine in Cancer

Stefania Morganti, Paolo Tarantino, Emanuela Ferraro, Paolo D'Amico, Bruno Achutti Duso, and Giuseppe Curigliano

Abstract

Following the completion of the Human Genome Project in 2003, research in oncology has progressively focused on the sequencing of cancer genomes, with the aim of better understanding the genetic basis of oncogenesis and identifying actionable alterations. The development of next-generation-sequencing (NGS) techniques, commercially available since 2006, allowed for a cost- and time-effective sequencing of tumor DNA, leading to a "genomic era" of cancer research and treatment. NGS provided a significant step forward in Personalized Medicine (PM) by enabling the detection of somatic driver mutations, resistance mechanisms, quantification of mutational burden, germline mutations which settled the foundation of a new approach in cancer care. In this chapter we discuss the history, available techniques and applications of NGS in oncology, with a particular referral to the PM approach and the emerging role of the research field of pharmacogenomics.

Keywords

Oncogenesis · Personalized medicine · Genomic era · Sequencing · Next-generation-sequencing (NGS) · Homologous recombination · Microsatellite instability · Tumor mutational burden · RNA sequencing · Liquid biopsy · Pharmacogenomics

2.1 Historic Background of DNA Sequencing and Introduction

Cancer is a genetic disease. This simple statement covers decades of research, which gradually unveiled the biological mechanisms leading to oncogenesis. Comprehensively, it is the accumulation of molecular alterations in somatic cells genomes that produces tumor progression [1]. Some of these alterations are inherited (germline mutations), but most of them randomly arise through time as a result of DNA replication errors during mitosis, or from exposure to

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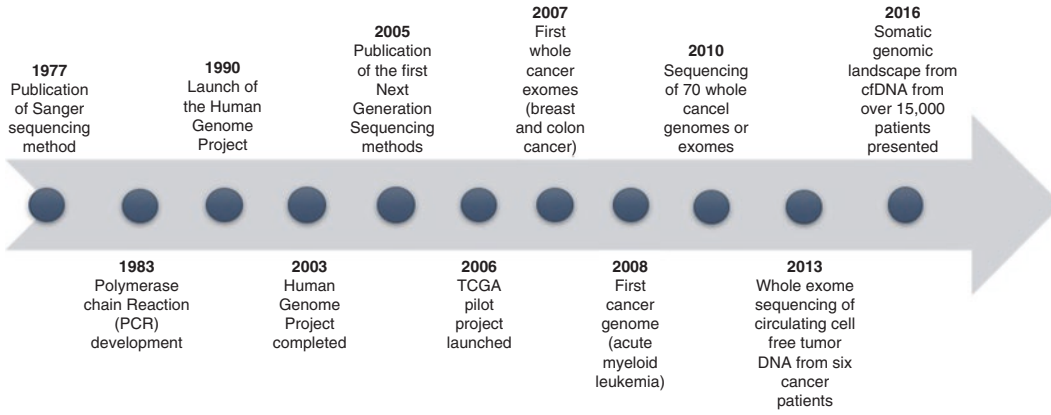
DNA-damaging carcinogens [2]. Moving from this awareness, research in oncology progressively focused on cancer cells genome, trying to find the leading cause(s) of the pathological proliferation which ultimately leads to cancer growth and spread. The pathogenic DNA alterations discovered in this process were divided into two main categories: oncogenes, whose activation/amplification leads to oncogenesis; and oncosuppressors, whose loss, instead, leads towards proliferation [3]. Following this principle, therapies in oncology gradually evolved from the generic cytotoxic compounds classically used, targeting every proliferating cell, to more sophisticated targeted therapies, directed to those genetic alterations found to be driving tumorigenesis. The early success of trastuzumab (approved in 1998 for metastatic breast cancer) and imatinib (approved in 2001 for chronic myeloid leukemia) showed that a more effective and less toxic way of treating cancer was possible, paving the way to Personalized Medicine (PM) [4].

In the early 2000s' we've started implementing genetic testing into clinical practice, to stratify patients according to their mutational status regarding those oncogenes/oncosuppressors. Nonetheless, the most commonly used tests were only aimed at those few mutations known to be useful and targetable in each specific tumor type, thus limiting the information provided on the disease. One more advancement was needed to understand the complex genetic scenario of cancer: a reference, "normal" genome sequence to compare with the abnormal ones found in tumors. The development of the Sanger sequencing method allowed to obtain such fundamental feature trough an international effort giving birth to the Human Genome Project, launched in 1990 and completed in 2003 [5]. During these fourteen years, cancer researchers kept accumulating knowledge on the basic mechanisms of cancer, identifying the majority of the most potent oncogenes and tumor suppressors. With a complete human genome reference in hand, it finally became possible to confirm those pathogenic alterations already known and to discover new ones, through a number of new large-scale sequencing projects applied to cancer genomes, such as the American TCGA (The Cancer Genome Atlas) [6] and the British Cancer Genome Project [7], launched in the first 2000s'. These successful landmarks gave birth to the "genomic era" of cancer research, and promoted the progressive development of more affordable and reliable sequencing methods: in 2004, 454 Life Sciences showcased a paralleled form of sequencing called pyrosequencing, enabling to decrease sequencing expenses at six-fold contrasted with mechanized Sanger sequencing. This breakthrough led to the first of many so-called Next-Generation Sequencing (NGS) platforms, which made DNA sequencing dramatically simpler and faster by employing microscopic, spatially separated DNA templates to massively parallelize the capture of data. Under quantitative perspective, while Sanger method could sequence 10^5 base pairs per run, the current highest throughput commercial instruments can generate nearly one terabase per run (10^{12} base pairs), thus allowing the sequencing of all the coding exons of genome (Whole Exome Sequencing, WES) and even the sequencing of a full genome (Whole Genome Sequencing, WGS) in a short time and at an affordable price. With data generation becoming relatively easy, the bottleneck for sequencing experiments now lies in the data analysis step, gradually up to an ever challenging framework.



2.1.1 The NGS Revolution in the Context of PM

In addition to broadly improving our knowledge on cancer conceptions, NGS promoted the development of PM, giving oncologists one powerful instrument to understand each patient's disease and its unique genetic features. PM, in a nutshell, refers to the actual tailoring of medical therapy according to the individual characteristics of each subject and its condition. It is not a new concept, but the use of NGS and the consequent availability of large-scale human genome databases have created an opportunity for significant onward movement of this approach. With the development of sequencing techniques, we've progressively moved from a One-size-fits-all Medicine, that wouldn't take patient's characteristics into account, to a Stratified Medicine, which groups patients according to their disease subtype, clinical features and biomarkers. The idea of PM takes the personalization of treatments even further, by taking into account patient's environment and behaviors, their medication history and the complete genome mutational scenario obtainable with NGS [8].

PM in oncology usually involves identifying mutations in cancer genomes predicting response or resistance to therapies. This is a crucial issue not only in clinical practice, but also in research, since many potentially useful drugs with profound activity in a subset of patients are abandoned during the development process because of inactivity in a large percentage of patients. Therefore, identifying predictive biomarkers may enable to distinguish the right cohorts of patients for more rational clinical trials. The research field of pharmacogenomics tries to address this problem, identifying efficacy and safety biomarkers, mainly through NGS platforms. Today, about 10% of labels for Food and Drug Administration (FDA)-approved drugs contain pharmacogenomics information [9].

As for the clinical practice, for a long time tumor biomarkers have been tested with Sanger sequencing or PCR. The development of NGS, however, gave the opportunity of screening a broader set of genes in one comprehensive test, able to identify alterations even in the scarce biopsy tissue often available in the everyday practice. As sequencing costs drop and new biomarkers are clinically validated for each

tumor, it progressively gets more convenient to apply NGS techniques instead of only testing a small number of alterations [10]. Moreover, NGS allows not only to identify the most common known alterations, but also the long tail of rare mutations that occur each in less than 1% of the patients, which sometimes provide important information on drug sensitivity.

Most of these tests are performed on tumor tissue, usually obtained by collection of fresh biopsies; notwithstanding, these procedures pose risks and discomfort to patients, making it unethical and unsafe to perform as multiple biopsies at each recurrence may be required for thorough interpretation. Because of the aforementioned limitations, other sources of neoplastic cells were considered, including circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA), both obtainable by noninvasive blood draws (the so called "liquid biopsies"). With NGS sequencing it is possible to obtain extensive genetic information from these sources, providing an instrument to capture the intrinsic intratumoral heterogeneity and identify both prognostic and predictive factors as well as imminent resistance mechanisms [11]. It was recently proposed to incorporate this instrument into cancer staging, shifting to a TNM-B cancer staging system to be assessed the diagnosis of every cancer and at every successive stage of the disease [12].

While the concept of PM is already considered a standard for some cancer types, the application of NGS in everyday practice is still facing big challenges, such as the high costs and the limited availability of approved targeted drugs. New types of clinical trial, better described further in the present chapter, are being developed to face these challenges, and to demonstrate the potentials of NGS in clinical practice.

2.2 Technical Aspects

2.2.1 Before NGS: The Sanger Method

Sanger sequencing is the first method developed to sequence DNA. Created by Frederick Sanger and colleagues in 1977 [13], it relies on random inhibition of DNA replication by incorporation of dideoxynucleotides (ddNTP). This generates DNA strands of various lengths that are later separated by electrophoresis.

Basic elements of a Sanger sequencing reaction are:

– DNA template;

It consists of a single-stranded DNA sample. It's usually previously amplified by PCR to generate many identical copies of the DNA of interest.

– DNA polymerase enzyme;
– Primers;

Primers are short sequences (≈20 nucleotides) complementary to DNA template. They bind to the template DNA and act as a starter for the DNA polymerase.

– Deoxynucleotides (dATP, dCTP, dGTP, dTTP);
– Dideoxynucleotides (ddNTP).

ddNTP are analogous to dNTP, but without the –OH group at 3′ and 5′ carbon positions. They serve as base-specific chain terminators.

A Sanger sequencing process actually consists of four parallel reactions. Each of them contains DNA template, primers, DNA polymerase and dNTP, but only one between ddATP, ddGTP, ddCTP or ddTTP.

Once the primer has bound to DNA, the DNA polymerase starts a replication process, adding consecutively the specific dNTP complementary to the template. Chain elongation continues until a dideoxynucleotide is randomly incorporated instead of the analogous deoxynucleotide. Without a 3'-OH group, it can't indeed realize a phosphodiester bond with another nucleotide, causing termination of chain elongation.

This process is repeated for several cycles, allowing that a dideoxynucleotide could be virtually incorporated at every single position of target DNA. At the end, each of the four parallel reactions contains a collection of DNA fragments

of different lengths. These fragments are finally separated by length through an electrophoresis reaction, allowing DNA sequencing by reading in which position of every reaction the ddNTP has been inserted. To permit automate reading, ddNTP or the primer are labelled.

2.2.2 The Technique

NGS is a relatively new technique, firstly launched on market by 454 Life Sciences in 2005 [14]. Since then, many progresses have been made to improve accuracy and reduce costs, and several platforms based on different techniques are now available for research and clinical applications. Despite the platform used, every NGS process can be summarized in 3 phases:

- Library preparation (± amplification);
- Sequencing;
- Data analysis.

2.2.2.1 Library Preparation and Amplification

The first step in NGS is creating a library from a DNA template. In order to achieve this goal, the sample material is first fragmented by endonuclease, mechanically or enzymatically. Fragment's lengths need to be compatible with the sequencer that will be used. After this step, DNA can eventually be enriched. It's the case of gene-panels or whole-exome-sequencing, where an enrichment passage is necessary to isolate and sequence only those genes of interest, whereas for whole-genome sequencing this is not performed. DNA fragments are later end-repaired to ensure that each molecule is free of overhangs and contains 5′ phosphate and 3′ hydroxyl groups. Finally, these platform-specific adapters are attached to the ends of each fragment to each other.

Most NGS platforms require also an amplification step before sequencing. This is necessary for many imaging systems, which are not able to detect single fluorescence events. Having many thousands of identical copies of a DNA fragment in a defined area ensures that the signal can be distinguished from background noise.

The larger part of NGS platforms uses an emulsion PCR amplification (454 Life Science, SOLiD, Ion Torrent). Alternative techniques are solid-phase bridge amplification (Illumina) and rolling circle amplification (Complete Genomics (BGI)).

2.2.2.2 Sequencing

Current NGS technology can be divided in two major categories: short- and long-read sequencing.

Short-read sequencing instruments, with a read length range between 100 and 600 bp, are the most frequently used today. They're cheaper and have a higher accuracy. Two different approaches exist: sequencing by synthesis and sequencing by ligation. Long-read sequencing, also known as "single molecule real-time", can instead obtain reads longer than 2.5 kb (PacBio) or >10 Kb (MinION), but with high costs and low accuracy. Nevertheless, long-read technique is more suitable for sequencing of really complex regions, and it's mainly used for identification of structural variations, analysis of different DNA transcripts from alternative splicing, and for resolving repetitive or heterozygous sequences.

Sequencing by Synthesis (Illumina, Ion Torrent, 454 Life Science)

NGS "by synthesis" identifies a group of sequencing methods in which a single base per cycle is added. Illumina, Ion Torrent and 454 Life Science are the major platforms created using this technique, developed in different ways.

Illumina [15]

After library preparation, DNA fragments are loaded into a flow cell where they're immobilized by bonding to slide-linked adapters. Bridge amplification PCR takes place, generating isolated clusters of identical single strand DNA fragments.

Sequencing reaction starts when specific primers hybridize with the unbound library adapter of every clone, generating a dsDNA segment where DNA polymerase can attach.

A mixture of all four modified nucleotides is then added to the flow cell. They're fluorescent-

labelled dNTP that contain a terminator, i.e. a 3'-O-azidomethyl group that prevents the addition of subsequent nucleotides.

During each cycle, only one nucleotide can be incorporated because of 3′-block. Unbound dNTPs are then removed and the slide is imaged to identify which dNTP has been incorporated in each cluster position. The fluorophore is then cleaved and the 3-OH regenerated to begin a new cycle.

Ion Torrent [16]

DNA libraries are amplified through emulsion PCR. Each fragment is immobilized on one hydrogel bead by hybridization between a specific DNA sequence coated on and the library adapter. In every tube reaction there're million of droplets containing each a bead with a library fragment, but after PCR amplification million clonal fragments are immobilized on a single bead.

Differently from Illumina, Ion Torrent uses unmodified, not 3′-blocked dNTPs and only one signal for sequencing. dATP, dTTP, dCTP, and dGTP are added iteratively, with a wash between each of them. If the specific nucleotide is complementary to the base on DNA template, the DNA polymerase incorporates it, and a proton is released during this reaction. It's the "signal" for the sequencer that detects and records a pH change. In case of homopolymers, the pH change will be proportional to the number of identical nucleotides added during the same cycle.

454 Life Science

454 Life Science uses a sequencing technology called "pyrosequencing", that is very similar to Ion Torrent method. Also in this case dNTPs, not labelled nor 3′-blocked, are flowed sequentially into the sequencer, and only one signal is used. Instead of pH changes, pyrosequencing detects the chemoluminescent light emitted each time a nucleotide is incorporated. Every time a dNTP binds the complementary base of template DNA, a pyrophosphate molecule is released. The PPi molecule is then transformed by ATP sulfurylase into ATP. ATP, in turn, is a cofactor for the conversion of luciferin to oxyluciferin by luciferase, generating a fluorescent signal. Also in this case, the signal is proportional to the amount of analogous nucleotides incorporated in each cycle.

454 Life Science was acquired by Roche in 2007, and shut down in 2013 when its technology became non-competitive.

Sequencing by Ligation (SOLiD [17])

Differently from methods exposed so far, sequencing by ligation uses segments of oligonucleotides that hybridize to DNA template at each cycle. DNA ligase replaces therefore DNA polymerase as major enzyme responsible for sequencing reaction.

At first, a known sequence (adapter) is connected to the single-strand DNA template. This sequence is complementary to an anchor sequence, with which it hybridizes providing a site to initiate ligation.

Each labelled probe of oligonucleotides consists of one or two known bases and a series of degenerated/universal bases. In the ligation step a probe connects to 5′ extremity of the anchor, hybridizing to DNA template. Complementarity is guaranteed only for the 1 or 2 known bases of the probe. After ligation, the fluorophore is cleaved from the probe along with several bases by a cleavage agent, revealing a 5′ phosphate. The template is then imaged, the known base or bases in the probe are identified and DNA ligase can bind another probe in 5′-position.

In SOLiD platform, each "probe extension" cycle consists on ten rounds of hybridization, ligation, imaging and cleavage, allowing the identification of 2 out of every 5 bases. After a probe extension, a reset step takes place. All probes and anchors are removed and the cycle begins again with a new anchor at n-1 position.

Long-Read Sequencing (PacBio [18], MinION [19])

Extensive employment of NGS technology during last decades allowed an unprecedented comprehension of genome complexity, and revealed the limits of this technology at the same time. Specifically, it appeared clearly how these techniques are unsuitable for resolving large structural features and repetitive sequences regions.

Development of long-read sequencing technology overcame this critical issue, marking the transition to "third-generation sequencing" approaches.

The first long-reads sequencer available on market was the PacBio RS, launched by Pacific Bioscience in 2010 and updated in 2013 as PacBio RS II. It's based on a "Single Molecule, Real-Time (SMRT)" sequencing technology, that doesn't need any amplification step.

Sequencing is based on real time detection of a fluorescent signal, emitted when a nucleotide incorporation occurs. A single molecule of DNA template is putted into a SMRT cell, each containing a nanostructures called zeromode waveguides (ZMWs), a DNA polymerase enzyme and dNTPs labelled with 4 different fluorescent colours. Whenever a nucleotide is incorporated, it releases a luminous signal that is recorded by sensors. The ordered series of lights recorded is later translated into a specific DNA sequence.

A further promising third-generation sequencing technology, alternative to SMRT, is based on nanopore sequencing. MinION is the first prototype of a nanopore sequencer, available since 2014.

During sample preparation a hairpin adapter is linked to the 2 strands of DNA molecule by one end. The DNA fragment flows slowly through a nanopore, where a motor protein "reads" which nucleotide is passing by analysing the ionic current variation that it causes. This variations are recorded progressively and then interpreted to identify the sequence. Both the direct and the inverse strands are read, generating a highly accurate consensus sequence.

Another sequencing platform based on nanopore technology is now developing: the "PromethION". Not still commercially available, it's an evolution of MinION sequencer with 48 individual flow cells, each with 3000 pores (equivalent to 48 MinIONs). It's a very promising technology that will compete with PacBio RS II in terms of reads length and with Illumina for cost-effectiveness.

DNA amplification	
Emulsion PCR amplification	Single molecules of DNA template are captured by specific adapters onto microbeads by primer hybridization. These beads are incorporated into a controlled emulsion, containing also DNA template, primers and PCR reagents. A PCR amplification takes place, and finally each bead is coated with millions of clonally amplified molecules.
Solid-phase bridge amplification	DNA fragments are ligated to adapter sequences and immobilized on a solid support through the bound with specific primers. The interaction between the free ends of nearby primers creates a bridge structure, which acts as a template for PCR amplification.
Rolling circle amplification	A circular ssDNA template hybridizes with a short DNA or RNA primer, and a DNA polymerase is used to amplify this primer forming a long ssDNA. The final product is a concatemer containing hundreds of tandem repeats complementary to the circular ssDNA template.
Copy Number Alterations/ Variations (CNAs/CNVs)	Subcategory of structural variation (SV) characterized by gain or loss of many copies of a large DNA segment.
Coverage (depth)	It's the number of times a certain nucleotide is sequenced. It corresponds to the numbers of reads that includes a specific position into the reconstructed sequence.
Enrichment	Process by which specific regions of interest are captured by hybridization before sequencing.
Flow cell	Single-use sequencing chip/plate/ slide used by Illumina sequencers
Gene panel	A set of chosen genes of interest that are sequenced together from the same sample. Selection of regions of interest is done by designing specific probes or primers
Homopolymer	A DNA sequence composed by identical nucleotides
Indels	Structural DNA variations that consists on insertion or deletion of nucleotides

(continued)

DNA amplification	
Library	A comprehensive collection of cloned DNA fragments that represent together a genome of interest
Nanopore	Pores of nanometres size located in electrically insulating materials and used to study the physical properties of biomolecules by measuring changes in current as individual molecules transit
Pair-end sequencing	Both ends (5′ and 3′) of a DNA fragment are sequenced, allowing a better alignment with the reference genome
Quality score	Score that estimates the probability of a base call error
Reads	The nucleotide sequences "read" by the sequencer from a library; they essentially represent the data output of a sequencing reaction
Real-time sequencing	Sequencing strategy in which there is no pause after the detection of a base or series of bases, thus the sequence is derived in real-time
Single-end sequencing	DNA fragments are sequenced from only one end
Single Nucleotide Variations (SNVs)	It's a single base variation in the DNA sequence that occurs at a specific position in the genome. When at least 1% of population presents such variation this is called single nucleotide polymorphism (SNP)
Structural Variations (SVs)	Genomic rearrangements involving more than 50 bp. They include insertions, deletions, inversions, transpositions, translocations, tandem repeats, and copy number variations (CNVs).
Variant of Undetermined/ Unknown Significance (VUS)	A variation in a genetic sequence whose clinical significance is not known
Whole Exome Sequencing	Sequencing process that targets the exome, i.e. the protein-encoding parts of all the genes
Whole Genome Sequencing	Sequencing process by which the whole DNA sequence of an organism's genome is read at a single time, without using methods for sequence selection

2.2.2.3 Data Analysis

NGS data analysis pipeline can be divided into four operations: base calling, read alignment, variant identification (SNP, indel, CNV, SV), and variant annotation. Different pipelines are used depending on the sequencing approach (gene-panel, WES, WGS or RNA-seq).

Base Calling

Base calling is the process by which the signals provided during sequencing are translated into a sequence of bases, removing the noisy signals. Base-calling software are usually integrated into the sequencer itself, and generate a file output called "FASTQ". It's a text-file that stores both the nucleotide sequence and a quality score for each base, i.e. a score that reflects the probability of the base call being wrong or correct.

Read Alignment

During this process, the DNA of the sequenced sample is compared/aligned to a reference genome. Given that NGS generally produces millions of short reads, each read needs to find the corresponding part on reference genome. Several alignment algorithms exist, and "Burrows-Wheeler-Alignment" (BWA) is probably one of the most used. The BWA output is a "Sequence Alignment/Map" (SAM) format file. SAM files are then converted into a BAM format that is a binary compressed file more suitable for successive steps.

Variant Identification/Calling

Variant calling is the process by which variants from sequence data are identified. Four main classes of sequence variants exist (SNVs, indels, CNAs, and SVs), each requiring a different computational approach for sensitive and specific identification.

GATK, SAMtools and VCMM are the main workflows used to detect single nucleotide variations (SNVs), both as germline variant or somatic mutation. It has been reported that while all three methods call a large number of common SNVs, each tool also identifies SNVs not found by the other methods.

Alignment of indel-containing sequence reads is more challenging, and specific algorithms are necessary. Main approaches currently used are called "gapped alignment" and "split reads".

A gapped alignment-based indel detection requires that the aligner used in the previous phase was able to detect an indel into a specific read (a "gap"). Most of software used to identify SNPs are also able to infer small indels detecting these "gaps" (GATK, SAMtools).

Nevertheless, these tools are unsuitable for longer indels that aren't completely contained within a read. Split read methods (e.g., Pindel) are designed to re-align soft-clipped reads to facilitate the identification of medium-sized indels. They use algorithms that are able to map the two ends of a read interrupted ("split") by insertion or deletion.

Detection of CNAs is conceptually different from other variant calling, given that CNAs don't change the specific DNA sequence, which is instead under- or over-represented. A good sensor of CNAs could be represented by the number of reads mapping a specific region, after normalization to the average read depth. CNV-seq, ExomeCNV and VarScan2 are only few examples of tools specifically developed to detect CNAs.

Analysis of SVs also presents some challenges, essentially due to the complexity typical of structural variations. The main technologies used to identify SVs are called "read-pair" and "split-read" methods.

Read-pair (RP) methods are based on the evaluation of the span and orientation of paired-end reads, i.e. reads that are sequenced from both extremities in opposite directions. SVs are detected as significant differences between the fragments identified by the paired-end reads and the corresponding regions of the reference sequence. A read-pair that spans an isolated deletion maps to the corresponding regions of the reference, but the mapped distance is greater than the insert size. Oppositely, that mapped distance is shorter in case of insertions. Inversions are instead characterized by a different relative orientation between the sequenced and the reference genome.

Using a split-read (SR) methods, the presence of a SV breakpoint is instead suggested by a split sequence-read signature breaking the alignment to the reference genome. A gap in the read is a marker of a deletion while stretches in the reference reflect insertions.

BreakDancer, BreakPointer, CLEVER and GASVPro are the principal tools available for SVs identification.

Variant Annotation

Variant annotation process is finally used to distinguish "real" variants from sequencing artefacts, trying to identify which ones are potentially pathogenic and have a real clinical value.

Many annotation tools are available (for instance: ANNOVAR, AnnTools, SVA, VARIANT, VEP), usually developed as web applications. Whereas most of them provide annotation of SNPs and InDels, annotation of SVs is limited to CNVs and performed only by recently developed applications. In summary, all annotation tools provide a description of what is known about the variant mutation identified, and generate links to one or more public databases of known mutations.

Platform	Sequencing technique	Read length (bp)	Data output	N° of reads	Runtime	Error rate/type
First generation						
Sanger	NA	400–900	0.7–2.1 Mb	96	20 min–3 h	0.3%
Second generation						
454						
GS junior+	Pyrosequencing	700	70 Mb	0.1 M	18 h	1% indels
GS FLX titanium XL+	Pyrosequencing	700	700 Mb	1 M	23 h	1% indels
Illumina						
MiniSeq (high-output)	SBS	75 (SE)	1.65–1.875 Gb	22–25 M (SE)	7 h	<1% substitution
		75 (PE)	3.3–3.75 Gb	44–50 M (PE)	13 h	
		150 (PE)	6.6–7.5 Gb		24 h	
HiSeq2500 (v4) (dual flow cell)	SBS	36 (SE)	128–144 Gb	Up to 4 B (SE)	29 h	0.1% substitution
		50 (PE)	360–400 Gb	Up to 8 B (PE)	2.5 d	
		100 (PE)	720–800 Gb		5 d	
		125 (PE)	900 Gb–1 Tb		6 d	
NextSeq 550 (high output)	SBS	75 (SE)	25–30 Gb	Up to 400 M (SE)	11 h	<1% substitution
		75 (PE)	50–60 Gb	Up to 800 M (PE)	18 h	
		150 (PE)	100–120 Gb		29 h	
MiSeq (v3)	SBS	75 (PE)	3.3–3.8 Gb	44–50 M (PE)	21 h	0.1% substitution
		300 (PE)	13.2–15 Gb		56 h	
HiSeqX (dual flow)	SBS	150 (PE)	1.6–1.8 Tb	5.3–6 B	<3 d	0.1% substitution
NovaSeq	SBS	50 (PE)	134–167 Gb (S1)		13 h (S1)	NA
			333–417 Gb (S2)		16 h (S2)	
		100 (PE)	266–333 Gb (S1)	2.6–3.2 B (S1)	19 h (S1)	
			667–833 Gb (S2)	6.6–8.2 B (S2)	25 h (S2)	
		150 (PE)	400–500 Gb (S1)		25 h (S1)	
			1000–1250 Gb (S2)		36 h (S2)	
Ion torrent						
PGM	SBS	200–400 (SE)	30 Mb–2 Gb	400,000–5.5 M	2.3–7.3 h	1% indel
Proton	SBS	Up to 200 (SE)	Up to 10 Gb	60–80 M	2–4 h	1% indel
S5	SBS	200–600 (SE)	0.3–25 Gb	2–130 M	2.5–4 h	1% indel

SOLiD						
5500xl	SBL	50 (SE)	160 Gb			
		75 (SE)	240 Gb	~1.4 B	10 d	0.01% A-T bias
		50 (PE)	320 Gb			
Third generation						
PacBio						
RS II	SMRT	>15,000 (average)	500 Mb–1 Gb	Up to 55,000	30 min–4 h	15% indel
Sequel	SMRT	30,000 (average)	5–10 Gb	~ 400,000	30 min–20 h	15%
Oxford Nanopore						
MinION	SMRT	Up to 900 kb	10–20 Gb	Up to 1 M	Up to 48 h	5–10%

A-T adenine-thymine, *B* billion, *bp* base pairs, *d* days, *Gb* gigabase pairs, *h* hours, *indel* insertions-deletions, *Kb* kilobase pairs, *M* million, *Mb* megabase pairs, *min* minutes, *NA* not applicable, *PE* pair-end, *SBL* sequencing by ligation, *SBS* sequencing by synthesis, *SE* single-end, *SMRT* single-molecule-real-time, *Tb* terabase pairs

2.3 Detection of Driver Alterations and Resistance to Guide Therapy

NGS represents the most important innovation of the last decades in oncobiology, with a huge impact in cancer diagnosis and care. Thanks to exponential technological advances, whole cancer genome sequencing has become feasible, leading to the identification of genetic and epigenetic alterations potentially involved in the pathogenesis of the tumor as well as in the mechanisms of resistance to specific treatments. These alterations include point mutations, copy number variations and chromosomal rearrangements, regarding genes involved in cell proliferation, death and differentiation. Some mutations occurred in somatic cancer genome, known as "driver mutations", directly or indirectly confer a selective growth advantage to malignancies bearing them. Other alterations, as a result of increased mutation rates, arise in somatic cancer genome during the progression of a tumor, but do not contribute to its growth. These are called "passenger mutations". Detection of driver alterations that results in oncogene addiction is currently the primary application of NGS in current oncology and discriminating between driver and passenger alterations is a challenge point of translational research. Several statistical and computational techniques to characterize these mutations have been described, including variant effect prediction, recurrence/frequency assessment and pathway/network analysis. These techniques provide alternative strategies to filter the long list of somatic mutations, thus identifying an enriched subset of sub-clonal carriers who may undergo further functional validation. For further reading about different technical approaches, three reviews [20–22] published on Nature, Genome Medicine and Nature Reviews Genetics are strongly recommended**.**

Identification of driver events can guide the way to treatment with matched targeted therapies. Multiple recurrent driver alterations are the target for specific biological agents. They have been or are being investigated, including BRAF V600E, EGFR, KIT, ERBB2, PIK3CA, TSC1, FGFR3, AKT1 and ROS1 mutations, ERBB2 amplifications, and ALK translocations. In lung cancer, the detection for EGFR mutations and ALK and ROS1 rearrangement status are currently carried out in daily practice, using PCR and/or immunohistochemistry. NGS is the most comprehensive method to test several genes at the same time. The last MAP (Molecular Analysis for Personalised Therapy) consensus [23] recommends the use of NGS in the context of clinical trials. For non small cell lung cancer (NSCLC) at least 20 genes should be analyzed in specific panels including EGFR, BRAF, HER2, KRAS, PI3KCA, NTKR, ALK, MET (ex 14), AKT1, BRCA1/BRCA2, HRAS, NRAS; rearrangement status of ALK, ROS1, NTRK; amplification of RET, MET and EGFR; aberrations (mutations or amplifications) in FGFR1/2/3, NOTCH1/NOTCH2. Likewise, estrogen receptor (ER)-positive breast cancer should be tested using NGS assay for PIK3CA, ESR1, AKT1, ERBB2.

The treatment of lung cancer, for instance, represents a successful case of establishing new agents targeted to specific alterations. EGFR mutations and the anaplastic lymphoma kinase (ALK) rearrangements determine an aberrant expression or activity of these two tyrosine kinases. Activating EGFR mutations in the tyrosine kinase (TK) domain of the EGFR gene, most frequently exon 19 deletion mutations and the single-point substitution mutation L858R in exon 21, are predictive for response to the EGFR TK Inhibitors (EGFR-Is) gefitinib, erlotinib and afatinib. Crizotinib and second generation ALK inhibitors, have also been demonstrated to be highly effective against ALK positive NSCLC, showing impressive and prolonged responses with relatively low rates of toxicity [24].

Unfortunately, almost the totality of patients treated with targeted therapies will develop secondary resistance. NGS can be useful to identify the implicated mechanisms of resistance and to aid on following treatment choices. For example, NSCLC patients progressing to a prior treatment with EGFR-Is can acquire T790 M

mutations in around 50% of cases. The latter is the target for a new agent, osimertinib, an oral, potent, irreversible EGFR tyrosine-kinase inhibitor selective for EGFR tyrosine-kinase inhibitor sensitizing mutations, and the EGFR Thr790Met resistance mutation. The AURA 3 trial [25] demonstrated the great superiority of osimertinib to platinum-based chemotherapy in EGFR-Is pretreated patients with T790 M mutation, reporting a PFS of 10.1 months in osimetinib group vs. 4.4 months in the control group. The introduction of osimertinib has allowed to prolong as possible the chemo-free interval in EGFR-positive population.

Another case of secondary resistance is ESR1 mutations in ER-positive breast occurred after a prolonged aromatase inhibitor (AI) therapy, particularly in the metastatic setting [26, 27]. This population seem to have a shorter OS in comparison with patients with wild-type ESR1 in some studies, even after adjustment for the potential effects of previous hormone therapy, visceral disease, and performance status [27, 28]. This statement still lacks validation. Notwithstanding, a retrospective analysis of the BOLERO-2 trial suggested a PFS benefit from addition of everolimus, an mTOR targeted therapy, to exemestane for patients with D538G ESR1 mutations (21.1%), with overlapping outcomes when compared to wild-type patients. That benefit was not observed for patients with Y537S mutation alone or with both Y537S and D538G mutation [27]. In the PALOMA3 trial [29], in which patients who failed on prior endocrine therapy were randomized to fulvestrant in combination with the palbociclib or to fulvestrant and placebo, ESR1 mutations were detected in 29% of patients. The PFS benefit of patients in the combination arm was maintained in patients with ESR1 mutations. Prospective trials are needed to understand if ESR1 mutations could be tested to select a specific population who should be approached differently.

Furthermore, NGS has become a very useful instrument in the management of carcinoma of unknown primary site (CUP). Molecular profiling allows prediction of tumor origin by detecting site-specific gene expression profiles. A Prospective Trial of the Sarah Cannon Research Institute, published in 2013 on Journal of Clinical Oncology, showed that the molecular profiling assay predicted a tissue of origin in 98% of cases [30]. Considering the modest benefit achieved with a platinum/taxane containing empiric regimen, the accurate identification of the putative primary may substantially change the management and outcome of patients with CUP, particularly if a tumor more responsive to the best site-specific therapy is identified. On the aforementioned study, median survival of 12.5 months for patients who received site-specific therapy is considered favourable compared with previous results using empiric CUP regimens. Another point of research for CUP is detecting actionable mutations. Varghese et al. [31] evaluated the tissue samples of 333 patients with diagnosis of CUP performing MSK-IMPACT panel (a panel developed by Memorial Sloan Kettering Cancer group), and when there was no clear driver alteration, whole exon sequencing (WES) was performed. Thirty percent of patients had potentially targetable genomic alterations identified. Of these, 10% received targeted therapies. The most common driver alterations detected are: ERBB2 amplification, BRAF V600E mutation and PIK3CA mutations. We are eagerly waiting for the results of a french randomized phase III trial (NCT01540058) comparing a diagnostic and therapeutic strategy based on molecular analysis followed by suspected primary cancer tailored specific therapy, to an empiric strategy in patients with CUP.

Traditionally, drug development has been histology driven. The dissemination of basket trials, on which patients are assembled by the presence of common mutations, regardless of the organs involved, led to a new direction. Pembrolizumab was the first drug approved by FDA considering tumor's biomarker without accounting for its original location. This accelerated approval pathway was gained for unresectable or metastatic solid tumors

possessing a microsatellite instability high (MSI-H) biomarker. Larotrectinib, a potent and highly selective small-molecule inhibitor of tropomyosin receptor kinase (TRK) proteins, at present under FDA approval, is another emblematic case. 55 TRK fusion-positive patients were prospectively and consecutively enrolled, both adults and children, in three separate Phase 1–2 trials [32]. Data on efficacy and safety from these studies were evaluated into a single analysis, recently published on The New England Journal of Medicine. Overall response rate ranged from 75–80% and, at 1 year, 71% of the responses were ongoing and 55% of the patients remained progression-free.

2.4 Tumor Heterogeneity

Genetic variations are observed among tumor of different specimens, as well as between individuals with the same tumor type (intertumor heterogeneity). Within a primary tumor and its metastatic sites, subclonal diversity may be observed (intratumor heterogeneity). During the evolution of a malignancy, driver alterations that arise very early and are inexorable for neoplastic growth are better distributed throughout the tumor. On the other hand, alterations occurring later in cancer evolution are not homogenously localized and may be exclusive to limited tumor regions or single metastasis. Dysfunction of mechanisms that maintain genome integrity or exposure to exogenous mutagens could elevate the mutation rate and increase the heterogeneity of the tumor [33]. The intratumoral heterogeneity is difficult to assess because collection of extensive biopsies of different lesions is not feasible in routine clinical practice. The WES on cDNA could overcome these limitations by theoretically identifying all subclonal alterations as well as the quantification of heterogeneity, which could have direct therapeutic implications, including the prediction of response to immunotherapeutic agents [34].

2.5 Biomarkers

A biomarker is understood as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [35]. It's defined as "prognostic" biomarker when it provides information about disease outcome irrespective of treatment, whereas a "predictive" biomarker gives information on disease outcome related to a specific treatment. Several predictive biomarkers have been identified in the last 20 years, and all international guidelines demand their analysis to optimize treatment in solid and hematological tumors. HER2 amplification in breast cancer, EGFR mutation in lung cancer, BRAF mutation in melanoma or BCR-ABL amplification in myeloid chronic leukaemia and microsatellite instability (MSI) in colorectal cancer are a few examples. Beside these "classic" biomarkers, relatively new and more complex ones are currently under development in clinical practice. Homologous recombination deficiency (HRD) and tumor mutation burden (TMB) have the most robust data [36, 37] so far and are currently under validation in numerous settings.

2.6 HRD

Homologous recombination (HR) is a DNA repair mechanism responsible for repair of double-strand breaks (DSBs) [38, 39]. BRCA1-2 [40, 41], PALB2 [42], RAD51, ATM and other genes related to the Fanconi anemia-pathway (FANCA, FANCC, FANCD2, etc.) [43] are essential components of HR-mediated DNA repair. When mutations in this pathway occur, other mechanisms of DNA repair must take over, such as base-excision repair (BER) or non-homologous end joining (NHEJ) [44]. HR-deficient cells are consequently vulnerable to agents that targets these alternative pathways, such as poly-(ADP ribose) polymerase (PARP) inhibitors (e.g., veliparib, olaparib, rucaparib, niraparib), and to others DNA damaging drugs, such as

platinum regimens [45, 46]. This mechanism have been called "synthetic lethality".

The singularity of HRD as predictive biomarker lies on its complexity. Many genes need to be analysed in parallel, and the assay's results be expressed as a quantitative score that indicates if the HR pattern is impaired or not. Many panels based on NGS sequencing have been developed and are currently available to test HRD in different cancers [47]. For more comprehensive information about HRD, see the excellent review of O'Kane and collegues [47].

2.7 MSI

A microsatellite (MS) tract is a short (2– to 5–base pair) sequence of DNA tandemly repeated 10–60 times, and variations in these repeats are called "microsatellite instability" (MSI) [48]. This condition occurs when mismatch repair (MMR) pathway is impaired, mainly due to MS tendency to base-pair mismatching during DNA replication.

Like HRD, MSI can derive from both inherited and somatic mutations. The first is characteristic of Lynch syndrome, an hereditary condition characterized by a high risk of colon and endometrial cancer (the two most common types) as well as tumors of the ovaries, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin [49].

Several studies have analysed MS instability as a prognostic biomarker, with controversial results [50, 51]. Following the development of immune-checkpoint inhibitors (ICPI), a possible role as predictive biomarker has been recently reconsidered. Many authors demonstrated that high levels of MSI (MSI-H) predicts a good response to ICPI, whereas MSI stable (MSS) tend not to [52]. This evidence has led to the FDA approval of pembrolizumab for MSI-H cancers in May 2017, the first tumor-agnostic drug approval in history.

MSI can be detected through several methods, of which the most employed are PCR and IHC. Nevertheless, the use of NGS panels showed feasibility and accuracy [53] in this context, with the obvious advantage of providing many additional information to stand for the concept of therapy personalization at its best.

2.8 TMB

Tumor mutational burden (TMB) is defined by the total number of mutations per coding area of a tumor genome. Cancer associated to environmental DNA damage are typically highly mutated, whereas paediatric and well differentiated tumors have usually a lower TMB [54]. NSCLC and melanoma are classically associated with tumorigenesis caused by DNA damage, respectively due to smoke and UV radiations.

Most recently, TMB has been identified as a predictive biomarker of immunotherapy response. ICPI have shown to be more effective in cancers that present multiple neoantigens i.e. cancer-specific antigens that can be recognized as non-self by T lymphocytes. Since TMB correlates well with the number of neoantigens and consequently to potential efficacy of ICPI in reactivating immunity against cancer cells. In melanoma, NSCLC and urothelial cancer the correlation between high TMB and response to ICPI has already been proven [55–57].

TMB has been historically measured through WES. This approach is accurate, but expensive and unfeasible in clinical practice. Targeted sequencing panels have been recently developed to simplify TMB analysis in clinical routine, and specific trials showed their reliability [54, 58]. Their implementation could represent a possible solution to the unmet need of predictive biomarkers able to identify patients more likely to respond to immunotherapy.

2.9 RNA Sequencing and Liquid Biopsy: Beyond "Classic" DNA Sequencing

RNA sequencing (RNA-seq) is a relatively new application of NGS, developed to analyse the transcriptome. Even if DNA sequencing of tumors provides many information about cancer biology,

it's not able to describe what happens after DNA transcription. Conversely, RNA-seq can be used to quantify gene expression, to capture alternative transcripts originating from splicing variants and to detect chimeric gene fusions.

Analysis of gene expression through RNA profiling is already implemented in clinical practice. OncotypeDx [59] and Mammaprint [60] are both mRNA analysis based tests broadly validated and used to predict the risk of relapse in early breast cancer. The former uses qRT-PCR while the latter a microarray.

Detection for fusion transcripts and their quantification is also a validated application of transcriptome analysis. BCR-ABL1, the signature of chronic myeloid leukemia, is a fusion protein commonly detected through fluorescence-in situ hybridization (FISH). Quantification of BCR-ABL1 transcripts has, in its turn, been identified as a reliable marker to monitor treatment response [61]. EML4-ALK is instead a chimeric transcript sometimes found in approximately 5% of NSCLCs [62]. ExoDx Lung [63] is a RT-PCR based test used to detect this protein, guiding prescription of specific targeted TKi.

Finally, capturing alternative transcripts can be useful both from a diagnostic and therapeutic point of view. For instance, BRCA1 and BRCA2 are genes typically mutated in patients with a family history of breast and/or ovarian cancer. Nevertheless, it's not infrequent to meet families with a strong family history but without BRCA1/2-positive test for known pathogenic mutations. Using a RNA-based method, alternative BRCA1 transcripts have been recently identified in some families notably negative at conventional tests [64].

Alternative transcripts with clinical values have also been described in prostate cancer. AR-V7 is a well characterized variant of androgen receptor associated with castration-resistance [65, 66] and commonly identified with a RT-PCR test on CTCs.

Even if RNA analysis has clearly a wide applicability, RNA-seq is rarely used in clinical practice. Several studies have only recently demonstrated a high level of concordance between gene expression measurement by RNA-seq, RT-PCR and microarrays [67, 68]. Moreover, RNA-seq has shown to detect novel transcripts [69] as well as single nucleotide polymorphisms (SNPs) [70], allowing for a complete mutation analysis of exome with only one assay. Nevertheless, a better characterization of RNA-seq in terms of analytical and clinical validity is still warranted before a real implementation in clinical practice.

ctDNA is, essentially, fragmented DNA released into circulation from apoptotic or necrotic cancer cells. It's usually found in blood along with cell free DNA originated from normal cells. Analysis of ctDNA, together with intact CTCs, is the basic concept of liquid biopsy. Collection of fluid instead of classic tissue sample is gradually spreading throughout clinical practice for a few suitable reasons: first, it's technically easier to collect and it can be repeatedly performed without risks or side effects for patients. Second, since tumor genome is known to evolve rapidly, DNA analysis over time and during treatment is a compelling tool to look for mutational changes potentially responsible for resistance [71]. Also, ctDNA represents a wider picture of tumor clones, allowing a better representation of their heterogeneity [72, 73].

The principal methods available for ctDNA analysis are droplet digital PCR (ddPCR) and NGS itself. The first is most sensitive and cost-effective, and it allows for an absolute quantification of mutant and wild-type copies. Conversely, NGS is more complex and expensive, but it has a higher throughput which renders a more comprehensive detection of all known and unknown mutations, without preventive selection of any gene.

Liquid biopsy analyses by NGS have been already evaluated in many cancer types, with reassuring accuracy and reliability [74–76] for most of the population (problems with HER2 amplification, for example). Several ongoing studies are assessing its systematic application in clinical practice and if it could actually improve patients' outcomes by helping in the selection of the best treatment possible at the right time.

2.10 Pharmacogenomics

Pharmacogenomics (PGx) represents one of the main branches of PM. The term, which reflects the combination between pharmacology and genomics, refers to the study of relations between the human genome and drug response. In 1959 the German pharmacologist Friedrich Vogel firstly coined the term "*pharmacogenetics*", referring to polymorphism of specific genes inducing individual drug response or susceptibility to adverse drug reactions (ADRs) [77]. The availability of genome-wide sequence data, and the advent of the "*omics*" era, marked the evolution from "*pharmacogenetics*" to "*pharmacogenomics*", and now these terms are often used interchangeably in literature [78].

The aim of PGx is to optimize drug therapy, with maximal efficacy and safety, focusing on individual genetic variability. All kinds of genetic alterations can influence drug response, i.e. base-pairs substitutions, rearrangements, insertions, deletions and copy number alterations [78, 79]. These mutations can impact both on pharmacodynamics (e.g., modifying the interaction between the drug and its target) and pharmacokinetics (i.e., absorption, distribution, metabolism, elimination) of a specific drug.

Outside oncology PGx research is focused on germ-line variants of specific genes known to have relations to certain medications, involved in specific pathways or drug-resistance mechanisms. Among them, the cytochrome P450 (CYP) enzymes are the most prevalent and better characterized drug-metabolizing enzymes, with more than 57 CYP isoenzymes, 18 families and 44 subfamilies documented until now. CYP2D6 is probably the most studied of CYP genes, for which more than 80 variants have been reported [80]. There is strong evidence that the presence of defective instead of functional alleles can alter the metabolism of many drugs, decreasing efficacy (i.e., codeine, tamoxifen) [81, 82] or increasing toxicity (e.g., propafenone, perphenazine) [83, 84].

Mutation of DPYD gene is another example of germline variation related to drug metabolism, which has been implicated in many cases of chemotherapy toxicity. DPYD codify for the dihydropyrimidine dehydrogenase (DPD), involved in the degradation of fluoropyrimidines, like 5-fluorouracil, (5-FU), capecitabine, and tegafur. DPD dysfunction leads to an increased exposure to active metabolites, resulting (just so we don't abuse the "which") in severe or even fatal toxicity [85, 86].

In the field of cancer therapy, these alterations are important not only in drug-metabolism genes, but also in cancer cells themselves. For this, both inherited genome (germline) variations and somatically acquired genome variants must be explored. As previously explained, cancer proliferation is sustained by some "driver mutations" that alter intracellular signalling pathway, and the principle of targeted therapy is to hit these altered pathways, inhibiting cell proliferation. Mutations of proteins in these pathways define sensibility or resistance to specific targeted agents, and are consequently defined as "predictive biomarkers".

Since trastuzumab approval by FDA in 1998, hundreds of gene-based target-specific drugs have been developed for different tumors and are already applied as standards of care. Their employment is obviously subordinate to the demonstration of the genomic alteration for which they are purposely designed. In the majority of cases, these mutations are detected through specific tests, different for distinct kinds of alterations. Chromosomal rearrangements like BCR-ABL, ROS1 or EML4-ALK and amplifications like HER2 are mainly detected through FISH test; whereas mutations of k-RAS, n-RAS, BRAF, EGFR and many others genes are identified with RT-PCR-sequencing [87–89]. These "classic" assays have high sensitivity and specificity, they are relatively cheap and broadly used in clinical practice everywhere. Nevertheless, they have been created to look for specific alterations, and consequently they can be used only for this purpose. The advent of NGS radically changed the approach to cancer molecular analysis, allowing complete and unique molecular profiling for each tumor, without looking for any specific alteration. Moreover, differently from previous assays, with

NGS methods all genomic alterations can be simultaneously detected (i.e. base-pairs substitutions, rearrangements, insertions, deletions and copy number alterations) [90, 91]. Several NGS panels for personalized therapy in oncology are currently available. The biggest commercially available to date is FoundationOne, developed by Roche, that allows parallel analysis of 315 cancer-related genes. This assay was reported to detect at least one clinically actionable variant in 76% of samples (n = 2200), with an average of 1.57 clinically actionable variants detected per sample [91].

NGS panels have also been created to analyze genes related to drug response ad drug metabolism. These resources facilitated the creation and progressive enrichment of PharmGKB (http://www.pharmgkb.org/), a PGx knowledge database collecting clinical information, such as including dosing guidelines and drug labels, and annotating genetic variants as well as the gene–drug–disease relationship.

Despite continuous advances made by NGS methods in pharmacogenomics, several steps are still required before their implementation in clinical practice. First of all, technological limitations are posed by complexity and variability of pharmacogenes like CYP, for which NGS sequencing is frequently inadequate for coverage and accuracy because of the short lengths of reads [92, 93]. With widespread deep sequencing, new additional variants in pharmacogenes are continuously discovered and annotated in PGx databases. Nevertheless, the definition of their real functional effect is complex, and their clinical value, diagnostic and drug-response relevance frequently can't be precisely defined. Further technical progress and clinical studies need to be implemented before NGS applied to PGx becomes an integral part of PM.

2.11 NGS in Clinical Cancer Care Practice: Challenges and Limitations

The NGS-based technologies have already impacted in the decision-making process for treatment choice. Over the past decade, the application of NGS to cancer genomics projects have revealed outstanding new information about the genetic profile of cancer. Several factors will need to be addressed in the next years to improve applications of NGS in the clinical practice.

NGS offers multiple approaches to investigate genome, including sequencing of whole genome, exome, and transcriptome. Nevertheless, targeted panels are often used in the clinical setting for detecting specific genetic alterations. They can range from hotspot panels, focused on individual codons, to more comprehensive panels that include the coding regions of hundreds of genes [94]. The choice of NGS panel content depends on the intended use. Such panels are usually designed as pan-cancer panels and include a large number of genes with solid scientific evidence of therapeutic opportunity. Panels designed for diagnosis and to determine patients' prognosis are usually tumor specific, smaller in size, and contain only genes directly implicated in the biology of the respective malignancy [95]. When designing the NGS panel content, it is crucial to distinguish disease-causing genetic variants from the multitude of candidate genes. This process, known as variants prioritization, is complex and multidimensional and it is a necessary step to building panels [96]. Several prioritization tools and online resources based on large databases are available. Examples of clinical oncology database used for prioritization are the National Center for Biotechnology Information (NCBI), the Online Mendelian Inheritance in Man (OMIM), the Catalogue of Somatic Mutations in Cancer (COSMIC), the cBioPortal and the PharmGKB. The main effort of these institutions has being put towards creating international databases to increase level of evidence and expertise. In addition, the GENIE project must be mentioned here. This registry contains the existing CLIA-/ISO-certified genomic data obtained during routine practice at multiple international institutions. It is a dynamic tool, derived from a variety of cancer types including rare entities, and is enriched of late-stage disease; thus, it estimates a "real world" dataset. The information obtained by the registry could be not only an instrument of prioritization of variants,

but it is a precious resource for powering clinical and translational research, adding new mutations to existing drug labels or identifying new drug targets. For more information about the GENIE project, visit the web site http://www.aacr.org/Research/Research/Pages/aacr-project-genie.

In order to validate the utility of gene panels in clinical practice, some clinical trials were conducted. The SAFIR02 [97] and MOSCATO [98] trials were designed for testing drugs recommended by NGS results versus conventional therapy. These studies failed essentially for two reasons: drugs are not well matched to alterations and the method for driver identification is not optimal since validated and robust tools to interpret the whole biological parameters were not available at the time. The next step to overcome these limitations is represented by a new generation of softwares for target prediction and large databases, as described above.

In the era of NGS, the detection of new predictive biomarkers led to a new way of conceiving clinical trials in which the patients' enrolment is stratified based on biomarkers that could potentially predict and increase the response to targeted therapy. These genome forward or genome driven trials prospectively scrutinize the efficacy of new drugs in a population defined on the basis of specific biomarkers [99]. An interesting point of these trials is an early response evaluation in which strategic sequenced biopsies provide the opportunity to understand the biology of underlying responses as well as mechanism of resistance to new targeted agents. According to information obtained, the treatment is continued only in the subgroups of patients who benefit from the therapy. An example of a biomarker stratified trial is the I-SPY 2, an ongoing randomized phase II trial in the neoadjuvant setting, designed to assess the incremental benefit of new targeted agents added to conventional chemotherapy in patients with locally advanced breast cancer (NCT01042379). In the screening phase of I-SPY2, potential candidates undergo MammaPrint on breast tissue associated with the ER, PgR, and HER2 assessment. Patients who come out as ER+/MammaPrint high, ER−, or HER2+ are eligible for the study. These patients are then randomized to either standard neoadjuvant chemotherapy with weekly paclitaxel (plus trastuzumab for HER2+) or paclitaxel combined with one of several investigational agents followed by four cycles of doxorubicin/cyclophosphamide. Biomarkers are used to identify signatures for experimental arms. Regimens are dropped if they do not improve pCR rates for any biomarker signature. The adaptive design of this trial, selecting the subgroups of patients more responsive, allows accelerating the investigational agents' development into subsequent phase III trial, to improve the approval process in a specific setting and to limit the exposure to potentially harmful drugs.

It must be noted that the accessibility to both tests and drugs in this context is not yet guaranteed and regulated by international order. In the case of test accessibility, some countries have started initiatives to facilitate it, such as France Genomics 2025 and Genomics England 100 k Genomes. On the other hand, the accessibility of a not yet approved target agent for a patient positive for the specific mutation is possible only under a clinical trial. Therefore, a situation in which a patient carries a specific mutation but does not have the possibility to receive the targeted therapy could occur. For this reason, the development of tests and drugs should be done in parallel and contemplated when designing clinical trials.

New and clever study designs have emerged as an efficient way to expand access to targeted therapies for patients with particular alterations and to demonstrate clinical validity of new biomarkers as well as the therapeutic implication that follows.

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3 Pharmaco-epigenomics: On the Road of Translation Medicine

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Abstract

Epigenomics refers to the study of genome-wide changes in epigenetic mechanisms including DNA methylation, histone modifications and non-coding RNAs expression. The alterations in normal DNA methylation and histone acetylation/deacetylation patterns lead to deregulated transcription and chromatin organization resulting in altered gene expression profiles that facilitates tumor development and progression. In consequence, novel therapeutic strategies aimed at reversing aberrant epigenetic marks in cancer cells have been developed and used in recent molecular studies and clinical trials. Pharmaco-epigenomics is a research area, which refers to the study of epigenome changes in cancer development and how chemotherapeutic agents can reverse these aberrant epigenetic marks by targeting the epigenetic machinery. Besides, the effects of genome-wide polymorphisms in populations leading to variations in drug response are also study subject of pharmaco-epigenomics and are being studied extensively in cancer. Recent findings showed that drug response could be largely influenced by the presence of aberrant epigenetic marks of the whole genome. This implies that biological pathways and cellular processes are under the impact of epigenome status. However, data about the relationship between drug response and the epigenomic variations is still scarce mainly because the epigenome is highly variable between individuals. The present chapter reviewed the advances on the epigenetics changes mainly DNA methylation and histones modifications on cervical and breast human cancers. A special emphasis in how they could be used as targets for the development and use of novel drugs in cancer therapy is delineated.

Keywords

Epigenomics · DNA methylation · Histone modifications · Non-coding RNAs · Histone acetylation · Epigenetic marks · Cervical cancer · Breast cancer · Epigenetic biomarkers · Epigenetic therapies

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3.1 Introduction

Alterations of DNA methylation and histones acetylation are common epigenetics marks in cancer. DNA and histones modifications are highly regulated in normal tissues and its deregulation has a deep influence in the nucleosomes organization and finally in gene expression, DNA replication and DNA repair. Epigenetics changes accumulate during the early and late stages of carcinogenesis. Currently with the arrival of potent high throughput technologies, the discovery of epigenetics marks at genome-wide level have resulting in an accelerated understanding of the epigenetic regulation of oncogenes and tumor suppressor genes in human cancers. Epigenome changes on DNA and histones directly impacts in the chromatin state, which becomes more or less condensed. It has been well established that DNA hyper-methylation and histones hypo-acetylation are epigenetic marks resulting in a condensed form of chromatin, related to the gene promoter regions that should be inactive during a specific time or in response to a stimuli [1]. In contrast, DNA hypo-methylation and histones hyper-acetylation release the condensed chromatin state resulting in transcriptional machinery recruitment to promoters and gene activation. This implies that genes may become active or inactivated by epigenetic marks representing a potent and flexible cellular mechanism for fine-tuning in time and tissue specific gene expression regulation.

Recent studies have mapped the epigenetic modifications of histone code and CpG methylation affecting key genes and cellular pathways leading to carcinogenesis in diverse types of tumors. Numerous attempts to understand and detect the epigenetic changes that occur in early stages of carcinogenesis and how to revert them are currently ongoing. These research efforts may results in the discovery of novel treatments targeting the epigenome and the improvement of existing cancer therapies.

Pharmaco-genomics also focuses on the identification of genome sequence variants that influence drug effects via alterations in a drug's pharmacokinetics and pharmaco-dynamics in cancer [2]. Genome-wide studies of populations of patients have facilitated the of novel candidate gene related to specific drug response phenotypes. However, translation of pharmacogenomic data into clinical practice have been hampered by the lack of effective alternative therapies for those individuals with "high risk" genotypes, and requires improvements in health care systems and guides to properly utilize genetics to guide drug prescribing. In this chapter, we provide a general description of recent developments in the field of epigenetics and its impact on personalized medicine to manage cervical and breast cancers.

3.2 Epigenetic Regulation in Cancer

3.2.1 Histones Modifications

Histones are small alkaline proteins that package and order large strands of DNA into highly ordered structures called nucleosomes resulting in a compacted chromatin state that forms a barrier for gene transcription. There are four core histones H2A, H2B, H3 and H4, while histones H1/H5 acts as linkers. In the canonical structure, nucleosome is assembled from unmodified histones and a 147 base pairs fragment of DNA form a tight two-turn 'superhelix' around a wedgeshaped compact histone octamer composed of two copies each of the core histones [3]. Nucleosomes vary in the composition of their histones, as the incorporation of variant histones and post-translational modifications of histone amino acid side chains, which provide a special dynamics in the condensation of chromatin. Histones have "tails" at their NH2- and COOH-terminal ends that may be modified in charge through diverse posttranslational modifications. Histones modifications comprise acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation and SUMOylation resulting in a "histone code" that drives chromatin dynamics that is read in order to regulate gene expression [4]. Histone acetylation occurs mainly at lysine residues of the H3 and H4 resulting in the

neutralization of positive charges on histone tails leading to weakness electrostatic interactions between the histones and the negatively charged phosphate backbone of nucleic acids making RNA polymerase and general transcription factors easier to access the promoter region of genes [5]. Therefore, the acetylation of histone lysine is associated with euchromatin and transcriptional activation of gene expression, whereas the deacetylation of residues is associated with heterochromatin and results in transcriptional gene silencing.

Histone acetyl transferases (HATs) are the enzymes responsible for the addition of acetyl groups from lysine residues of core nucleosomal histones. HATs are a diverse set of enzymes that can be divided into two classes based on their subcellular localization. Type A HATs are located in the nucleus and are involved in the acetylation of nucleosomal histones. They contain a conserved bromodomain, which helps them recognize and bind to acetylated lysine residues on histones. Several examples of type A HATs are Gcn5, p300/CBP, and TAFII250 which cooperate with activators to stimulate gene transcription. On the other hand, type B HATs are mainly located in the cytoplasm of cells and they are responsible for acetylating newly synthesized histones prior to their export to nuclei. HATs can be also grouped on the basis of their catalytic domains [6]. For instance, Gcn5 is the founding member of the Gcn5 *N*-acetyltransferases (GNATs), which includes Gcn5, PCAF, Elp3, Hat1, Hpa2 and Nut1.The MYST HATs are named for the founding members of this family: Morf, Ybf2 (Sas3), Sas2 and Tip60. Other HATs include p300/CBP (CREB-binding protein), Taf1 activator and a number of additional nuclear receptor co-activators.

Histone deacetylases (HDACs) are transcriptional co-repressors responsible for the removal of the acetyl groups from lysine of core histones. HDACs have been grouped into four classes including Class I HDACs (HDAC1, 2, 3, and 8) that share high homology with the yeast transcriptional regulator RPD3, class II HDACs which are related to HDA1 (HDAC4, 5, 6, 7, 9, and 10), class III HDACs, also called sirtuins, that exhibits homology with Sir2 (SIRT1, 2, 3, 4, 5, 6, and 7), and class IV HDAC (HDAC11) which is related to class I and II enzymes class I, II and VI HDACs are also referred to as "classical" HDACs [7]. The coordinated activities of HATs and HDACs are responsible for the degree of histones acetylation and in consequence the degree of gene activation and repression of oncogenes and tumor suppressor genes during tumorigenesis [8]. Altered expression and mutations of HDACs genes have been frequently observed during tumor development since they both induce the aberrant transcription activation or repression of key genes regulating the hallmarks of cancer [9] such as cell proliferation, cell cycle, invasion, metastasis, apoptosis, and angiogenesis. For instance, the suppression of histone acetylation in tumor suppressor genes may result from inactivation of HATs activity through gene mutation (e.g. mutations of p300, CBP or pCAF) in hematological and solid tumors, whereas misdirection of HATs activities as a result of chromosomal translocations has been implicated in acute leukemia [10]. These and recent studies have shed light on the mechanisms of action of HATs and HDACs cancer. Interestingly, in some cases the altered expression profiles of the enzymes correlates with clinical data from cancer patients, although the contribution of specific HATs and HDACs isoforms in different cancer types needs to be still established. This will allow for the design and a more selective development of HDAC isoforms-specific inhibitors with a potential impact on the personalized therapeutic treatment of cancer patients.

3.2.2 DNA Methylation

The most widely studied epigenetic modification in human cancers is the DNA methylation. In general, DNA methylation occurs in the CpG dinucleotides located in regions dubbed as CpG islands which are defined as genomic regions >200 bases with a G + C content of at least 50%, and a ratio of expected CpG frequencies of at least 0.6 [11]. These CpG islands represent ~1% of human genome and about 60% of human gene

promoters are associated with CpG islands which are usually unmethylated in normal cells. Increased DNA methylation in gene promoters is commonly associated with gene silencing, whereas hypomethylation is related to gene activation. DNA methylation of CpG islands is catalyzed by the activities of DNA methyl transferases (DNMTs) enzymes including DNMT1, DNMT3a and DNMT3b. DNMT1 is required for maintenance of genome methylation during DNA replication in mitosis of normal cells, whereas DNMT3a and DNMT3b, referred as the novo methyl transferases, are implicated in the generation of DNA methylation patterns during embryogenesis and setting up genomic imprints during germ cell development [11]. These proteins are highly conserved and they contain a regulatory domain the N-terminus that allows recognizing DNA, and the C-terminus which have a catalytic domain responsible for the enzymatic activity [12]. DNMT3L has no catalytic activity, but is an important regulator of DNMT3A in the form of DNMT3L-DNMT3A hetero-tetramers that facilitates the methylation of cytosine residues. An outstanding report showed that DNMT2 is not a true DNA methylase as this enzyme methylates small transfer RNAs [13].

DNA methylation may modulate gene expression by diverse mechanisms.

Methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins. MBD family of proteins binds to methylated cytosines on DNA and greatly modifies transcription rates [14]. MBD proteins may recruit histone modifying and chromatin-remodeling complexes to DNA methylated sites in gene promoters. DNA methylation can also directly inhibit transcription by precluding the recruitment of DNA binding proteins from their target sites. For instance, MeCP2 protein contains transcription repression domain that interacts directly with DNMT1 forming a complex that recruits HDACs influencing both DNA methylation and histone acetylation [15]. In contrast, unmethylated CpG islands generate a relaxed chromatin structure associated to activation of gene transcription by recruiting Cfp1, which associates with histone methyl transferase Setd1, creating domains rich in specific histone trymethylation mark H3K4 (H3K4me3) [16].

Tumor cells frequently exhibit alterations in DNA methylation patterns during the development and progression of carcinogenesis. For instance, increased expression of DNMT1, DNMT3a and DNMT3b resulting in the inactivation of tumor gene suppressors by hypermethylation has been reported in diverse types of cancers [17]. DNMT1 overexpression correlates with aberrant DNA methylation in solid tumors, and frequently is associated with lymph node metastasis and poor prognosis in patients [18, 19]. Similarly, high expression levels of DNMT3A or DNMT3B have been found in a large number of patients, and increased DNMT3A expression is involved in hepatocellular carcinogenesis. Also increased DNMT3B and CTCF are critical in the epigenetic inactivation of *BRCA1* in sporadic breast tumors [20]. These and additional reports suggested that increased levels of DNMTs could represents an opportunity for drugs intervention in cancer.

3.3 Therapies Targeting the Epigenome in Cancer

Epigenetics-based therapies rely in the fact that aberrant modifications in DNA and histones accumulated during tumorigenesis can be reversed through intervention of the enzymes responsible for the aforementioned epigenetics marks (Fig. 3.1). Therefore, HATs and HDACs represent the most promising epigenetic targets for cancer treatment with clinical inhibitors [21]. The contribution of these enzymes activities in tumor biology has been deciphered from concise analyses of their expression profiles in cancer patients and their mechanism of action from cancer cell lines. In particular, two outstanding papers showed that the loss of acetylation and methylation of specific residues in core histones H3 and H4 represented a novel hallmark of cancer that can be significantly associated with clinical parameters of patients [22, 23]. Esteller and coworkers reported for the first time a loss of global of monoacetylation and trimethylation of

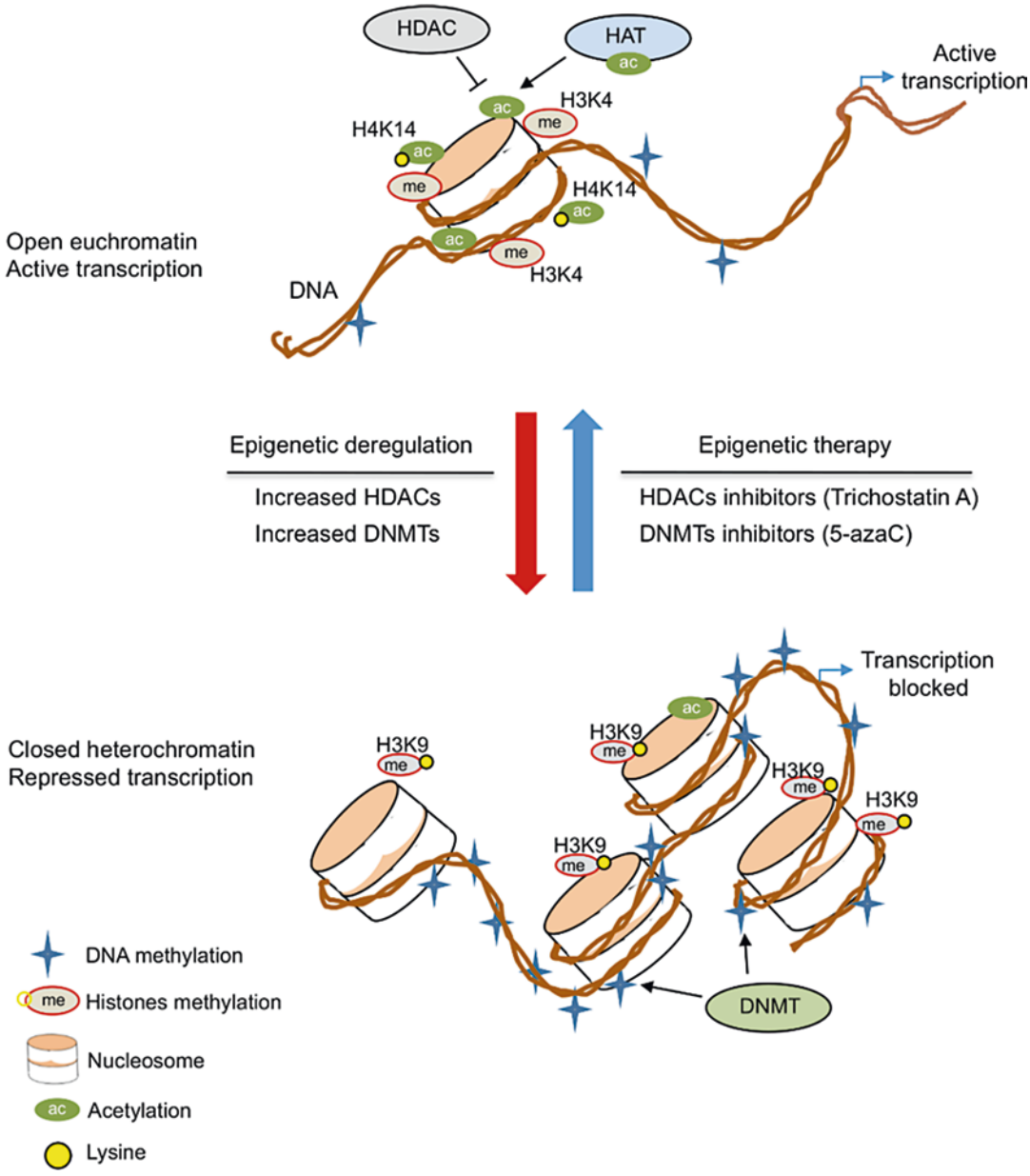

Fig. 3.1 Epigenetic crosstalk between enzymes, nucleosomes and DNA. The model indicates the two different status of chromatin: open and closed. The nucleosome core is wrapped by a nuclear DNA strand of 147 base pairs. Methylation of CpG islands is performed by DNA methyl transferases (DNMTs), whereas the histone tails acetylation is done by histone acetyl transferases (HAT)

In normal cells, low levels of DNA methylation and high levels of acetylation of histones results in an open chromatin an active transcription. In cancer cells, exist a pattern of hypermethylation of DNA and the trymethylation of lysine at the position 9 of histone 3 (H3K9me3) leads to a closed chromatin, which hampers the recruitment of transcriptional machinery to gene promoters. H3K4 methylation and H3K14 acetylation facilitates chromatin decondensation resulting in activation of cancer-related genes. Therapeutic inhibition of DNA methylation to restore normal methylation patterns could be achieved with DNMT inhibitors. Likewise, increased histone desacetylation can be hampered with histone deacetylases (HDACs) inhibitors

histone H4 in repetitive DNA sequences in cancer cells and in the mouse models of multistage skin carcinogenesis [22]. At the same time Selingson [23] and colleagues showed that changes in global levels of H3 and H4 histone acetylation and dimethylation were predictive of clinical outcome in prostate cancer patients. In addition, grouping of samples with similar patterns of modifications identified two disease subtypes with distinct risks of tumour recurrence in patients with low-grade prostate cancer. More recently was found that trymethylation of lysine at the position 9 of histone 3 (H3K9me3) can recruit the protein HP1 (heterochromatin protein 1) leading to a compacted chromatin, which hampers the recruitment of transcriptional machinery to gene promoters. In contrast, H3K4 methylation and H3K14 acetylation facilitates chromatin decondensation resulting in activation of cancer-related genes [24]. These and additional studies have established the functional and clinical relevance of epigenetic modifications in the disease which established the basis for clinical intervention.

Epigenetic enzymes are potential targets of several classes of inhibitors, including inhibitors of DNMT and HDAC, as well as the recently developed inhibitors of histone methyltransferases and HATs. Inhibitors of epigenetic enzymes used in pre-clinical treatments are so-called epidrugs. Several HDAC inhibitors have showed clinical efficacy in therapies of certain types of tumors. HDAC inhibitors are a group of small chemical molecules that inhibit the histone deacetylase activity reactivating gene expression [25]. Trichostatin A (TSA) is an organic compound that serves as an antifungal antibiotic and also as a epigenetic agent that selectively inhibits the class I and II mammalian HDAC families of enzymes, but not class III HDACs (i.e. sirtuina). TSA alone or in combination with chemo- or radiotherapy represent a therapeutic option for several types of cancer because of its ability for inducing apoptosis, cell cycle arrest, cell differentiation and autophagy. Other mechanisms of TSA actions include the ability to induce cell differentiation, thus inducing to mature some of the de-differentiated cells found in tumors. Other potent HDAC inhibitor is the suberoylanilide hydroxamic acid (SAHA or Vorinostat). SAHA act as a chelator for zinc ions in the active site of histone deacetylases by binding to the pocket of the catalytic site. The hydroxamic acid moiety of Vorinostat binds to a zinc atom, allowing the rest of the molecule to lie along the surface of the HDLP protein [26]. SAHA inhibits class I, II and IV of HDAC resulting in the accumulation of acetylated histones and acetylated proteins, including the core nucleosomal histones, proteins such as BCL6, p53, Hsp90 and other transcription factors crucial for the expression of genes associated to cell differentiation [27]. The antiproliferative effects of Vorinostat are due to the accumulation of acetylated proteins inducing growth arrest, differentiation or apoptosis in diverse tumor cells. Thus, the HDAC inhibitor Vorinostat blocks cancer cell proliferation both of cultured cells, and inhibits tumour growth in a variety of animal models with little or no toxicity to normal cells and has undergone evaluation in several Phase I and II clinical trials [3]. Early studies indicate that SAHA induced histones acetylation and activation of cyclin dependent kinase inhibitor p21 (WAF1) in bladder carcinoma cells [28] Vorinostat has been shown to inhibit the proliferation of a wide variety of transformed cells *in vitro*, including lymphoma, myeloma, leukemia, and non-small cell lung carcinoma [29]. Bellow, we summarize our current knowledge of the efforts in epigenetic therapies targeting the epigenetic machinery in cervical and breast cancer.

3.4 Epigenomics in Cervical Cancer

Cervical cancer is the fourth most common malignancy diagnosed in women mainly living in developing countries and is considered as a global health concern. Each year more than 528,000 new cases are diagnosed with an estimated annual

death rate of 266,000 deaths worldwide. In recent years cervical cancer incidence has decreased, however it has been predicted that there will be a 42% increase in incidence by the year 2020 [30]. Risk factors for cervical cancer comprises the socioeconomic status, smoking, nutrition and diet, early age at first sexual intercourse, multiparity, and use of oral contraceptives [31]. The majority of new cases of cervical cancer result from infection with the human papillomavirus (HPV) associated to other opportunistic pathogens. Development and progression of cervical cancer is a multistep mechanism represented by diverse types of lesion: (i) low-grade squamous intraepithelial lesion, (ii) high-grade squamous intraepithelial lesion, (iii) carcinoma in situ and (iv) invasive disease. Carcinogenesis involves the accumulation of genetic and epigenetic alterations in key regulatory genes and pathways controlling cell division, cell growth and apoptosis [32].

Epigenetic alterations due to aberrant DNA methylation patterns and histone modifications have been recently studied in cervical cancer and accepted as risk factors for disease progression. Early studies demonstrated that global DNA hypomethylation is an epigenetic event in cervical carcinogenesis and that the degree of DNA hypomethylation increased with the grade of cervical neoplasia suggesting that global methylation may serve as a biochemical marker of cervical cancer progression [33]. These findings were confirmed in a study showing a progressive demethylation in dysplastic and cancer cells compared to normal tissues [34]. Specific genes have been detected with aberrant DNA methylation patterns. For instance, the hypermethylation of the fragile histidine triad (FHIT) gene, a negative regulator of cell growth, was found reduced in cervical neoplasia in cervical cancer [35]. More recent studies searched for potential marker genes hypermethylated exclusively in neoplastic or carcinoma cells and unmethylated. For instance, Kitkumthorn and coworkers reported CCNA1 promoter hypermethylation in high-grade squamous intraepithelial lesions SIL (36.6%), microinvasive cancer (60%) and invasive squamous cell carcinomas SCC (93.3%) but not in normal epithelial cells [36]. These results suggest the potential clinical use of CCNA1 methylation as a molecular marker for the early diagnosis of invasive cancer. Others genes have found with a hypermethylation including CDH1, DAPK, RARB, and HIC1. Interestingly, promoter methylation was higher in advanced stages of disease, and methylation of RARB and BRCA1 predicted worse prognosis [37].

On the other hand, DAPK pro-apoptotic gene a positive mediator of γ-interferon induced programmed cell death, was found consistently hypermethylated in cervical cancer; therefore it may serve as a marker for detection of this malignancy. RASSF1A hypermethylation leading to low transcription was reported in HPVnegative but not in HPV positive cervical cancer cell lines and primary cervical tumors [38]. Genome-wide sequencing of cervical tumors confirmed a small proportion of mutations in classical tumor suppressor genes such as TP53 and RB1 that are inactivated by high-risk HPV E6 and E7 proteins [39, 40]. Interestingly, frequent mutations in genes such as ARIDIA and EP300, which are associated with chromatin modulation, were found in cervical tumors. These data highlights the role of DNA modifications in cervical cancer biology. Several studies also showed the importance of chromatin remodeling in cervical cancer prognosis. For instance, in a panel of 250 patients with cervical cancer, the acetylation of histone H3 acetyl K9, associated to active regions at enhancers and promoters, was correlated with low grading, low FIGO status, negative N-status and low T-status in cervical cancer. In addition, this epigenetic mark showed a higher expression in adenocarcinoma than in squamous cell carcinoma [41]. Moreover, the status of histone H3 acetyl K9 was also found to be an independent marker of overall survival. Histone H3 tri methyl K4, which is also associated to active transcription, was correlated with poor prognosis and it was found to be an independent marker of relapse-free survival [41].

Additional reports have showed the potential of epigenetic focused therapies in cervical therapies. Trichostatin A (TSA) HDAC inhibitor induced apoptosis of cervical cancer cells by

decreasing DNA-methyltransferase 3B [42]. Other study showed that TSA activates the expression of p21WAF1/CIP1 through release of the repression by c-myc from the p21WAF1/CIP1 promoter in Hela cancer cells indicating that it's a promising drug in cervical cancer therapy [43]. Additional epigenetic alterations in a number of genes related to cellular signaling and metabolism, migration, development and differentiation, cell cycle regulation, apoptosis, and DNA repair have been reviewed by Szalmás A and Kónya J [44].

On the other hand, many of the natural occurring dietary compounds such as polyphenols (e.g., epicatechins, stilbenes, benzoquinones, acetophenones, flavonoids, phenolic acids, proanthocyanidins and anthocyanins) have shown to influence DNA methylation by targeting the epigenetic machinery. Traditionally, these phytochemicals have been used in the treatment of various human diseases, because their antioxidant or anti-inflammatory properties. Bioactive phytochemicals such as flavonoids present in fruits, vegetables, and beverages modulate DNA methylation and are therefore promising natural agents for cancer prevention [45]. For instance, it was reported that dietary patterns influences LINE1 methylation and risk of developing cervical intraepithelial neoplasia [46]. In addition, genistein (4′,5,7-trihydroxyisoflavone) the major isoflavone present in soy bean, significantly reduced the expression and enzymatic activity of both DNMTs and HDACs. Genistein was able to restore the expression of important tumour suppressor genes such as MGMT, RARβ, p21, E-cadherin, and DAPK1 [47]. Likewise, timedependent exposure to sulforaphane decreases the expression of DNMT3B and HDAC1 and significantly reduces the enzymatic activity of DNMTs and HDACs in HeLa cervical cancer cells, which was associated with the increased expression of DNMT3B, HDAC1, RARβ, CDH1, DAPK1, and GSTP1 genes [48]. Other study showed that the treatment of a squamous cervical cancer cell line SiHa with both curcumin and genistein resulted in demethylation of promoter and reactivation of the RARβ2 gene [49]. These data suggested that polyphenols could be an important approach in the development of potential epigenetic-based therapies in cervical cancer.

3.5 Epigenomics in Breast Cancer

Breast cancer is the neoplasia with the highest incidence and mortality affecting women worldwide [50]. Breast carcinomas represent a heterogeneous group of tumors that are diverse in behavior, outcome, and response to therapy [51]. Extensive studies on epigenome changes in breast cancer have been undertaken. Aberrant genetic and epigenetic alterations have been studied in order to understand the role of epigenetics in cancer and to develop novel epigenetic therapies. In particular, since epigenetic alterations in DNA methylation and histones modifications are reversible, they might represent potential targets for breast cancer therapy. Therefore, identification of epigenetic modifications regulating key genes involved in the hallmarks of breast cancer is of critical importance. Remarkably, breast tumors present a global DNA hypomethylation in up to 50% of cases. Early studies showed that DNA hypomethylation is increased in breast carcinomas and it could be an independent prognostic parameter in tumor progression [52]. Hypomethylation was associated with the disease stage, tumor size, and histologic grade playing a potentially important role in tumor development [53]. DNA hypomethylation in breast tumors may affects repetitive DNA sequences and pericentromeric satellite DNA, which are normally heavily methylated in non-malignant cells. For instance, long interspersed nuclear elements (LINEs) retrotransposons are hypomethyated in breast cancer inducing transcriptional reactivation [54]. Also, they can integrate into other sites of genome, leading to insertional mutagenesis and genomic instability [55]. Other hypomethylated genes include the gene encoding the plasminogen activator uPA (PLAU), the melanoma associated cancer/testis antigens MAGE, the breast cancer specific protein 1/synuclein-γ (SNCG),

and the multidrug resistance 1 (MDR1) genes which are methylated and silenced in adult tissues, but hypomethylated and expressed in breast cancer cells [56–59].

In addition to global DNA hypomethylation, which is responsible in part for altered gene expression patterns, hypermethylation of promoter regions of tumor suppressor genes have been found in breast tumors [60]. Locke and coworkers propose an interesting model of progression of the breast cell epigenome from progenitor to malignancy. They claim that during progression from normal progenitor cell to differentiated epithelium, cells exhibit an increasing and differential level of DNA methylation. During early stages of tumorigenesis, much of genomic methylation is lost, with the exception of a small subset of genomic loci that exhibit DNA hypermethylation. Remarkably, the methylome of malignant lesions is similar between early lesions like ductal carcinoma *in situ* and advanced invasive ductal carcinoma [60]. Besides, several hyper methylated genes have been well described in breast cancer. For instance, it was reported that the adenomatous polyposis coli (APC) gene, a tumor suppressor gene associated with both familial and sporadic cancer, was methylated in 34 of 77 breast cancer tumors and cell lines (44%). In most cell lines tested, a concordance between promoter methylation and gene silencing was found. In addition, demethylation with 5-aza-2'deoxycytidine restored APC expression in all methylated cell lines tested [61]. In other study it was found that hypermethylation of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene occurred in breast cancer cell lines MCF7, MDAMB157, MDAMB231, T47D, and ZR75-1. Also in 28 of 45 (62%) primary mammary carcinomas, the promoter of RASSF1A was highly methylated. In agreement with hypermethylation status, the expression of RASSF1A was lower in tumors compared with normal tissues. Other genes such as p16ink4a (CDKN2A), CCND2, NES1 (KLK10), RARB, and HIN-1 (SCGD3A1) also exhibited hypermethylation in breast cancer.

3.6 Epigenetic Therapies Targeting DNMTs and HDACs in Breast Cancer

DNA methyl transferases inhibitors are classified in two broad categories: i) nucleoside analogues and ii) non-nucleoside analogues. 5-Azacytidine (5-azaC, Vidasa), and 2′- deoxy analog (5-azadC, decitabine) are well-known examples of nucleoside analogues. They are incorporated into the DNA during replication and form covalent bonds with DNMTs blocking their functions in this way [62]. These small compounds are first converted into the active triphosphate form by different kinases and then incorporated into DNA and/or RNA. 5-Azacytidine is considered as a global inhibitor of DNA methyl transferases that is primarily converted to 5azaC monophosphate (5-azaC-MP) by uridine-cytidine kinase and is incorporated into DNA and then to diphosphate (5-azaCDP) and triphosphate (5-azaC-TP) by pyrimidine monophosphate and diphosphate kinases, respectively [62]. When 5azaC-TP is incorporated into RNA molecules the drug may interferes with protein translation. An outstanding study provides insights in the mechanisms underlying the effect of DNA methyl transferases inhibitors at gene transcription level. Tao and coworkers showed that treatment of cancer cells with 5-azacytidine leads to a release of RNA polymerase II stalling at genes with DNA-hypermethylated regions suggesting that this is the fundamental mechanism underlying tumor suppressor genes reactivation in breast cancer cells [63].

Zebularine (1-(β-D-ribofuranosyl)-2(1H)-pyrimidinone) is another cytidine analog described as a potent inhibitor of both cytidine deaminase and DNA methylation [64]. Zebularine also is incorporated into the DNA of tumor cells, which is facilitated by the overexpression of pyrimidine kinases in cancer. Zebularine is an orally active inhibitor with minimal cytotoxicity both in vivo and in vitro, and induce selective depletion of DNMT1 over DNMT3A and DMT3B in cancer cells. Its mechanism of action is similar to the one

for aza-analogs and involves the formation of a covalent adduct with DNMTs via thiol addition (Cys81) at the C6 position of the pyrimidinone ring, followed by proteasomal pathway-mediated degradation of the protein [64]. In a genetically engineered MMTV-PyMT transgenic mouse model of breast cancer, zebularine (5 mg/mL) induced a significant delay in the growth of mammary tumors. After 48 days of zebularine treatment, the tumors were predominantly necrotic compared with untreated animals. In addition, a high apoptotic index was observed as early as 13 days following treatment. Also a depletion of DNMT1 and partial depletion of DNMT3b was found after zebularine treatment [65].

3.7 Conclusions

Now become clear how aberrant epigenetic alterations can be used as predictive markers for the outcome of conventional chemotherapies or as targets of novel therapies in cancer. Discovery of novel drugs that target proteins from the epigenetic machineries provide better therapeutic opportunities, and utilization of such inhibitors for DNA methylation, histone modifications, and expression of non-coding RNAs for several cancer types is underway. These epigenetic therapies or biomarkers might change the daily clinical practice for beneficial for ontological patients.

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4 Design and Implementing Pharmacogenomics Study in Cancer

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Abstract

The advances in technology has shifted healthcare from a "one size fits all" model to focus on personalized therapy. Understanding the relationship of genome variations and its effect on drug response has led to individualized drug selection, maximizing drug efficacy and improving toxicity profile. The developments in pharmacogenomics has led to the discovery of predictive and prognostic biomarkers, and has transformed cancer research leading to the creation of pharmacogenomics databases. While challenges associated with the implementation of pharmacogenomics based medicine exist, integrating data amongst collaborative networks will be crucial for researchers to identify all the functional elements of the human genome sequence. Future advances in the area of pharmacogenomics research will eventually lead to the identification of the right therapeutic drug for the right patient.

Keywords

Pharmacogenetics · Health-care system · Personalized medicine · Computer-based systems · Point-of-care method · Germline mutations, somatic mutations · Whole exome sequencing · Randomized trials · Molecular tumor board

4.1 Introduction

Recently, healthcare has been shifting from a "one size fits all" model to a precise personalized regimen [1]. Precision medicine may broadly be defined as the tailoring of medical treatment to the individual characteristics of patients [2]. Pharmacogenomics (PGx) informed prescribing is one of the first applications of genomics in medicine [3].

The main priority of PGx is to optimize treatment by understanding the underlying biological mechanisms and utilizing genomic contributions to treatment response to predict and individualize therapy and improve treatment outcomes [4–6]. Pharmacogenomic approaches attempt to refine the aim of personalized medicine by utilizing an individual's germline and somatic DNA signatures to guide treatment [3, 7]. Pharmacogenetics has been used to refer to the effect of genetic variation in one gene on drug metabolism and

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disposition. PGx, on the other hand, generally refers to how the entire genome can influence the response to drugs [7, 8].

PGx is a key component of precision medicine that can be used to select an optimal dosage for patients, more precisely identify individuals who will respond to a treatment and avoid serious drug-related toxicities [2]. The use of PGx could decrease the overall cost of healthcare owing to the reduction in adverse drug reactions (ADR), number of failed trials, time taken to obtain drug approval, length of medication, number of medications taken, and the effects of disease on the body [1]. Integration of these genetic data into the clinical decision-making process has the potential to significantly advance the practice of precision medicine and, in the case of PGx, ultimately affect every patient [6].

Cancer PGx have contributed a number of important discoveries to current cancer treatment, changing the paradigm of treatment decisions. Both somatic and germline mutations are utilized to better understand the underlying biology of cancer growth and treatment response [4].

PGx testing is currently available for a wide range of health problems including cardiovascular disease, cancer, diabetes, autoimmune disorders, mental health disorders and infectious diseases. Tangible benefits to patients are currently being observed. Since many cancers are not viewed as a single disease, but rather as a group of several subtypes, each with a distinct molecular signature, identifying the genomes of the malignancy and of the patient can aid in effective and safe treatment [9].

More recently, numerous targeted therapies, which benefit smaller, molecularly defined subsets of patients have been approved and are now being used clinically. These targeted drugs often require pharmacogenomic tests to identify the appropriate patient population for whom the drug is indicated. Although the current landscape of molecularly targeted therapies is largely limited to oncology, targeted therapies in non-oncology fields are rapidly expanding [2]. In cancer, both inherited and somatically acquired variants can influence a patient's response to treatments. Once a pharmacogenomic relationship has been discovered and validated, there are many obstacles to translating it into clinical practice [8].

Although approximately 15% of medications approved by these agencies (FDA and EMA) have PGx information on their drug labels, only a handful have made it to clinical consideration. In terms of therapeutic area, oncology has the most biomarker information in FDA drug labels, followed by psychiatry and infectious diseases [7].

The inclusion of pharmacogenomic information in labeling is useful to optimize dosing for drugs that exhibit variable pharmacokinetics secondary to polymorphic drug metabolism, activation, or transport, and to optimize patient selection for drugs that may have poor efficacy or poor tolerability in certain genetic subgroups. Since the pharmacokinetic properties of biologic drugs are not impacted by known pharmacogenomic factors, the pharmacogenomic information in biologic drug labeling generally describes the impact of PGx on the drug's safety or efficacy profile. Of the currently marketed biologic drugs with pharmacogenomic information in the product labeling, the majority of pharmacogenomic information describes the impact of a molecular alteration in the drug target or biological pathway on efficacy [2]. However, not all biomarker information included in drug labeling leads to a required or recommended action [7]. The Table 4.1 shows the pharmacogenomic biomarkers in FDA drug labeling in Oncology.

Traditionally, clinical PGx testing has been performed reactively, when a patient requires a medication for which PGx data can guide prescribing practices. Most often, this has been accomplished through testing of a single gene. A drawback of this approach is that some therapies need to be initiated as soon as possible, and testing requires time [10].

To be practical for use in prescribing decisions, pharmacogenetic test results should ideally be available preemptively, it means that the test result is available in the medical record as a pre-prescription patient characteristic: the test result has not been ordered because a specific pharmacogenetically high-risk drug is being contemplated but rather is available because a broad screening of multiple genes has already

Table 4.1 Pharmacogenomic Biomarkers in Drug Labeling in Oncology

Drug	Biomarker	Labeling Text
Abemaciclib VERZENIO LVD: 09/28/17	ESR, PGR (Hormone Receptor) ERBB2 (HER2)	**Indications and usage:** Is indicated: • in combination with fulvestrant for the treatment of women with HR-positive, HER2-negative advanced or metastatic breast cancer with disease progression following endocrine therapy. • as monotherapy for the treatment of adult patients with HR-positive, HER2-negative advanced or metastatic breast cancer with disease progression following endocrine therapy and prior chemotherapy in the metastatic setting [37].
Ado-Trastuzumab Emtansine KADCYLA LVD: 07/25/16	ERBB2 (HER2)	**Indications and usage:** As a single agent, is indicated for the treatment of patients with HER2-positive, metastatic breast cancer who previously received trastuzumab and a taxane, separately or in combination. Patients should have either: •received prior therapy for metastatic disease, or • developed disease recurrence during or within 6 months of completing adjuvant therapy. **Warnings and precautions/HER2 testing:** Detection of HER2 protein overexpression or gene amplification is necessary for selection of patients appropriate for KADCYLA therapy because these are the only patients studied for whom benefit has been. In the randomized study, patients with breast cancer were required to have evidence of HER2 overexpression defined as 3+ IHC by Dako Herceptest™ or evidence of overexpression defined as FISH amplification ratio ≥ 2.0 by Dako HER2 FISH PharmDx™ test kit. Only limited data were available for patients whose breast cancer was positive by FISH and 0 or 1+ by IHC. Assessment of HER2 status should be performed by laboratories with demonstrated proficiency in the specific technology being utilized. Improper assay performance, including use of suboptimally fixed tissue, failure to utilize specified reagents, deviation from specific assay instructions, and failure to include appropriate controls for assay validation, can lead to unreliable results [38].
Afatinib GILOTRIF LVD: 01/12/18	EGFR mutation-positive	**Indications and usage:**• First-line treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors have non-resistant epidermal growth factor receptor (EGFR) mutations as detected by an FDA-approved test. **Limitation of use:** Safety and efficacy of GILOTRIF were not established in patients whose tumors have resistant EGFR mutations • treatment of patients with metastatic, squamous NSCLC progressing after platinum-based chemotherapy [39].
Alectinib ALECENSA LVD: 06/05/18	ALK	**Indications and usage:** Is indicated for the treatment of patients with anaplastic lymphoma kinase (ALK)-positive metastatic non-small cell lung cancer (NSCLC) as detected by an FDA-approved test. **Patient selection:** For the treatment of metastatic NSCLC with ALECENSA based on the presence of ALK positivity in tumor specimens [40].
Anastrozole ARIMIDEX LVD: 05/02/14	ESR, PGR (hormone receptor)	**Indications and usage:** Is indicated for adjuvant treatment of postmenopausal women with HR-positive early breast cancer, for the first-line treatment of postmenopausal women with hormone receptor-positive or hormone receptor unknown locally advanced or metastatic breast cancer; and for the treatment of advanced breast cancer in postmenopausal women with disease progression following tamoxifen therapy. Patients with ER negative disease and patients who did not respond to previous tamoxifen therapy rarely responded to ARIMIDEX.
Atezolizumab TECENTRIQ LVD: 06/19/18	CD274 (PD-L1)	**Indications and usage:** Is a programmed death-ligand 1 (PD-L1) blocking antibody indicated for the treatment of patients with: • locally advanced or metastatic urothelial carcinoma who: • are not eligible for cisplatin-containing chemotherapy, and whose tumors express PD-L1 (PD-L1 stained tumor-infiltrating immune cells [IC] covering ≥5% of the tumor area), or • are not eligible for any platinum-containing chemotherapy regardless of level of tumor PD-L1 expression, or • have disease progression during or following any platinum-containing chemotherapy, or within 12 months of neoadjuvant or adjuvant chemotherapy. This indication is approved under accelerated approval based on tumor response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials. • metastatic NSCLC who have disease progression during or following platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA approved therapy for these aberrations prior to receiving TECENTRIQ [41].

(continued)

Table 4.1 (continued)

Drug	Biomarker	Labeling Text
Avelumab **BAVENCIO** LVD: 10/12/17	CD274 (PD-L1)	**Indications and usage:** Is a programmed death ligand-1 (PD-L1) blocking antibody indicated for the treatment of • Adults and pediatric patients 12 years and older with metastatic Merkel cell carcinoma (MCC). This indication is approved under accelerated approval based on tumor response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials. • Patients with locally advanced or metastatic urothelial carcinoma (UC) who: ♣ have disease progression during or following platinum-containing chemotherapy (1.2) ♣ have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. This indication is approved under accelerated approval based on tumor response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials [42].
Brigatinib **ALUNBRIG** LVD: 04/28/17	ALK	**Indications and usage:** Is indicated for the treatment of patients with anaplastic lymphoma kinase (ALK)-positive metastatic NSCLC who have progressed on or are intolerant to crizotinib. This indication is approved under accelerated approval based on tumor response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial [43].
Cabozantinib **COMETRIQ** LVD: 01/12/18	RET	**Indications and usage:** Is indicated for the treatment of patients with progressive, metastatic medullary thyroid cancer (MTC) [44].
Capecitabine **XELODA** LVD: 12/14/16	DPYD	**Warnings and precautions:** Dihydropyrimidine dehydrogenase deficiency based on postmarketing reports, patients with certain homozygous or certain compound heterozygous mutations in the DPD gene that result in complete or near complete absence of DPD activity are at increased risk for acute early-onset of toxicity and severe, life-threatening, or fatal adverse reactions caused by XELODA (e.g., mucositis, diarrhea, neutropenia, and neurotoxicity). Patients with partial DPD activity may also have increased risk of severe, life-threatening, or fatal adverse reactions caused by XELODA. Withhold or permanently discontinue XELODA based on clinical assessment of the onset, duration and severity of the observed toxicities in patients with evidence of acute early-onset or unusually severe toxicity, which may indicate near complete or total absence of DPD activity. No XELODA dose has been proven safe for patients with complete absence of DPD activity. There is insufficient data to recommend a specific dose in patients with partial DPD activity as measured by any specific test. **Patient counseling information:** Dihydropyrimidine dehydrogenase deficiency patients should be advised to notify their healthcare provider if they have a known DPD deficiency. Advise patients if they have complete or near complete absence of DPD activity they are at an increased risk of acute early onset of toxicity and severe, life-threatening, or fatal adverse reactions caused by XELODA (e.g., mucositis, diarrhea, neutropenia, and neurotoxicity) [45].
Ceritinib **ZYKADIA** LVD:12/21/17	ALK	**Indications and usage:** Is a kinase inhibitor indicated for the treatment of patients with metastatic NSCLC whose tumors are anaplastic lymphoma kinase (ALK)-positive as detected by an FDA-approved test. **Patient selection:** For treatment of metastatic NSCLC with ZYKADIA based on the presence of ALK positivity in tumor specimens [46].

Cetuximab **ERBITUX** LVD: 06/05/18	EGFR RAS	**Indications and usage**: Is an epidermal growth factor receptor (EGFR) antagonist indicated for treatment of: Head and neck Cancer • locally or regionally advanced squamous cell carcinoma of the head and neck in combination with radiation therapy. • recurrent locoregional disease or metastatic squamous cell carcinoma of the head and neck in combination with platinum-based therapy with fluorouracil. • recurrent or metastatic squamous cell carcinoma of the head and neck progressing after platinum-based therapy. ∗Because expression of EGFR has been detected in nearly all SCCHN tumor specimens, patients enrolled in the head and neck cancer clinical studies were not required to have immunohistochemical evidence of EGFR tumor expression prior to study entry. Colorectal Cancer K-Ras wild-type, EGFR-expressing, metastatic colorectal cancer as determined by an FDA-approved test • in combination with FOLFIRI for first-line treatment, • in combination with irinotecan in patients who are refractory to irinotecan-based chemotherapy, • as a single agent in patients who have failed oxaliplatin- and irinotecan-based chemotherapy or who are intolerant to irinotecan. Determine EGFR-expression status using FDA-approved tests prior to initiating treatment. Also confirm the absence of a Ras mutation prior to initiation of treatment with ERBITUX. Information on FDA-approved tests for the detection of K-Ras mutations in patients with metastatic CRC. **Limitations of use:** ERBITUX is not indicated for treatment of Ras mutant colorectal cancer or when the results of the Ras mutation tests are unknown [47]. **Increased tumor progression, increased mortality, or lack of benefit in patients with Ras-mutant mCRC** ERBITUX is not indicated for the treatment of patients with CRC that harbor somatic mutations in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either K-Ras or N-Ras and hereafter is referred to as “Ras” or when the Ras status is unknown.
Cisplatin LVD: 01/19/17	TPMT	**Adverse reactions:** Ototoxicity (…) genetic factors (e.g., variants in the thiopurine S-methyltransferase [TPMT] gene) may contribute to cisplatin-induced ototoxicity; although this association has not been consistent across populations and study design [48].
Cobimetinib **COTELLIC** LVD: 01/26/18	BRAF	**Indications and usage:** Is a kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with a BRAF V600E or V600K mutation, in combination with vemurafenib. **Dosage and administration** • confirm the presence of BRAF V600E or V600K mutation in tumor specimens prior to initiation of COTELLIC [49].
Crizotinib **XALKORI** LVD: 02/07/18	ALK ROS1	**Indications and usage:** Is an inhibitor of receptor tyrosine kinases including ALK, hepatocyte growth factor receptor (HGFR, c-met), ROS1 (c-ROS), and Recepteur d’Origine Nantais (RON) Indicated for the treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors are anaplastic lymphoma kinase (ALK) or ROS1-positive as detected by an FDA-approved test. **Patient selection:** For the treatment of metastatic NSCLC with XALKORI based on the presence of ALK or ROS1 positivity in tumor specimens with FDA-approved tests [50]. ∗The ROS1 status of NSCLC tissue samples was determined by laboratory-developed break-apart FISH (96%) or RT-PCR (4%) clinical trial assays. For assessment by FISH, ROS1 positivity required that ≥15% of a minimum of 50 evaluated nuclei contained a ROS1 gene rearrangement.

(continued)

Table 4.1 (continued)

Drug	Biomarker	Labeling Text
Dabrafenib **TAFINLAR** LVD: 05/04/18	BRAF G6PD RAS	**Indications and usage:** Is a kinase inhibitor indicated as a single agent for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E mutation as detected by an FDA-approved test. TAFINLAR is indicated, in combination with trametinib, for: • the treatment of patients with unresectable or metastatic melanoma with BRAF V600E or V600K mutations as detected by an FDA-approved test. • the adjuvant treatment of patients with melanoma with BRAF V600E or V600K mutations, as detected by an FDA-approved test, and involvement of lymph node(s), following complete resection. • the treatment of patients with metastatic non-small cell lung cancer (NSCLC) with BRAF V600E mutation as detected by an FDA-approved test. • the treatment of patients with locally advanced or metastatic anaplastic thyroid cancer (ATC) with BRAF V600E mutation and with no satisfactory locoregional treatment options. **Limitations of use:** TAFINLAR is not indicated for treatment of patients with wild-type BRAF melanoma, wild-type BRAF NSCLC, or wild-type BRAF ATC. **Patient selection:** Confirm the presence of BRAF V600E mutation in tumor specimens prior to initiation of treatment. **Warnings and precautions:** Glucose-6-phosphate dehydrogenase deficiency TAFINLAR, which contains a sulfonamide moiety, confers a potential risk of hemolytic anemia in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Monitor patients with G6PD deficiency for signs of hemolytic anemia while taking TAFINLAR, TAFINLAR may promote the growth and development of malignancies with activation of RAS through mutation or other mechanisms. **Patient counseling information:** Inform patients of the following confirmation of BRAF V600 mutation. TAFINLAR may cause hemolytic anemia in patients with G6PD deficiency [51].
Durvalumab **IMFINZI** LVD:02/16/18	CD274 (PD-L1)	**Indications and usage:** Is a programmed death-ligand 1 (PD-L1) blocking antibody indicated for the treatment of patients with: • locally advanced or metastatic urothelial carcinoma who: Have disease progression during or following platinum-containing chemotherapy, have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. This indication is approved under accelerated approval based on tumor response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials. • Unresectable, stage III NSCLC whose disease has not progressed following concurrent platinum-based chemotherapy and radiation therapy [52].
Erlotinib **TARCEVA** LVD:10/18/16	EGFR	**Indications and usage:** Is a kinase inhibitor indicated for: • the treatment of patients with metastatic NSCLC) whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test receiving first-line, maintenance, or second or greater line treatment after progression following at least one prior chemotherapy regimen. • first-line treatment of patients with locally advanced, unresectable or metastatic pancreatic cancer, in combination with gemcitabine. Limitations of use: • safety and efficacy of TARCEVA have not been established in patients with NSCLC whose tumors have other EGFR mutations. • TARCEVA is not recommended for use in combination with platinum-based chemotherapy. **Selection of patients:** Based on the presence of EGFR exon 19 deletions or exon 21 (L858R) substitution mutations in tumor or plasma specimens [53].

Everolimus AFINITOR LVD: 04/10/18	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** Is a kinase inhibitor indicated for the treatment of: • postmenopausal women with advanced hormone receptor-positive, HER2 negative breast cancer in combination with exemestane after failure of treatment with letrozole or anastrozole. • adults with progressive neuroendocrine tumors of pancreatic origin (PNET) and adults with progressive, well-differentiated, non-functional neuroendocrine tumors (NET) of gastrointestinal (GI) or lung origin that are unresectable, locally advanced or metastatic. **Limitation of use:** AFINITOR is not indicated for the treatment of patients with functional carcinoid tumors. • adults with advanced renal cell carcinoma (RCC) after failure of treatment with sunitinib or sorafenib. Everolimus is an inhibitor of mammalian target of rapamycin (mTOR), a serine-threonine kinase, downstream of the PI3K/AKT pathway. Constitutive activation of the PI3K/Akt/mTOR pathway can contribute to endocrine resistance in breast cancer [54].
Fluorouracil LVD: 07/29/16	DPYD	**Warnings and precautions:** Increased risk of serious or fatal adverse reactions in patients with low or absent Dipyrimidine dehydrogenase (DPD). Activity based on postmarketing reports, patients with certain homozygous or certain compound heterozygous mutations in the DPD gene that result in complete or near complete absence of DPD activity are at increased risk for acute early-onset of toxicity and severe, life-threatening, or fatal adverse reactions caused by fluorouracil (e.g., mucositis, diarrhea, neutropenia, and neurotoxicity). Patients with partial DPD activity may also have increased risk of severe, life-threatening, or fatal adverse reactions caused by fluorouracil. Withhold or permanently discontinue fluorouracil based on clinical assessment of the onset, duration and severity of the observed toxicities in patients with evidence of acute early-onset or unusually severe toxicity, which may indicate near complete or total absence of DPD activity. No fluorouracil dose has been proven safe for patients with complete absence of DPD activity. There is insufficient data to recommend a specific dose in patients with partial DPD activity as measured by any specific test. **Patient counseling information** • patients to notify their healthcare provider if they have a known DPD deficiency. Advise patients if they have complete or near complete absence of DPD activity, they are at an increased risk of severe and life-threatening mucositis, diarrhea, neutropenia and neurotoxicity [55].
Fulvestrant FASLODEX LVD:11/14/17	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** Is an estrogen receptor antagonist indicated for the: • treatment of HR-positive, HER2-negative advanced breast cancer in postmenopausal women not previously treated with endocrine therapy. • treatment of HR-positive advanced breast cancer in postmenopausal women with disease progression following endocrine therapy. • treatment of HR-positive, HER2-negative advanced or metastatic breast cancer in combination with palbociclib or abemaciclib in women with disease progression after endocrine therapy [56].
Gefitinib IRESSA LVD: 07/13/15	EGFR	**Indications and usage:** IRESSA is indicated for the first-line treatment of patients with metastatic NSCLC whose tumors have EGFR exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test. **Limitation of use:** Safety and efficacy of IRESSA have not been established in patients with metastatic NSCLC whose tumors have EGFR mutations other than exon 19 deletions or exon 21 (L858R) substitution mutations. **Patient selection:** Based on the presence of EGFR exon 19 deletion or exon 21 (L858R) substitution mutations in their tumor [57].
Imatinib mesylate GLEEVEC LVD:09/29/17	Kit (CD117) positive PDGFRB	**Indications and usage:** Is an inhibitor of the receptor tyrosine kinases for platelet-derived growth factor (PDGF) and stem cell factor (SCF), c-kit, and inhibits PDGF- and SCF-mediated cellular events. Is indicated in patients with Kit (CD117) positive unresectable and/or metastatic malignant gastrointestinal stromal tumors (GIST) • adjuvant treatment of adult patients following resection of Kit (CD117) positive GIST [58]. **Clinical studies:** Dermatofibrosarcoma protuberans (DFSP) is a cutaneous soft tissue sarcoma, characterized by a translocation of chromosomes 17 and 22 that results in the fusion of the collagen type 1 alpha 1 gene and the PDGF B gene. An open-label, multicenter, phase 2 study was conducted testing Gleevec in a diverse population of patients with life-threatening diseases associated with Abl, Kit or PDGFR protein tyrosine kinases [58].

(continued)

Table 4.1 (continued)

Drug	Biomarker	Labeling Text
Irinotecan CAMPTOSAR LVD: 12/19/14	UGT1A1	Is a topoisomerase inhibitor. **Dosage and administration**: Dosage in patients with reduced UGT1A1 activity when administered in combination with other agents, or as a single-agent, a reduction in the starting dose by at least one level of CAMPTOSAR should be considered for patients known to be homozygous for the UGT1A1∗28 allele. However, the precise dose reduction in this patient population is not known, and subsequent dose modifications should be considered based on individual patient tolerance to treatment. **Warnings and precautions:** Individuals who are homozygous for the UGT1A1∗28 allele (UGT1A1 7/7 genotype) are at increased risk for neutropenia following initiation of CAMPTOSAR treatment [59].
Lapatinib YKERB LVD: 04/06/17	ERBB2 (HER2) ESR, PGR (hormone receptor) HLA-DQA1, HLA-DRB1	**Indications and usage:** is indicated in combination with: • capecitabine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER2 and who have received prior therapy including an anthracycline, a taxane, and trastuzumab. **Limitation of use:** Patients should have disease progression on trastuzumab prior to initiation of treatment with TYKERB in combination with capecitabine. • letrozole for the treatment of postmenopausal women with hormone receptor-positive metastatic breast cancer that overexpresses the HER2 receptor for whom hormonal therapy is indicated. TYKERB in combination with an aromatase inhibitor has not been compared to a trastuzumab-containing chemotherapy regimen for the treatment of metastatic breast cancer **Pharmacogenomics:** The HLA alleles DQA1∗02:01 and DRB1∗07:01 were associated with hepatotoxicity reactions in a genetic substudy of a monotherapy trial with TYKERB (n = 1194). Severe liver injury (ALT >5 times the upper limit of normal, NCI CTCAE grade 3) occurred in 2% of patients overall; the incidence of severe liver injury among DQA1∗02:01 or DRBI∗07:01 allele carriers was 8% versus 0.5% in non-carriers. These HLA alleles are present in approximately 15% to 25% of Caucasian, Asian, African, and Hispanic populations and 1% in Japanese populations. Liver function should be monitored in all patients receiving therapy with TYKERB regardless of genotype [60].
Letrozole FEMARA LVD: 04/05/18	ESR, PGR (hormone receptor)	**Indications and usage:** Is an aromatase inhibitor indicated for: • adjuvant treatment of postmenopausal women with hormone receptor positive early breast cancer. • extended adjuvant treatment of postmenopausal women with early breast cancer who have received prior standard adjuvant tamoxifen therapy. • first and second-line treatment of postmenopausal women with hormone receptor positive or unknown advanced breast cancer [61].
Neratinib NERLYNX LVD: 07/17/17	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** Is a kinase inhibitor indicated for the extended adjuvant treatment of adult patients with early stage HER2-overexpressed/amplified breast cancer, to follow adjuvant trastuzumab-based therapy [62].
Niraparib ZEJULA LVD: 03/27/17	BRCA	**Indications and usage:** Is a poly(ADP-ribose) polymerase (PARP) inhibitor indicated for the maintenance treatment of adult patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer who are in a complete or partial response to platinum-based chemotherapy. **Clinical studies:** The NOVA trial demonstrated a statistically significant improvement in PFS for patients randomized to ZEJULA as compared with placebo in the gBRCA Mut cohort and the non-gBRCA mut cohort [63].

Nivolumab **OPDIVO** LVD:04/16/18	BRAF CD274 (PD-L1) Microsatellite instability, mismatch repair	**Indications and usage:** OPDIVO is a programmed death receptor-1 (PD-1) blocking antibody indicated for the treatment of: • patients with BRAF V600 wild-type unresectable or metastatic melanoma, as a single agent. • patients with BRAF V600 mutation-positive unresectable or metastatic melanoma, as a single agent. • patients with unresectable or metastatic melanoma, in combination with ipilimumab. • patients with melanoma with lymph node involvement or metastatic disease who have undergone complete resection, in the adjuvant setting. • patients with metastatic non-small cell lung cancer and progression on or after platinum-based chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving OPDIVO. • patients with advanced renal cell carcinoma who have received prior antiangiogenic therapy. • patients with intermediate or poor risk, previously untreated advanced renal cell carcinoma, in combination with ipilimumab. • patients with recurrent or metastatic squamous cell carcinoma of the head and neck with disease progression on or after a platinum-based therapy. • patients with locally advanced or metastatic urothelial carcinoma who: • have disease progression during or following platinum-containing chemotherapy • have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. • adult and pediatric (12 years and older) patients with microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan. • patients with hepatocellular carcinoma who have been previously treated with sorafenib [64].
Olaparib **LYNPARZA** LVD: 10/23/17	BRCA	**Indications and usage:** Is a PARP inhibitor indicated for the treatment of adult patients with deleterious or suspected deleterious germline BRCA-mutated advanced ovarian cancer who have been treated with three or more prior lines of chemotherapy. **Patient selection:** Based on the presence of deleterious or suspected deleterious germline BRCA-mutations [65].
Olaratumab **LARTRUVO** LVD:10/19/16	PDGFRA	**Indications and usage:** Is a platelet-derived growth factor receptor alpha (PDGFR-α) blocking antibody indicated, in combination with doxorubicin, for the treatment of adult patients with soft tissue sarcoma (STS) with a histologic subtype for which an anthracycline-containing regimen is appropriate and which is not amenable to curative treatment with radiotherapy or surgery. (1) this indication is approved under accelerated approval. Continued approval for this indication may be contingent upon verification and description of clinical benefit in the confirmatory trial [66]
Osimertinib **TAGRISSO** LVD: 04/18/18	EGFR	**Indications and usage:** Is a kinase inhibitor indicated for • the first-line treatment of patients with metastatic NSCLC whose tumors have EGFR exon 19 deletions or exon 21 L858R mutations, as detected by an FDA-approved test. • the treatment of patients with metastatic EGFR T790 M mutation-positive NSCLC, as detected by an FDA-approved test, whose disease has progressed on or after EGFR TKI therapy. **Patient selection:** Select patients for the first-line treatment of metastatic EGFR-positive NSCLC with TAGRISSO based on the presence of EGFR exon 19 deletions or exon 21 L858R mutations in tumor specimens. Select patients for the treatment of metastatic EGFR T790 M mutation-positive NSCLC with TAGRISSO following progression on or after EGFR TKI therapy based on the presence of an EGFR T790 M mutation in tumor or plasma specimens. Testing for the presence of the T790 M mutation in plasma specimens is recommended only in patients for whom a tumor biopsy cannot be obtained. If this mutation is not detected in a plasma specimen, re-evaluate the feasibility of biopsy for tumor tissue testing [67].
Palbociclib **IBRANCE** LVD: 02/06/18	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** Is a kinase inhibitor indicated for the treatment of HR-positive, HER2-negative advanced or metastatic breast cancer in combination with: • an aromatase inhibitor as initial endocrine based therapy in postmenopausal women; or • fulvestrant in women with disease progression following endocrine therapy [68].

(continued)

Table 4.1 (continued)

Drug	Biomarker	Labeling Text
Panitumumab **VECTIBIX** LVD: 06/29/17	EGFR RAS	**Indications and usage:** Is indicated for the treatment of patients with wild-type RAS (defined as wild-type in both KRAS and NRAS as determined by an FDA-approved test for this use) metastatic colorectal cancer (mCRC) [• as first-line therapy in combination with FOLFOX • as monotherapy following disease progression after prior treatment with fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy. **Limitation of use:** Vectibix is not indicated for the treatment of patients with RAS-mutant mCRC or for whom RAS mutation status is unknown. **Patient selection:** Prior to initiation of treatment with Vectibix, assess RAS mutational status in colorectal tumors and confirm the absence of a RAS mutation in exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of both KRAS and NRAS [69].
Pazopanib **VOTRIENT** LVD: 05/31/17	UGT1A1 HLA-B	**Pharmacogenomics:** Pazopanib can increase serum total bilirubin levels. In vitro studies showed that pazopanib inhibits UGT1A1, which glucuronidates bilirubin for elimination. A pooled pharmacogenetic analysis of 236 Caucasian patients evaluated the TA-repeat polymorphism of UGT1A1 and its potential association with hyperbilirubinemia during pazopanib treatment. In this analysis, the (TA)7/(TA)7 genotype (UGT1A1∗28/∗28) (underlying genetic susceptibility to Gilbert's syndrome) was associated with a statistically significant increase in the incidence of hyperbilirubinemia relative to the (TA)6/(TA)6 and (TA)6/(TA)7 genotypes. In a pooled pharmacogenetic analysis of data from 31 clinical studies of pazopanib administered as either monotherapy or in combination with other agents, ALT >3 X ULN (NCI CTC grade 2) occurred in 32% (42/133) of HLA-B∗57:01 allele carriers and in 19% (397/2101) of non-carriers and ALT >5 X ULN (NCI CTC grade 3) occurred in 19% (25/133) of HLA-B∗57:01 allele carriers and in 10% (213/2101) of non-carriers. In this dataset, 6% (133/2234) of the patients carried the HLA-B∗57:01 allele. Liver function should be monitored in all subjects receiving pazopanib, regardless of genotype [70].

Pembrolizumab KEYTRUDA LVD: 06/19/18	BRAF CD274 (PD-L1) Microsatellite instability, Mismatch repair	**Indications and usage:** Is a programmed death receptor-1 (PD-1)-blocking antibody indicated in: Melanoma • for the treatment of patients with unresectable or metastatic melanoma. NSCLC • as a single agent for the first-line treatment of patients with metastatic NSCLC whose tumors have high PD-L1 expression [(tumor proportion score (TPS) ≥50%)] as determined by an FDA-approved test, with no EGFR or ALK genomic tumor aberrations. • as a single agent for the treatment of patients with metastatic NSCLC whose tumors express PD-L1 (TPS ≥1%) as determined by an FDA-approved test, with disease progression on or after platinum-containing chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving KEYTRUDA.• in combination with pemetrexed and carboplatin, as first-line treatment of patients with metastatic nonsquamous NSCLC. Head and neck squamous cell cancer (HNSCC) • for the treatment of patients with recurrent or metastatic HNSCC with disease progression on or after platinum containing chemotherapy. Urothelial carcinoma • for the treatment of patients with locally advanced or metastatic urothelial carcinoma who are not eligible for cisplatin-containing chemotherapy and whose tumors express PD-L1 [Combined positive score (CPS) ≥10], or in patients who are not eligible for any platinum-containing chemotherapy regardless of PD-L1 status. • for the treatment of patients with locally advanced or metastatic urothelial carcinoma who have disease progression during or following platinum-containing chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with platinum containing chemotherapy. Microsatellite instability-high cancer • for the treatment of adult and pediatric patients with unresectable or metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient ∗ solid tumors that have progressed following prior treatment and who have no satisfactory alternative treatment options, or ∗ colorectal cancer that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan. • limitation of use: The safety and effectiveness of KEYTRUDA in pediatric patients with MSI-H central nervous system cancers have not been established. Gastric Cancer • for the treatment of patients with recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma whose tumors express PD-L1 [combined positive score (CPS) ≥1] as determined by an FDA-approved test, with disease progression on or after two or more prior lines of therapy including fluoropyrimidine- and platinum containing chemotherapy and if appropriate, HER2/neu-targeted therapy. Cervical Cancer • for the treatment of patients with recurrent or metastatic cervical cancer with disease progression on or after chemotherapy whose tumors express PD-L1 (CPS ≥1) as determined by an FDA-approved test. **Patient selection:** For treatment of NSCLC, gastric Cancer, or cervical Cancer, select patients based on the presence of positive PD-L1 expression. If PD-L1 expression is not detected in an archival gastric cancer specimen, evaluate the feasibility of obtaining a tumor biopsy for PD-L1 testing [71].
Pertuzumab PERJETA LVD: 12/20/17	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** PERJETA is a HER2/neu receptor antagonist indicated for: • use in combination with trastuzumab and docetaxel for treatment of patients with HER2-positive metastatic breast cancer (MBC) who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease. • use in combination with trastuzumab and chemotherapy as ∗ neoadjuvant treatment of patients with HER2-positive, locally advanced, inflammatory, or early stage breast cancer (either greater than 2 cm in diameter or node positive) as part of a complete treatment regimen for early breast cancer. ∗ adjuvant treatment of patients with HER2-positive early breast cancer at high risk of recurrence. **Patient selection:** Based on HER2 protein overexpression or HER2 gene amplification in tumor specimens. Assessment of HER2 protein overexpression and HER2 gene amplification should be performed using FDA-approved tests specific for breast cancer by laboratories with demonstrated proficiency [72].

(continued)

Table 4.1 (continued)

Drug	Biomarker	Labeling Text
Ribociclib **KISQALI** LVD: 03/13/17	ESR, PGR (hormone receptor) ERBB2 (HER2)	**Indications and usage:** KISQALI is a kinase inhibitor indicated in combination with an aromatase inhibitor as initial endocrine-based therapy for the treatment of postmenopausal women with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced or metastatic breast cancer [73].
Rucaparib **RUBRACA** LVD: 04/06/18	BRCA CYP2D6 CYP1A2	**Indications and usage:** Is a poly (ADP-ribose) polymerase (PARP) inhibitor indicated: • for the maintenance treatment of adult patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer who are in a complete or partial response to platinum-based chemotherapy. • for the treatment of adult patients with deleterious BRCA mutation (germline and/or somatic)-associated epithelial ovarian, fallopian tube, or primary peritoneal cancer who have been treated with two or more chemotherapies. **Patient selection:** for the treatment of advanced ovarian cancer with Rubraca based on the presence of a deleterious BRCA mutation (germline and/or somatic). **CYP enzyme polymorphism:** Based on population pharmacokinetic analyses, steady-state concentrations following rucaparib 600 mg twice daily did not differ significantly across CYP2D6 or CYP1A2 genotype subgroups [74].
Tamoxifen LVD:03/09/06	ESR, PGR (hormone receptor)	**Indications and usage:** Estrogen and progesterone receptor levels 10 fmol or greater. Metastatic breast Cancer: Tamoxifen is effective in the treatment of metastatic breast cancer in women and men. In premenopausal women with metastatic breast cancer, tamoxifen is an alternative to oophorectomy or ovarian irradiation. Available evidence indicates that patients whose tumors are estrogen receptor positive are more likely to benefit from tamoxifen therapy. Adjuvant treatment of breast Cancer: Is indicated for the treatment of node-positive breast cancer in women following total mastectomy or segmental mastectomy, axillary dissection, and breast irradiation. Tamoxifen is indicated for the treatment of axillary node-negative, breast cancer in women following total mastectomy or segmental mastectomy, axillary dissection, and breast irradiation. The estrogen and progesterone receptor values may help to predict whether adjuvant tamoxifen therapy is likely to be beneficial. Tamoxifen reduces the occurrence of contralateral breast cancer in patients receiving adjuvant therapy for breast cancer. Ductal carcinoma in situ (DCIS): Following breast surgery and radiation, tamoxifen is indicated to reduce the risk of invasive breast Reduction in breast Cancer incidence in high risk women: Tamoxifen is indicated to reduce the incidence of breast cancer in women at high risk for breast cancer. "High risk" is defined as women at least 35 years of age with a 5-year predicted risk of breast cancer ≥1.67%, as calculated by the Gail model. **Warnings**: There is evidence of an increased incidence of thromboembolic events, including deep vein thrombosis and pulmonary embolism, during tamoxifen therapy. When tamoxifen is coadminstered with chemotherapy, there may be a further increase in the incidence of thromboembolic effects. For treatment of breast cancer, the risks and benefits of tamoxifen should be carefully considered in women with a history of thromboembolic events. In a small substudy (N = 81) of the NSABP P-1 trial, there appeared to be no benefit to screening women for factor V Leiden and prothrombin mutations G20210A as a means to identify those who may not be appropriate candidates for tamoxifen therapy [75].

Trametinib **MEKINIST** LVD:05/04/18	BRAF G6PD RAS	**Indications and usage:** Is a kinase inhibitor indicated as a single agent for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E or V600K mutations. MEKINIST is indicated in combination with dabrafenib, for: • the treatment of patients with unresectable or metastatic melanoma with BRAF V600E or V600K mutations. • the adjuvant treatment of patients with melanoma with BRAF V600E or V600K mutations, and involvement of lymph node(s), following complete resection. • the treatment of patients with NSCLC with BRAF V600E mutation. • the treatment of patients with locally advanced or metastatic anaplastic thyroid cancer (ATC) with BRAF V600E mutation and with no satisfactory locoregional treatment options. Limitations of use: MEKINIST is not indicated for treatment of patients with melanoma who have progressed on prior BRAF-inhibitor therapy. **Patient selection**: melanoma: Confirm the presence of BRAF V600E or V600K mutation in tumor specimens prior to initiation of treatment NSCLC: Confirm the presence of BRAF V600E mutation in tumor specimens prior to initiation of treatment. • ATC: Confirm the presence of BRAF V600E mutation in tumor specimens prior to initiation of treatment. **Patient counseling information:** Evidence of BRAF V600E or V600K mutation within the tumor specimen is necessary to identify patients for whom treatment with MEKINIST is indicated. **Clinical trials experience:** MEKINIST administered with Dabrafenib: The trials excluded patients with known history of G6PD deficiency, among others. **Warnings and precautions:** New primary malignancies non-cutaneous malignancies, based on its mechanism of action, dabrafenib may promote growth and development of malignancies with activation of RAS through mutation or other mechanisms [76].
Trastuzumab **HERCEPTIN** LVD: 04/27/17	ERBB2 (HER2) ESR, PGR (hormone receptor)	**Indications and usage:** Herceptin is a HER2/neu receptor antagonist indicated for: Adjuvant breast Cancer treatment of HER2 overexpressing node positive or node negative (ER/PR negative or with one high risk feature breast cancer • as part of a treatment regimen consisting of doxorubicin, cyclophosphamide, and either paclitaxel or docetaxel. • as part of a treatment regimen with docetaxel and carboplatin. • as a single agent following multi-modality anthracycline based therapy. Metastatic breast Cancer • in combination with paclitaxel for first-line treatment of HER2-overexpressing metastatic breast cancer • as a single agent for treatment of HER2-overexpressing breast cancer in patients who have received one or more chemotherapy regimens for metastatic disease. Metastatic gastric Cancer is indicated, in combination with cisplatin and capecitabine or 5-fluorouracil, for the treatment of patients with HER2-overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma who have not received prior treatment for metastatic disease. **Patient selection:** Based on HER2 protein overexpression or HER2 gene amplification in tumor specimens [77].
Vemurafenib **ZELBORAF** LVD: 11/06/17	BRAF RAS	**Indications and usage:** Is a kinase inhibitor indicated for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E mutation as detected by an FDA-approved test. **Limitation of use:** ZELBORAF is not indicated for treatment of patients with wild-type BRAF melanoma **Patient selection:** For the presence of BRAF V600E mutation in melanoma tumor specimens prior to initiation of treatment. **Warnings and precautions**: May promote malignancies associated with activation of RAS through mutation or other mechanisms. Monitor patients receiving ZELBORAF closely for signs or symptoms of other malignancies [78].

LVD: level version date
Modified from Table of Pharmacogenomic Biomarkers in Drug Labeling Last Updated: 12/2017 [79]

been performed. This preemptive approach may counteract many of the disadvantages of reactive pharmacogenetic testing. The recent availability of high-quality genotyping arrays and other multiplex approaches that are oriented to pharmacogenetics and reasonably priced makes preemptive genotyping financially feasible. Unlike pharmacogenetic testing for individual genes, array-based preemptive testing can include a large number of relevant pharmacogenes that cover most, if not all, pharmacogenetically high-risk drugs. The test results are then available prior to any prescribing decision involving these high-risk drugs, consistent with the vision that in every such decision, patient genomic variation will be considered as an inherent patient characteristic, as are age, weight, renal function, and allergy status [11].

4.2 Global State of Implementation

All countries are at different stages of clinical implementation, such as sequencing, implementing into the electronic health record (EHR), or currently undergoing implementation projects to improve their strategy. Many countries are undergoing research in the field of genomic medicine, showing their capabilities such as using genotyping and/or genome or exome sequencing for disease prediction, diagnosis, prevention, and treatment as well as family counseling. Most of the countries currently involved in PGx research show the availability of clinical sequencing resources for cancer treatment, rare disease diagnosis, and microbial pathogen identification in specialized centers only [1].

The US and European countries are focusing on implementing variants into the EHR and developing the clinical decision support system (CDSS), as well as gene-drug pair discovery and standardization of the language used and regulations. In Europe, EuroGentest and Genomics England are trying to sequence 100 K whole genes and link to the records of patients of rare diseases and cancer [1, 3].

The Ubiquitous Pharmacogenomics Consortium (U-PGx) is an established network of European experts equipped to address the remaining challenges and obstacles for clinical implementation of PGx into patient care. Funded by the European Commission, the U-PGx Consortium will implement pre-emptive PGx testing involving a panel of pharmacogenes into routine care to guide drug and dose selection for 43 drugs. U-PGx uses a multifaceted approach consisting of four components to achieve this objective, the first component is enabling tools consist of information technology (IT) solutions, PGx testing infrastructure, educating healthcare professionals in PGx, and translating the existing Dutch Pharmacogenetics Working Group (DPWG) guidelines, which were updated only in Dutch language, to six other local languages. The second component will implement pre-emptive genotyping of a panel of 50 variants in 13 pharmacogenes into clinical practice, of which only 5 actionable drug-gene pairs implemented in routine care are cancer related: Capecitabine-DPD, Fluorouracil-DPD, Irinotecan-UGT1A1, Tamoxifen-CYP2D6, and Tegafur-DPD. A third component applies innovative methodologies such as next-generation sequencing (NGS), pharmacokinetic modelling and systems pharmacology to discover additional variants associated with drug response and to elucidate drug-drug-gene interactions. The final, fourth, component will focus on ethical issues of the project and implications for PGx, and spearheads outreach and educational activities to influential stakeholders [3].

Several institutions in the US have ongoing PGx projects involving the development of a model or workflow process. In addition to these institutes there are several groups, for example, National Institutes of Health (NIH) Clinical center, Electronic Medical Records and Genomics (eMERGE), Implementing Genomics in Practice (IGNITE), and the Pharmacogenomics Research Network (PGRN), working in collaboration with institutes focused on a wide base of projects to improve the clinical implementation of PGx [1, 12].

eMERGE and IGNITE focuses on incorporating data into the EHR and developing CDS systems for PGx as well as for educating the stakeholders. Projects from other institutions (CLIPMERGE, 1200 patients project, PG4KDS) focus on developing certain aspects of the clinical implementation process, such as the CDS, education, or clinical implementation model development [1, 3].

The Targeted Agent and Profiling Utilization Registry (TAPUR) Study is a non-randomized clinical trial that aims to describe the safety and efficacy of commercially available, targeted anticancer drugs prescribed for treatment of patients with advanced cancer that has a potentially actionable genomic variant. TAPUR will study Food and Drug Administration (FDA)-approved targeted therapies that are contributed by collaborating pharmaceutical companies, catalogue the choice of molecular profiling test by clinical oncologists and develop hypotheses for additional clinical trials. The study start date was March, 2016, they try to recruit 1140 patients older than 12 years, with are divided into 14 groups according to their molecular profiling, and then were treated based on these results Primary outcome is objective response rate, the secondary outcome is overall survival. Final data collection date for primary outcome measure it's estimated to be done at March 2019 [13].

At this moment the trial continues to expand and now has more than 600 participants enrolled on study drug, more than 101 sites, in 20 states of America, and it's collaborating with Canadian Cancer Trials Group (CCTG) and WIN Consortium (Worldwide innovative networking in personalized cancer medicine). Both CCTG and WIN Consortium are leading studies similar to TAPUR, and the American Society of Clinical Oncology (ASCO) aims to collaborate with them to share study results to accelerate learning. WIN Consortium—a global network of renowned academic cancer centers, pharmaceutical and diagnostic companies, and patient advocacy organizations spanning 16 countries and four continents—will offer an opportunity to expand data to more than a dozen countries outside of North America. WIN will be deploying WIN-TAPUR in the following countries: Brazil, China, Denmark, France, India, Israel, Japan, Jordan, Luxembourg, Russian Federation, Singapore, South Korea, and Spain [14].

Canadian profiling and targeted agent utilization trial (CAPTUR/PM.1) is a pan-Canadian trial leveraging existing clinical genomic profiling platforms and the research capabilities of the CCTG) to evaluate targeted drug-genetic variant matches in patients with advanced cancers. It's a multicentre, open label, phase II basket trial, Patients must have incurable metastatic solid tumours, multiple myeloma, or B cell non-Hodgkin lymphoma, must have no standard treatment options known to prolong life and must have an actionable genomic variant known to be a target of, or predict sensitivity to, the commercially available targeted anticancer drug.

A drug-variant match is assigned based on protocol specified matching criteria or input of the MTB. Drug matches are drawn from a list of 17 commercially available anticancer agents. Determination of the best treatment is then made by physician and patient based on drug-specific eligibility requirements. Cohorts are defined by tumour type, genomic alteration and matched drug treatment. The primary endpoint is response rate, as determined by disease-appropriate objective criteria [15].

CAPTUR/PM.1 was developed in collaboration, and plans to share data, with ASCO's TAPUR and the Netherland's DRUP trials. The trial was activated November 2017 and is open to enrolment. The purpose of the study is to learn from the real world practice of prescribing targeted therapies to patients with advanced cancer whose tumor harbors a genomic variant known to be a drug target or to predict sensitivity to a drug [16]. The Table 4.2 compares the treatment based on the molecular profiling in the TAPUR and CAPTUR/PM.1 studies. Canada has several institutes involved in genomic medicine research, such as Ontario Genomics and Genome Canada.

Japan focuses on cancer, construction of large biobanks and large-scale Genome wide association studies (GWAS) by RIKEN, and it's building a large DNA database for PGx analysis. Japan is involved in several international projects, where

Table 4.2 Treatment based on the molecular profiling in the TAPUR and CAPTUR/PM.1 studies

TAPUR STUDY Molecular Profiling Test	TAPUR STUDY Treatment	CAPTUR STUDY Molecular Profiling Test	CAPTUR STUDY Treatment
VEGFR mutation, amplification or overexpression	Axitinib/Inlyta	VEGFR1, VEGFR2, VEGFR3	Axitinib/Inlyta 5 mg po tid
Bcr-abl, SRC, LYN, LCK mutations	Bosutinib/Bosulif	Bcr-abl, SRC	Bosutinib/Bosulif 500 mg po qd
ALK, ROS1, MET mutations	Crizotinib/Xalkori	ALK, ROS1, MET	Crizotinib/Xalkori 250 mg po tid
CDKN2A, CDK4, CDK6 amplifications	Palbociclib/Ibrance	CDKN2A, CDK4, CDK6, CCND1	Palbociclib/Ibrance 125 mg po qd for 21 d followed by 7 d off to comprise 28 d cycle
CSF1R,PDGFR,VEGFR	Sunitinib/Sutent	CSF1R, PDGFRA, PDGFRB,VEGFR1, VEGFR2, VEGFR3, KIT FLT3, RET, FGFR1, FGFR2, FGFR3, VHL	Sunitinib/Sutent 50 mg po qd on a schedule of 4 weeks on treatment followed by 2 weeks off
mTOR, TSC mutations	Temsirolimus/Torisel	AKT1, AKT2, AKT3, FBXW7, FLCN, mTOR, NF1, NF2, NTRK3, PIK3CA, PIK3R1, PTEN, RHEB, STKII, TSC1, TSC2	Temsirolimus/Torisel 25 mg infused over a 30–60 min period once a week
ERBB2 amplifications	Trastuzumab and pertuzumab/Herceptin and Perjeta	ERBB2	Trastuzumab/Herceptin = 3-weekly dose schedule. The initial loading dose is 8 mg/kg followed by 3-weekly maintenance dose of 6 mg/kg Pertuzumab/Perjeta = 840 mg, followed every 3 weeks thereafter by a dose of 420 mg
BRAFV600E mutations	Vemurafenib and Cobimetinib/Zelboraf and Cotellic	BRAFV600	Vemurafenib/Zelboraf = 960 mg (four 240 mg tablets) po tid Cobimetinib/Cotellic = 60 mg (three 20 mg tablets) po qd for 21 day, followed by 7 day of rest
KRAS, NRAS and BRAF wildtype	Cetuximab/Erbitux		
Bcr-abl, SRC, KIT, PDGFRB, EPHA2, FYN, LCK, YES1 mutations	Dasatinib/Sprycel	KIT, PDGFRA, PDGFRB, ABL1	Dasatinib/Sprycel 100 mg administered po qd

RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFRβ, RAF-1, BRAF mutations/amplifications	Regorafenib/Stivarga		
Germline or somatic BRCA1/BRCA2 inactivating mutations; ATM mutations or deletions	Olaparib/Lynparza	BRCA1, BRCA2	Olaparib/Lynparza 400 mg tid
POLE/POLD1;high mutational load	Pembrolizumab/Keytruda	High mutational burden, POLE, POLD1	Nivolumab/Opdivo combination phase – 1 mg/kg IV every 3 weeks for the first 4 doses + ipilmumab 3 mg/kg IV followed by the single-agent phase. Single-agent phase – 3 mg/kg IV every 2 weeks
MSIH, high mutational load and others	Nivolumab and Ipilimumab/Opdivo and Yervoy		
		NRG, EGFR, ERRB2, ERRB3, ERRB4	Afatinib/Gilotrif 40 mg qd
		EGFR	Erlotinib/Tarceva 150 mg po qd
		PTCH1, SMO	Vismodegib/Erivedge 150 mg po qd

Modified from TAPUR: clinicaltrials.gov NCT02693535 and CAPTUR: clinicaltrials.gov NCT03297606

RIKEN has been the most involved institutions in projects such as the International Cancer Genome Consortium (ICGC), where the aim is to understand the genomic changes in many forms of cancers. Chinese Ministry of Health wants to develop biomarkers for tuberculosis and cancer.

The Korean Genome Project is improving the next generation sequencing (NGS) for use in cancer gene analysis. In Singapore, the major Project, POLARIS, is a pilot project that focuses on TGFBI testing for disease diagnosis and family risk assessment in stromal corneal dystrophies, and further implementation of a 90 gene panel for gastrointestinal cancers.

Notably, several low- and middle-income countries have startup projects in genomics, such as Mexico's new initiative, the Mexico Genome Variation Project, in which all data on ethnically diverse populations are collected. This project is conducted at the Mexico National Institute of Genomic Medicine (INMEGEN). Together with the Mexican HapMap Project, the genetic structure of different populations and genomic medicine research capacity and infrastructure can be facilitated in the early stages of genomic medicine.

It is difficult for one country alone to conduct the research to create personalized medicine. Therefore several groups have joined to form international projects, including the Encyclopedia of the Human DNA Elements (ENCODE) project which identifies all functional elements in the human genome sequence. The Golden helix Institute of Biomedical research is an organization with interdisciplinary research and educational activities in genome medicine which spans across Europe, Asia and Latin America [1].

4.3 Prerequisites to Implementing a Pharmacogenomics Program

The successful clinical implementation of a PGx program at a large health-care system requires alignment of clinical and administrative stakeholders, including senior executive leadership (chief executive officer/president, chief medical officer, chief information officer, chief financial officer, and chief legal officer), senior clinical leaders (for clinical divisions, nursing, and pharmacy); pathology, pharmacy, and therapeutics committee members; patient advocates; and third-party payers. Prior to implementation, committees should be established representing these stakeholders. Alignment of common interests and concerns must be obtained within and across administrative and clinical stakeholder groups. This alignment is crucial if the PGx initiative is to be funded and clinically adopted into the standard of care [17].

Implementation research requires transdisciplinary teams that include expertise in genomics, clinical engineering, informatics, health services research, economics, and organizational science, as well as operational partners including administrators, clinicians, HIT professionals, payers, and patients [12].

Several significant challenges surround the implementation of PGx-based medicine on a wider scale, including reimbursement for genetic testing; development of infrastructure and standardized processes for storing, accessing, and interpreting genomic data; evidence of clinical utility; ethical and legal concerns; and prescriber uncertainty about the clinical and financial benefits of genome-guided therapy [6].

The Indiana Institute of Personalized Medicine (IIPM) in 2014, made an implementation team who create strategically important working groups: Clinical Implementation, Laboratory Implementation, Education and Marketing and IT/Workflow [17], to conduct a prospective trial to Assess Cost and Clinical Outcomes of a Clinical Pharmacogenomic Program (INGenious) [3, 17, 18]. The primary end point of this trial is to evaluate the financial impact on the total cost of patient care resulting from implementation of a PGx testing program within a safety-net and academic healthcare system. Final data collection date for primary outcome measure is June, 2018 [19].

For the IIPM a high priority in the early planning stages must be selecting PGx tests that are relevant to the patient population. These tests must provide clear, evidence-based, well-documented direction for therapy changes. In

addition, the PGx program must be dynamic; ongoing scientific and clinical reviews of the literature must be iteratively incorporated into the program. Once implemented, it is also important to continue to capture data on medication changes, adverse events, and costs associated with targeted medication efficacy failures, as these data can be used to validate the cost effectiveness of a PGx program.

Their Clinical Implementation working group comprised scientists, physicians, nurses, and clinical pharmacists. This team's objective was to select which gene–drug pairs would be implemented and to prepare the clinical direction to be provided. Clinical Pharmacogenetics Implementation Consortium guidelines on gene–drug pairs were used to help select targeted medications and the microarray architecture [17].

Another comprehensive, reproducible, and adaptable model was used by the Mayo Clinic to implement PGx in the clinical setting, and it was based on eight highly interrelated functional components.

Institutional leadership support to initiating, driving, and maintaining a successful implementation program. Because PGx testing was not widely reimbursable, institutional leadership regarded PGx implementation as an investment in good patient care and the future of medicine.

PGx governance: task force of experts overseeing all aspects of the implementation and coordinating efforts and resources was essential for PGx implementation. This team include genomic medicine, primary and specialty-care clinics, pharmacy, laboratory, education, research, informatics, information technology (IT), and administration. Routine meetings provided a structured forum to facilitate communication and decision making with regard to the selection, prioritization, development, and implementation of specific drug–gene interventions. A coordinated and dedicated multidisciplinary effort is critical for successfully facilitating the clinical adoption of this kind of models [6]. In Fig. 4.1 we describe a possible model of implementation of a PGx program, based in the models reported and the solution to the barriers that the different centers found in the implementation.

And other components as clinical approval, laboratory results, PGx education, PGx knowledge, CDSS-EHR implementation, and a long-term maintenance strategy to maintain and update the data, knowledge, interfaces, and CDSS-EHR applications [6].

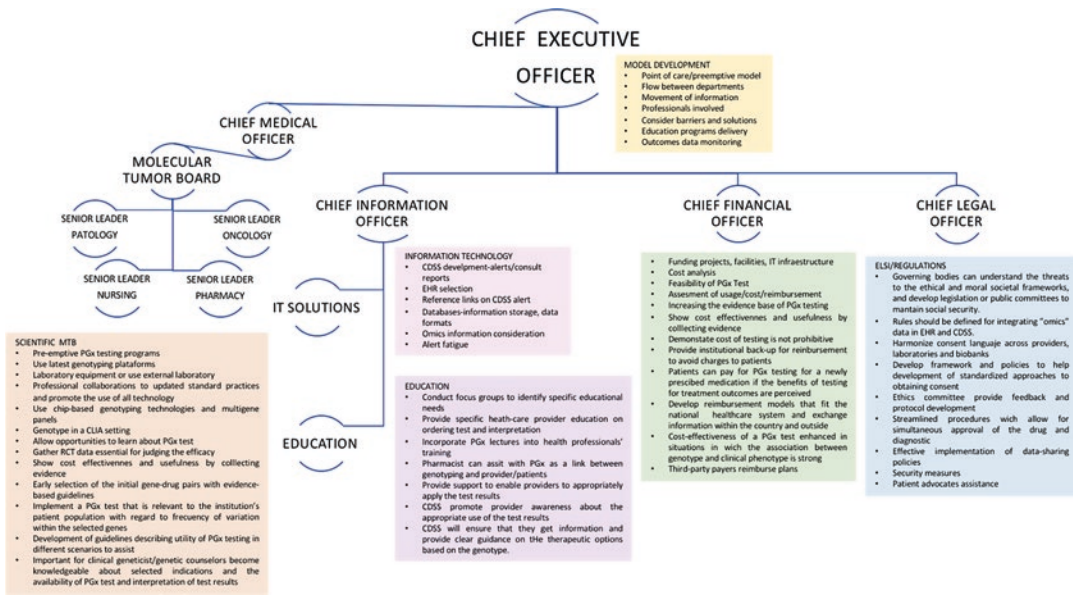

Fig. 4.1 Model of implementation of a PGX program. (Modified from Refs. [1, 3, 6, 17, 20])

In this subject German university hospitals have started to establish molecular tumor boards (MTB) in order to enable physicians to make molecularly guided therapy decisions. Disciplines which offer genetic testing at these hospitals are: neuropathology, pathology, hematology, oncology, human genetics, institute of molecular medicine, neurological clinic, central laboratory, and the NCT Heidelberg institute for clinical chemistry and laboratory medicine [20].

As part of Indiana University, the IIPM has access to faculty and graduate students from the IU Kelly School of Business, who assisted in financial modeling of the program, thus helping to cost-justify the establishment of the new laboratory (space, equipment, and staff) [17].

4.4 Information Technology (IT) Solutions

Information technology (IT) solutions must consider clinical workflows and facilitate the process for caregivers to order appropriate PGx tests. In addition, test results have to be integrated into the medical record system. PGx testing must be integrally linked to the electronic medical record (especially for International Classification of Diseases, Ninth Revision (ICD-9) diagnosis coding) and to the pharmacy ordering system (linking prescription orders to alerts for PGx tests) [17].

The electronic health record (EHR) provides a wealth of information to study questions at the population level. Large patient cohorts can be quickly created for a variety of diseases and drug response phenotypes to better evaluate drug safety. With the establishment of biobanks concurrent with the adoption of EHR, studies of genomic associations of drug response may become more comprehensive [4].

Pharmacogenomic CDSS are computer-based systems which support health care providers in prescribing drugs at the point of care. Pharmacogenomic CDSS link a patients' genotype to biomedical knowledge in order to assist physicians in assessing cancer status, in making a diagnosis, in selecting an appropriate cancer therapy or in making other molecularly guided decisions. A pharmacogenomic CDSS can either be integrated into the local hospital information system or used as a separate program such as a web service or mobile application. CDSS can provide a web-based access to the pharmacogenomic test results in a password protected online portal for physicians or patients [21].

Despite a lack of specific functionality in commercially available EHR to manage genomic data, we must be able to adapt existent functionality to deliver synchronous interventions as a clinician is interacting with the EHR (i.e., pop-up alert in the order entry system advising the clinician to order a PGx laboratory test based on a drug order or a pop-up alert prompted by a specific drug–gene interaction) and asynchronous interventions (i.e., inbox message or e-mail when new PGx test results are available) [6]. Each post-test alert can refer physicians to additional information about related genes or drugs. Them also can link the physicians to full guideline texts and original references. The physicians may prefer an option to prescribe the recommended medication within the alert. Alert fatigue has been mentioned as one of the main challenge for using alerts within pharmacogenomic CDSS [18, 21].

A variety of interventions in the EHR can be implemented designed to: remind clinicians if PGx testing was required based on current clinical guidelines, detect unreadable PGx test results and trigger a manual review process to validate discrete data, document relevant genotypes/phenotypes in the problem list, notify ordering clinicians of new PGx test result(s) with an inbox message containing specific drug–gene information; use available PGx results to alert prescribers of potential drug–gene interactions and suggest changes to the order (pop-up alert advising drug change, dose change, or a calculated dose); and provide links in the CDSS interventions to facilitate access to web-based and easy-to-use educational resources in a workflow friendly format [6].

The passive or post-test alerts can represent a problem definition to the physician, as the presentation of the raw genotype in the pharmacogenomic CDSS. The interpretation of

pharmacogenomic test result (phenotype) can be include in each problem definition. Beside the phenotype presentation, all pharmacogenomic CDSS can also indicate the clinical impact of a potential drug-gene interaction in their interpretation of the results [21].

Many institutes still use the point-of-care (POC) method, wherein they order the test when the patient comes for a consultation. Due to the test being ordered after the first consultation, the reactive method has the limitation of delayed treatment, compared to the preemptive approach where the test is done prior hospital admission and is available at any time [1].

Similar to the pre-test and post-test alerts, the reports could contain a genotype results section and a result interpretation (phenotype) section. Additionally, the clinical impact of potential drug-gene interactions can be explained, dosage-adjusted medication options based on the pharmacogenomic results can be recommended. Drugs which should be avoided can be displayed in the recommendation section of pharmacogenomic CDSS.

Another feature of some pharmacogenomic CDSS was the ability to store pharmacogenomic results in the EHR. Raw pharmacogenomic results in form of genotype results must be stored in the lab section of the EHR, to get an overview of all PGx tests ordered for this patient so far, and also provided a phenotype interpretation in the EHR, in the lab section. Pharmacogenomic test results remain relevant to the medical treatment of a patient over his/her lifetime, and it need to be stored in a way that they will not be forgotten or lost.

Inbox messages can inform physicians about new pharmacogenomic test results and enable them to request pharmacogenomic consultations. Search engines enable physicians to compare medical treatment options based on a patient's genotype. Hinderer review, 2017, to make readily available all the online references in an attempt to facilitate compliance with the recommendations [6].

Future pharmacogenomic CDSS will likely include prediction models to recommend preemptive genotyping for patients exceeding particular risk thresholds [21].

4.5 Establishment of a Molecular Tumor Board

The German model of MTB focus on cancer patients with progression after standard treatment, rare tumor entities and resistances to molecular targeted therapies. This mainly focus on exploratory genetic testing like run genetic tests to compare the patient's genes to a panel of known gene mutations. Panel sequencing is a method to perform next-generation sequencing (NSG). The MTB aimed at treating patients for whom a case-related cancer therapy according to guidelines has been ineffective. It might be necessary to include such patients earlier into the MTB instead of waiting until a therapy according to guidelines is ineffective and the cancer had an avoidable progress, as the experts have recommended.

Exploratory genetic testing requires a more comprehensive, advanced and time-consuming interpretation of the raw genetic results compared to routine genetic testing. These hospitals used different panel sizes to detect gene mutations in the genes of a patient, ranging from 8 to 160 gene mutations. Only one hospital performed whole-exome-sequencing (WES) and whole-genome-sequencing (WGS), which are both further methods of NSG. Overall, smaller panels were used for routine testing for an entity-related tumor board, whereas both smaller and greater panels or even whole-exome-sequencing were used for exploratory genetic testing for the MTB.

After analyzing and interpreting the results of the genetic test, physicians of the diagnostic departments of all five hospitals describe their interpretation of raw data in a medical report. In all hospitals, the final medical report only consists of a narrative textual description [20].

Part of the work of the MTB must be coordinate standard definitions for different genotypes and phenotypes among different laboratories and to optimize delivery of structured PGx test results from the laboratories to the EHR [6].

The medical reports can comprise the following sections: indication for genetic testing, diagnosis, tumor cell concentration, examined genes and gene sequences including splice sites, UTR,

promoter etc., description of genetic, description of non-synonym variants (mutations) including gen name, cDNA, associated protein, allele frequency and corresponding pathogenicity, copy number variation and the critical interpretation of the genetic mutations [20].

4.6 Clinical Laboratory Testing

Cancer is a disease associated with disruption of normal cellular circuitry and processes that leads to abnormal or uncontrolled proliferative growth, characterized by a complex spectrum of biochemical alterations that affects biological processes at multiple scales from the molecular activity and cellular homeostasis to intercellular and inter-tissue signaling [22].

Perhaps the most promising area of personalized medicine is the ability to tailor cancer treatments to the molecular profile of an individual's cancerous tumor [7]. The use of genomics in oncology significantly impacted treatment decisions for many patients. Somatic mutations are often attributed to treatment efficacy, whereas germline mutations are used to identify patients at highest risk of developing serious adverse events. Somatic mutations have highlighted the importance of understanding the underlying biology of cancer with discoveries elucidating the primary genetic changes driving tumorigenesis providing molecular drug targets. Prospective tumor sequencing is being increasingly utilized, changing the paradigm of cancer treatment from site specific cytotoxic treatment, to molecularly targeted treatment [4]. Screening tumors for a range of predictive and prognostic genetic biomarkers is now a hallmark of many cancer therapy regimens [7].

Genomic technologies and approaches have transformed cancer research and have led to the production of large-scale cancer genomics compendia. The resulting molecular characterization and categorization of individual samples from such compendia has driven development of molecular subtypes cancers as well as enhanced understanding of the molecular etiologies of carcinogenesis. The development of novel and effective targeted therapies has proceeded in parallel with and been accelerated by deeper, faster, and broader genomic characterization, enabling early application of molecular characterization at the point of care to inform clinical decision-making and to address resistance to primary therapy [22].

In oncology, germline mutations play a significant role in the treatment response to both chemotherapy and targeted anti-cancer agents. These mutations are often associated with the pharmacokinetics of a drug contributing to treatment related adverse events experienced by patients. In this regard, germline pharmacogenomic markers can identify patients at highest risk of developing serious adverse events that could subsequently lead to treatment discontinuation and failure. In addition to adverse events and pharmacokinetics of a drug, germline mutations may influence drug efficacy. The field of PGx has uncovered an abundance of actionable and clinically relevant markers including both somatic and germline (Table 4.3) mutations [4].

A wide variety of testing platforms have been used clinically but, in general, testing can be divided into two broad categories—genotyping tests and sequencing tests. Genotyping tests query specific, pre-defined positions in the genome to identify known variants that are associated with drug response or toxicity and, based on the variant(s) identified, a phenotype is predicted. Genotyping tests are particularly useful for genes with common variants known to impact protein function. Often in the United States, the variants included are those that are common among Caucasian populations. The frequency of pharmacogenomically important variants differs among populations, and variants that are common among non-Caucasian populations may or may not be included. Similarly, genotyping tests cannot detect rare or novel variants that were not included as part of the original test design and, if a variant is not detected, the patient is considered to have the wild-type genotype (i.e. $*1/*1)*1/*1$) and is predicted to have an enzyme or transporter that is functioning similar to the population average (i.e. normal activity/metabolizer, previously known as "extensive").

Table 4.3 Selected somatic and germline pharmacogenomic markers

Pharmacogenomic marker	Drug (s)	Genome	Outcome
ABL	Bosutinib, dasatinib, imatinib, nilotinib, ponatinib Crizotinib	Somatic	Efficacy
ALK	Vemurafenib	Somatic	Efficacy
BRAF EGFR	Afatinib, cetuximab, erlotinib, panitumumab, vandetinib	Somatic	Efficacy
FcγR HER2	Cetuximab, rituximab, trastuzumab	Somatic	Efficacy
KRAS KIT	Lapatinib, pertuzumab, trastuzumab, trastuzumab emtansine	Somatic	Efficacy
MET	Cetuximab, panitumumab Imatinib	Somatic	Efficacy
ABL	Trametinib	Somatic	Efficacy
BIM	Imatinib	Germline	Efficacy
CYP2B6	Cyclophosphamide	Germline	Toxicity
CYP2D6	Tamoxifen	Germline	Efficacy
DPYD	Capecitabine, fluorouracil	Germline	Toxicity
MLH1,MSH2, MSH6, PMS2	Fluorouracil	Germline	Efficacy
SLCO1B1	Methotrexate	Germline	Toxicity
SLC28A3	Anthracyclines	Germline	Toxicity
TCL1A	Aromatase inhibitors	Germline	Toxicity
TPMT	Mercaptopurine, thioguanine, cisplatin	Germline	Toxicity
UGT1A1	Imatinib	Germline	Toxicity

Modified from Filipski 2014

In contrast, sequencing tests can detect variants located anywhere in the sequenced region, including variants that have not previously been identified. These tests are particularly useful for highly polymorphic genes that are known to contain many rare variants. Although sequencing alleviates some of the limitations associated with genotype-based testing by allowing for all variants to be detected, the interpretation can still be challenging, and testing may identify new variants with unknown function and impact on drug disposition.

Repurposing the sequencing data generated for one application to benefit the patient in other ways allows for the data to have more value; one such purpose is preemptive pharmacogenomics [10].

There are three types of NGS: whole exome sequencing, whole genome sequencing, and targeted gene panels. Unlike genotyping of candidate genes and genome wide association studies, WGS can identify rare variants that maybe very important in the genomic contribution to treatment response and toxicity [4].

Until recently, PGx testing has been expensive, often in the range of hundreds of dollars to over $1000 to test a single gene and well into the thousands of dollars for gene panels. Therefore, the widespread clinical adoption of PGx-based medicine has been partially prohibited by cost. However, rapidly improving technology has allowed testing costs to drop significantly, such that gene panels are becoming increasingly affordable to patients. Due to the increasing affordability of panels, the transition from reactive to preemptive testing has begun [10].

At the Indiana University model the Laboratory Implementation working group included individuals experienced in PGx testing and in establishing a clinical genetics laboratory. The team, after extensive research, selected state-of-the-art microarray, automated DNA extraction, and sample handling systems [17].

4.7 Resources for Interpreting Variants

Precision genomic oncology—applying high throughput sequencing (HTS) at the point- of-care to inform clinical decisions—is a developing precision medicine paradigm that is seeing increasing adoption. However, interpreting individual variants remains a significant challenge, relying in large part on the integration of observed variants with biological knowledge. Provide an organized set of biological knowledge bases with relevance to the interpretation of small variants, defined as single nucleotide variants or short (on the order of 20 base pairs or fewer) insertions and deletions.

Because of the vast quantities of genomic data and, specifically, DNA variants, there is a tension between providing rich, highly curated information about individual variants and producing the largest possible catalog of variants with manageable levels of curation [22, 12]. (Table 4.4).

Comprehensive catalogs of germline variants inform decisions about the frequency of variants as seen in the general population as well as to identify variants that are annotated as cancer associated. Additional germline databases that catalog disease-associated variants can be useful to begin to address familial risk and potentially pharmacogenomic loci. Perhaps the oldest of the variant catalogs, dbSNP contains 325,658,303 individual variant records While the vast majority of variants in dbSNP have been observed in individuals without cancer, somatic variants are included and annotated in the database. Because dbSNP is driven by community submission of variants, levels of evidence vary among individual variants.

Whereas databases of germline variants are useful to filter out variants unlikely to be directly involved in carcinogenesis, data-bases of somatic variants are useful to identify variants and their frequencies as observed in tumors. The Catalog of Somatic Mutations in Cancer (COSMIC) database is perhaps the largest and best-known cancer variant database [22].

The German hospitals mainly used the COSMIC2, ClinVar3, and dbSNP4 resources to analyze and annotate the results of a genetic test [20].

Determining the clinical relevance of experimentally observed cancer variants remains a challenge in the application of HTS in clinical practice. Difficulties in differentiating driver and passenger mutations, lack of standards and guidelines in reporting and interpretation of genomic variants, lack of clinical evidence in associating genomic variants to clinical outcome, lack of resources to disseminate clinical knowledge to the cancer community, and the precise definition of actionability have been reported to contribute to the bottleneck [22].

Table 4.4 Catalogs of germline and somatic variants[22]

Resource	Variant Type	URL
dbSNPa	Germline and somatic	www.ncbi.nlm.nih.gov/projects/SNP/
COSMICa	Somatic	cancer.sanger.ac.uk/cosmic
ClinVara	Germline predisposition and somatic	www.ncbi.nlm.nih.gov/clinvar/intro/
gnomADb	Germline	gnomad.broadinstitute.org/
69 genomes from CGIc	Germline	www.completegenomics.com/public-data/69-genomes/
Personalized genome project	Germline	www.personalgenomes.org
NCI genomic data commons	Germline and somatic	portal.gdc.cancer.gov/
cBioPortal	Somatic	www.cbioportal.org
Intogen (partial TCGA dataset)	Somatic	www.intogen.org/search

Modified from Tsang 2014

4.7.1 PGx Knowledge

The first ethical challenge in the implementation of PM is thus to determine when evidence has reached a sufficient level of certainty to warrant clinical introduction. In considering the available evidence, relevant factors include the scope of estimated benefit, existence of alternative treatments, nature and scope of potential harms, and the overall quality of evidence [23]. The level of evidence required to fully translate pharmacogenomic discoveries into the clinic has relied heavily on randomized control trials [4]. Two key drivers that are holding back the adoption of PGx testing are the lack of expansive, strong clinical evidence supporting the routine and prospective use of genetic testing and the void of health economic data linking genetic testing with reductions in cost of care [1, 17].

Although randomized controlled trials (RCTs) are recognized as the gold standard by which to evaluate the clinical utility of a new drug, this study design is not ideal for measuring the benefit of pharmacogenetic testing, as clinically significant genetic variants are often present in only a small percentage of a given patient population [4, 24].

RCTs in patients with specific genetic polymorphisms may be precluded on ethical grounds. For example, it would be unethical to as sign patients who are homozygous for nonfunctional variants of the thiopurine S-methyltransferase gene (TPMT) to receive normal versus reduced doses of thiopurines, as the mechanism of TPMT variation is related to pharmacokinetics and it is known that normal doses of the drugs could result in lethal toxicity [12, 24].

Due to the challenges of performing pharmacogenetic RCTs (e.g., the high numbers of participants needed, ethical and cost issues), knowledge must be derived from non-RCT sources such as observational studies (e.g., case reports, cross-sectional studies, case-controlled studies) and pharmacokinetic and pharmacodynamic studies, including in vivo and in vitro studies, aimed at linking drug effects to genetic variation. Including the ability to compare larger numbers of subjects at a lower cost and with few ethical concerns [24].

Conventional approaches to clinical trials design may be inadequate due to molecular heterogeneity of tumors derived from a single primary tissue, leading to the adoption of basket, umbrella, and hybrid trials designs [22].

This is being achieved through advances such as a Foundation One report which provides whole exome sequencing of known cancer genes to identify drivers and prognostic markers of cancer, provide insight into the complex biology of why certain therapies work for some individuals and not others and define which patients are most appropriate for a particular clinical trial. Foundation Medicine recently launched Foundation ACCESS™ Trial Navigator, a service to health care providers to refer patients to appropriate clinical trials based on their clinical and genomic profiles (http://www.foundationone.com) [7].

Trials such as the National Cancer Institute'sMPACT trial is prospectively evaluating tumor mutations for actionable variants and then randomizing patients to receive a drug targeting the aberrant pathway or standard of care regardless of tumor site [4].

4.8 Clinical Practice Guidelines

As new tests enter clinical practice, appropriate support of clinician and patient choice is an ethical concern. Rarely is there a single right way to use a test, and appropriate use of any intervention must be individualized to the circumstances of the patient, including comorbidities and social factors contributing to quality of life. Given the evidence gaps that are common when tests are introduced into practice, clinicians and patients also need to consider what uncertainties they are most comfortable with and how those judgments should determine the use of a new PGx intervention [23].

The National Institute for Health and Clinical Excellence reported that a new clinical procedure can take up to 3 years to become standard of care. The time required for clinical translation (research to clinical adoption) often exceeds 10 years (range of 10–25 years) [17].

Because anticancer drugs usually show high toxicity and/or narrow therapeutic index, the potential of PGx biomarkers in anticancer therapy is particularly high. PGx information may be used in clinical practice at diverse levels: to stratify patient populations (patient selection biomarkers) into those who should or should not receive a given drug, as a biomarker of clinical response, with drug dose adjustment based on PGx tests or to substitute therapeutic drug monitoring for pharmacogenomic information [5]. The clinical utility of a biomarker test can be generally defined as "the conclusion that a given use of a biomarker test will lead to a net improvement in health outcome or provide useful information about diagnosis, treatment, management, or prevention of a disease [25]."

When incorporating PGx testing into routine clinical practice, we should bear in mind that the genotype is a surrogate biomarker of the metabolic status or the clinical response of a determined individual, with a determined drug, at a determined dose, and in a particular situation. Another issue is ethnic variability because commonly used genotyping tests may not be well suited to all human populations [5, 12].

The Clinical Pharmacogenetics Implementation Consortium (CPIC), the PGx Knowledge Base (PharmGKB), or the St. Jude Children's Research Hospital as well as other international initiatives, which use standardized approaches to evaluate the literature are particularly active in providing guidelines for a growing number of gene–drug pairs and therapeutic recommendations based on pharmacogenomic testing, therefore they are essential for the implementation of PGx into routine clinical genetic test results [5, 24].

The PGx Research Network (PGRN), was founded in 2000 and funded by NIH, is a group of investigators who lead research in the discovery of how genomic variation affects therapeutic and adverse drug effects.

The PharmGKB is a National Institutes of Health (NIH)–funded comprehensive online resource established in 2000 and managed by a scientific team at Stanford University that collects, curates, and disseminates knowledge about the impact of human genetic variation on drug responses [11, 24].

In 2008 and 2011, the Dutch Pharmacogenetics Working Group (DPWG) provided a listing of pharmacogenetics recommendations to address the need for clinical practice guidelines that facilitate the translation of genetic laboratory test results into actionable prescribing recommendations for specific drugs [3, 24].

In 2009, the Clinical Pharmacogenetics Implementation Consortium was organized established as a shared project of PharmGKB, with the first guideline published in 2011 [17, 24].

The CPIC membership now includes over 160 pharmacogenetics experts (clinicians and scientists) from 86 institutions and 16 countries as well as multiple observers from NIH and the Food and Drug Administration (FDA). To date, CPIC has published 19 gene–drug guidelines, 6 of which were recently updated [24].

These guidelines are based on the assessment of a known gene–drug relationships, the identification of content experts and the formation of writing committee, retrieval, summarization and presentation of the evidence linking genotype to drug variability, development of therapeutic recommendation and assignment of strength of the recommendation, internal and external review, and periodic review and guideline updates [5]. Thus there is now more detailed gene–drug guidance available to assist clinicians in interpreting a genetic test result and altering therapy based on that result [24].

CPIC guidelines are designed to help clinicians understand how available genetic test results should be used to optimize drug therapy not whether ordering a genetic test is appropriate. The underlying assumption governing CPIC guidelines is that genomic testing results will increasingly be available and clinicians will be faced with having a patient's relevant pharmacogenetic genotype available even if they did not order a test with a specific gene or drug in mind. Therefore, the question will become not whether to test but how to effectively use the pharmacogenetic information that is becoming increasingly available [24].

For the implementation of all the models they used the CPIC as the main source of peer-reviewed clinical guidelines addressing specific drug–gene interactions. And complement them with clinical guidelines published by medical societies and other professional groups and original publications [6].

There are 3 CPIC guidelines of interest for Oncology untill now: The Guideline for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing [26], the Guideline for CYP2D6 and Tamoxifen Therapy [27], and the Guideline for CYP2D6 Genotype and Use of Ondansetron and Tropisetron [28]. And they have planned to do the Guideline for Gene Encoding UDP Glucuronosyltransferase Family 1 member A1 (UGT1A1) and Irinotecan [24].

The Guideline for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing was updated in 2017, the purpose of this guideline is to provide information for the interpretation of clinical dihydropyrimidine dehydrogenase (DPYD) genotype tests so that the results can be used to guide dosing of fluoropyrimidines, 5-fluorouracil (5-FU) and capecitabine that are widely used in the treatment of solid tumors including colorectal and breast cancer, and cancers of the aerodigestive tract. Approximately 10–40% of fluoropyrimidine-treated patients develop severe and some- times life-threatening toxicity (neutropenia, nausea, vomiting, severe diarrhea, stomatitis, mucositis, hand-foot syndrome). Only 1–3% of the administered 5-FU is metabolized to cytotoxic metabolites, with all most 80% of the administered administered dose being degraded and the rest excreted in the urine. DPYD is the first and rate-limiting step in the catabolic pathway converting 5-FU to dihydrofluorouracil (DHFU). DPYD levels show high inter- and intraindividual variation, which influences 5-FU exposure. Reduced activity of DPYD results in reduced clearance and increased half-life of 5-FU, and it can cause profound dose-related toxicities. In a meta-analysis combining data from eight cohort studies the association of four DPYD variants with severe fluoropyrimidine-related toxicity was demonstrated.

They divide patients in 3 different phenotypes: DPYD normal metabolizer if they have normal DPYD activity and "normal" risk for fluoropyrimidine toxicity, DPYD intermediate metabolizer if they have decreased DPYD activity (leukocyte DPYD activity at 30% to 70% that of the normal population) and increased risk for severe or even fatal drug toxicity when treated with fluoropyrimidine drugs; and DPYD poor metabolizer if they have complete DPYD deficiency and increased risk for severe or even fatal drug toxicity when treated with fluoropyrimidine drugs. Based on genotype they asses dossing recommendations, for a normal metabolizer there is no indication to change dose or therapy (Use label-recommended dosage and administration), for an intermediate metabolizer reduce starting dose based on activity score followed by titration of dose based on toxicity or therapeutic drug monitoring (if available). Activity score 1: Reduce dose by 50% Activity score 1.5: Reduce dose by 25–50%. And for a poor metabolizer with activity score 0.5: Avoid use of 5-FU or 5-FU prodrug-based regimens. In the event, based on clinical advice, alternative agents are not considered a suitable therapeutic option, 5-FU should be administered at a strongly reduced dosed with early therapeutic drug monitoring. Activity score 0: Avoid use of 5-FU or 5-FU prodrug-based regimens.

The benefit of DPYD genotyping has been demonstrated in a prospective study, which showed a reduced occurrence of severe 5-FU-related toxicity and no toxicity-related deaths in carriers of c.1905 + 1G > A after genotype-guided dose reduction. Conversely, not all carriers of DPYD decreased/no function variants develop severe toxicity at standard doses. As a consequence, some carriers of such variants may not receive the full benefit of fluoropyrimidine therapy with the recommended dose reductions [26].

Guideline for CYP2D6 and Tamoxifen Therapy, the purpose of this guideline is to provide clinicians information that will allow the interpretation of clinical CYP2D6 genotype tests so that the results can be used to guide prescribing of tamoxifen. Tamoxifen, a selective estrogen

receptor modulator (SERM), has been studied and utilized in breast cancer for more than 40 years. When administered to women with ER-positive breast cancer for 5 years after surgery, tamoxifen almost halves the annual recurrence rate and reduces the breast cancer mortality rate by one-third in both pre- and postmenopausal women.

Tamoxifen undergoes extensive primary and secondary liver metabolism by cytochrome P450 enzymes via two major pathways: N-demethylation and 4-hydroxylation. The predominant metabolic pathway (considered to contribute to over 90% of tamoxifen metabolism) is the demethylation of tamoxifen to N-desmethyltamoxifen primarily mediated by CYP3A4, followed by CYP2D6-mediated oxidation to 4-hydroxy-N-desmethyltamoxifen (endoxifen). A minor metabolic pathway is hydroxylation of tamoxifen (mediated mainly by CYP2D6 but also catalyzed by CYP3A4 and CYP2C19) to 4-hydroxytamoxifen (4HT), which can then be further metabolized to endoxifen.

The hydroxylation of either tamoxifen or N-desmethyltamoxifen is considered to bioactivate tamoxifen. Both 4HT and endoxifen exhibit nearly 100-fold greater antiestrogenic potency than the parent drug. Maximum inhibition of estrogen-induced stimulation and ER transcription is achieved with endoxifen concentrations ranging between 100–1000 nanomolar (nM).

Clinical studies to evaluate the association between endoxifen concentrations and CYP2D6 polymorphisms with tamoxifen outcome have yielded conflicting results. Initial and follow-up data, demonstrated that CYP2D6 poor metabolizer (PMs) had a two–threefold higher risk of breast cancer recurrence (compared to CYP2D6 normal metabolizer (NMs) and led an FDA special emphasis panel to recommend a tamoxifen label change to incorporate data that CYP2D6 genotype was an important biomarker associated with tamoxifen efficacy.

Regarding the role of measurement of endoxifen concentrations, Madlensky et al. identified an association between low endoxifen (lowest quintile) and recurrence. In a separate study of premenopausal patients, Saldores et al. demonstrated similar findings that patients with low endoxifen concentrations (<14 nM) exhibited a higher risk for distant relapse or death compared with those with high concentrations (>35 nM).

The potential benefit of using CYP2D6 genotype to guide tamoxifen use is that patients with genotypes that are associated with a higher risk of breast cancer recurrence and worse event-free survival [e.g., CYP2D6 intermediate metabolizer (IMs) and PMs] may be identified and alternative doses (e.g., 40 mg) and agents administered. Given that the alternative drug treatments (aromatase inhibitors either with or without ovarian function suppression) have been demonstrated to be superior to tamoxifen, and that CYP2D6 PMs switched from tamoxifen to anastrozole do not exhibit an increased risk of recurrence, it is expected that the risks to use CYP2D6 genotyping to guide hormonal treatment would be low [27].

Guideline for CYP2D6 Genotype and Use of Ondansetron and Tropisetron, it purposes to provide information to allow the interpretation of clinical CYP2D6 genotype tests so that the results can be used to guide use of the 5-hydroxytryptamine type 3 (5-HT3) receptor antagonists, ondansetron and tropisetron. The 5-HT3 receptor antagonists are used in the prevention of chemotherapy-induced, radiation-induced, and postoperative nausea and vomiting. CYP2D6 polymorphisms can influence the metabolism of some of these drugs, thereby affecting drug efficacy. The 5-HT3 receptor antagonists are generally well tolerated; mild headache, constipation, and transient elevations in liver enzymes are common side effects. Ondansetron has also been associated with cardiac adverse events such as corrected QT prolongation. Ondansetron is metabolized to four inactive metabolites by multiple CYP enzymes, including CYP3A4, CYP1A2, and CYP2D6, followed by glucuronide conjugation to metabolites not clinically relevant for pharmacologic activity. Tropisetron is extensively metabolized by CYP2D6 to inactive metabolites

and further conjugated to glucuronides and sulfates. The potential benefit of using CYP2D6 genotype to guide ondansetron and tropisetron use is that patients with genotypes that are associated with a decreased response (e.g., CYP2D6 UMs) may be identified and alternative antiemetics administered. At this time, the evidence does not justify increasing the dose in CYP2D6 UMs because dose adjustments based on CYP2D6 UMs have not been studied and a detailed recommendation of dosing for the different CYP2D6 phenotypes is missing [28].

4.8.1 PGx Education

In prospect of whole genome sequencing, the discovery of new gene-drug interaction pairs is very likely and will further increase the pharmacogenomic knowledge base. However, translating this pharmacogenomic knowledge into clinical routine has been slow and is hindered by the lack of the physicians' knowledge and experience in pharmacogenomic testing [21].

Providers lack PGx knowledge, leading to problems with ordering and understanding the results of PGx testing and communicating the clinical impact of these results to their patients [3, 6].

It is also imperative to identify and to align key clinicians in support of the pharmacogenomics endeavor. Because clinical pharmacogenomics encompasses knowledge from a broad spectrum of medical subspecialties, identifying clinicians with interest and expertise in this field is a challenge. These individuals played a critical role in the education of their colleagues [18].

In some models the basic education on clinical genetics and the evolving science of PGx was provided to the clinical teams, education was designed not only for busy clinicians (e.g., physician, residents, nurse practitioners, and physician's assistants) but also for pharmacists who were responsible for responding to inquiries from both clinicians and patients [6, 17]. Clinicians who treat outpatients must also clearly understand the tests that are available, when to order them, and what to do in the event of receiving "clinical alerts" [17].

General information about genomic medicine and PGx principles can be delivered via conferences, newsletters, and other means. These can include lectures, recorded grand rounds, short educational videos, blended learning courses, video conferences, targeted emails, and competency-based online training for pharmacists [6].

4.9 Cost-Effectiveness of PGX Implementation

Demonstration of PGx cost-effectiveness would facilitate acceptance and implementation by hospital systems and payers. The eMERGE-PGx Project implemented a PGx-sequencing panel at multiple sites, and explored diverse approaches to designing and implementing PGx-based CDS alerts and collecting outcomes. Although no cost-effectiveness analyses were conducted in this preliminary effort, the collection of clinic/facility-level economic outcomes and expanded development of well-validated instruments to assess implementation outcomes particularly relevant to PGx should be considered in the future. In the meantime, the sites are collaborating to report descriptive metadata and define quantitative and qualitative outcomes across many domains pertinent to cost-effectiveness analyses. More extensive economic analyses for pharmacogenomics is underway at Vanderbilt University [12].

It is important to study whether PGx testing has direct patient-care benefits. Outcomes of interest would include decreasing common and rare ADRs, lowering the need for outpatient visits, reaching intended therapeutic effects faster or in a greater percentage of the population, and reducing the cost of care.

Preemptive genotyping of patients likely to receive PGx-relevant drugs represents the most efficient method of PGx implementation and

obviates the need for clinicians to initiate the testing [12].

4.10 Uses of PGx in Current Oncology

Testing for inherited cancer risk offers another important benefit of genomic research. Although the etiology of most cancer is multifactorial, several inherited cancer syndromes have been identified that result in high lifetime risks for specific cancers. Examples include hereditary breast ovarian cancer syndrome, conferring risk of breast and ovarian cancer, and Lynch syndrome, conferring risk of colorectal cancer. Discovery of the genes associated with these cancer syndromes has enabled genetic testing to identify individuals at risk, who can then be offered a range of preventive interventions. As with PGx, active ongoing research is identifying additional genes associated with cancer risk, which will increase opportunities for this testing approach over time [23].

One of the most recent developments in the field of precision oncology Pembrolizumab (Keytruda), an anti-PD-1 antibody that functions as a checkpoint inhibitor. On May, 2017, the US Food and Drug Administration (FDA) granted it accelerated approval for the treatment of adult and pediatric patients with unresectable or metastatic solid tumors that have been identified as having microsatellite instability-high (MSI-H) or MMR deficient (dMMR). This is a historical approval as the first time a cancer treatment is approved based on a common biomarker rather than the anatomic location in the body where the tumor originated, it allows a drug to be used in a non-tissue specific context [22, 29].

The genomes of mismatch repair-deficient tumors all harbor hundreds to thousands of somatic mutations, regardless of their cell of origin. Le, et at, evaluated the efficacy of PD-1 blockade in a range of different subtypes of mismatch repair-deficient cancers. Twelve different cancer types were enrolled in the study and all enrolled patients had evidence of mismatch repair-deficiency as assessed by either polymerase chain reaction or immunohistochemistry. For most cases, germline sequencing of MSH2, MSH6, PMS2 and MLH1 was performed to determine if the mismatch repair-deficiencies were associated with a germline change in one of these genes. Objective radiographic responses were noted in 53% of patient, with 21% achieving a complete radiographic response. Disease control was achieved in 77%. The estimates of PFS at 1- and 2-years were 64% and 53%, respectively. The estimates of OS at 1- and 2- years were 76% and 64%.

To estimate the fraction of cancer patients to which the results of this study might be applicable, they evaluated 12,019 cancers and found that >5% of adenocarcinomas of the endometrium, stomach, small intestine, colon and rectum, cervix, prostate bile duct and liver, as well as neuroendocrine tumors, non-epithelial ovarian cancers and uterine sarcomas, were dMMR. Because genetic and immunohistochemical tests for dMMR are already widely available, these results tie immunity, cancer genetics, and therapeutics together in a manner that will likely establish a new standard-of-care and in the future, testing for MMR- deficiency in patients' refractory to other treatments might be considered in order to identify those who may benefit from PD-1 pathway blockade, regardless of tumor type [30].

Similarly, nivolumab, also a PD-1 inhibitor, has shown efficacy on MSI-H/dMMR tumors too. The phase 2 Checkmate-142 study evaluated nivolumab in patients with MSI-H or dMMR metastatic colorectal cancer(CRC). Overall, 32% patients responded to nivolumab, including 2.7% with a complete response. Among patients who had progressed after receiving prior treatment with fluoropyrimidine-, oxaliplatin-, or irinotecan-based chemotherapy, 28% responded to nivolumab. On August, 2017, the FDA granted an accelerated approval for nivolumab as a treatment for patients with MSI-H or dMMR metastatic CRC after progression on standard chemotherapy [29].

Oncogenic TRK fusions directly induce cancer cell proliferation and activate various downstream signaling path- ways. These TRK fusions occur rarely, but in a diverse spectrum of histological tumor types. Larotrectinib (LOXO-101) is the first selective small-molecule pan-TRK inhibitor. Larotrectinib blocks the ATP-binding

site of the TRK family of receptors, TRKA, TRKB, and TRKC. TRK-fusion status was prospectively determined by a variety of local testing methods, including DNA sequencing, RNA sequencing, next-generation sequencing (NGS), fluorescent in situ hybridization (FISH), and IHC analyses.

Larotrectinib could be the first targeted therapy developed in a tissue type-agnostic manner to address patients with NTRK-fusion tumors [29]. The U.S. Food and Drug Administration (FDA) has accepted the New Drug Application (NDA), and granted Priority Review for larotrectinib for the treatment of adult and pediatric patients with locally advanced or metastatic solid tumors harboring a neurotrophic tyrosine receptor kinase (NTRK) gene fusion [31].

Cetuximab, an anti-EGFR monoclonal antibody is used in the treatment of colorectal cancer, however only a subset of patients responded to treatment. Tumor samples from cetuximab treated patients were retrospectively analyzed identifying mutations in the KRAS gene that were associated with response to therapy. Patients that are mutation positive do not respond to cetuximab, leading to the development of a companion diagnostic for KRAS testing and changes to the prescribing information for cetuximab to only be used in KRAS mutation negative patients [4].

Of squamous cell adenocarcinomas, approximately 50% are thought to be driven by mutations in EGFR, ALK, or KRAS genes, while 80% squamous cell carcinomas are thought to be driven by mutations or amplifications in PIK3CA, FGFR1, and PTEN. Over the past decade, several genetically targeted therapies have been developed, including erlotinib, afatinib, gefitinib, and osimertinib to treat EGFR mutation specific cancers, and crizotinib, ceritinib, and alectinib to treat cancers positive for ALK gene rearrangement [7].

Molecularly targeted therapies like crizotinib have replaced cytotoxic therapy as standard of care in several cancer types including breast cancer, NSCLC, and melanoma. Recently a germline mutation in the proapoptotic gene BIM was associated with the resistance to tyrosine kinase inhibitors in chronic myeloid leukemia (CML) and epidermal growth factor receptor (EGFR) mutant NSCLC [4].

The 21-gene recurrence-score assay (Oncotype DX, Genomic Health) is one of several commercially available gene-expression assays that provide prognostic information in hormone-receptor–positive, human epidermal growth factor receptor type 2 (HER2)-negative, axillary node-negative breast cancer with tumors of 1.1–5.0 cm in the greatest dimension for a tumor of any grade or 0.6–1.0 cm in the greatest dimension and intermediate or high tumor grade. The Trial Assigning Individualized Options for Treatment (TAILORx) was designed to address whether chemotherapy is beneficial for that women. The recurrence score based on the 21-gene assay ranges from 0 to 100 and is predictive of chemotherapy benefit when it is high, whether a high score is defined as 26 or higher; when the recurrence score is low (0–10), it is prognostic for a very low rate of distant recurrence (2%) at 10 years that is not likely to be affected by adjuvant chemotherapy [32].

The women with a mid-range recurrence score of 11–25 were randomly assigned to receive either chemoendocrine therapy or endocrine therapy alone. Chemotherapy were docetaxel–cyclophosphamide and anthracycline-containing regimens. The endocrine therapy regimens among postmenopausal women most commonly included an aromatase inhibitor; among premenopausal women, endocrine therapy regimens most commonly included either tamoxifen alone or tamoxifen followed by an aromatase inhibitor, and suppression of ovarian function was used in 13% of premenopausal women. At 9 years, the two treatment groups had similar rates of invasive disease–free survival (83.3% in the endocrine-therapy group and 84.3% in the chemoendocrine – therapy group), freedom from disease recurrence at a distant site (94.5% and 95.0%) or at a distant or local–regional site (92.2% and 92.9%), and overall survival (93.9% and 93.8%), with some benefit of chemotherapy found in women 50 years of age or younger with a recurrence score of 16 to 25. The results of this trial suggest that the 21-gene assay may identify up to 85% of women with early breast cancer who can be spared adjuvant chemotherapy, especially

those who are older than 50 years of age and have a recurrence score of 25 or lower, as well as women 50 years of age or younger with a recurrence score of 15 or lower. It was a prospective clinical trial, a type of trial that provides the highest level of evidence supporting the clinical usefulness of a biomarker [33].

Some treatment decisions are less clear cut. For example, as we know patients with two defective CYP2D6 alleles are more likely to experience recurrence of breast cancer after treatment with tamoxifen. However, it is unclear whether the best alternative is another drug (a different selective estrogen-receptor modulator, for instance) or an altered dose of tamoxifen, particularly in premenopausal women for whom there is a shortage of data to support alternative treatments. Although clear from pharmacogenomic testing that the drug or drug dose is suboptimal in a patient with the high-risk genotype, a lack of clinical data for alternative therapies make it difficult to recommend other medications [8, 27].

SNPs in the ESR1 gene were shown to be associated with thromboembolic events in women treated with tamoxifen. These events are relatively rare (1.7–8.4%) therefore using this large practice-based cohort, investigators were able to capture enough events to evaluate the underlying genetic difference in women with and without thromboembolic events [34].

Severe musculoskeletal pain has been reported in up to half of women treated with aromatase inhibitors contributing to a treatment discontinuation rate of about 10%. Ingle et al. found four single nucleotide polymorphisms (SNPs) mapping to the T-cell leukemia 1A (TCL1A) gene were associated with the development of musculoskeletal adverse events in patients receiving adjuvant aromatase inhibitors [35].

A GWAS identified SNPs annotated to the transporter gene, SLCO1B1 that were associated with methotrexate clearance. Deep resequencing of the gene was done and Ramsey et al. found that rare variants, only identified through deep sequencing accounted for 17.8% of the genes effect on clearance [5].

For capecitabine, fluorouracil, tegafur and DPYD, recommendations are to consider alternative drugs for homozygous individuals, or to reduce by about a 50% the starting dose and to adjust doses based on the toxicity or the pharmacokinetics [5, 26].

Cisplatin is an effective and widely used chemotherapeutic agent for the treatment of solid tumors. Dose-limiting side effects of cisplatin include ototoxicity usually bilateral and irreversible. Reports demonstrate some degree of hearing loss in 75–100% of cisplatin-treated patients. The mechanism for cisplatin ototoxicity is the acute and chronic generation of reactive oxygen species (ROS), in addition to DNA damage. Glutathione-S-transferase subclasses M1, T1 and P1 have been demonstrated to influence the outcome of chemotherapy with platinum compounds and are directly involved in their detoxification, the GSTM1 positivity have showed detrimental effects related to ototoxicity, as the megalin A allele SNP rs2075252, the genetic variants rs1220119 in thiopurine-S-methyl-transferase (TMPT) and rs9332377 catechol-O-methyl-transferase COMT, and (xeroderma pigmentosum type C) XPC [36].

4.11 Ethical Considerations

Patients may vary in their awareness and acceptance of cancer prevention or other measures recommended as a result of a PGx test. Information aids will likely need to be developed for different media and using a range of examples and illustrations such that they can be tailored to specific patient needs, including health literacy levels and acceptable formats. Failure to do so may inadvertently result in PGx bypassing population groups.

An additional issue arises when a PGx test identifies a risk that is shared among family members. Clinicians and healthcare systems have an ethical duty to inform patients of this family risk, and to counsel them about contacting family members to inform them of the benefits of testing. How far this duty extends, however, is a matter of debate. Arguably the duty is assumed by the patient once he or she has been counseled about the importance of informing family members. Even so, many patients are unlikely to be able to discharge this duty without assistance from healthcare systems.

Failure to create clinical practice guidelines with a broader, and electronic, implementation view in mind may mean that smaller clinics, community-based clinics, and clinics in rural locales may not be able to incorporate the guidelines or decision support into their EHR systems. Because these clinics often serve populations more diverse than large academic medical centers, such a lacuna has the possibility of excluding those populations from the health benefits of genomic medicine [23].

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5 Onco-omics Approaches and Applications in Clinical Trials for Cancer Patients

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Abstract

Omics technologies have revolutionised fundamental and medical research. Oncology is perhaps the field where these technologies have been most rapidly adopted and where they have had their biggest impact, dramatically transforming clinical practice guidelines over a very short period of time. Along with this transformation has come an even larger array of technologies, tools and jargon, that make following the most recent developments in the field a truly daunting task for those not involved in it. This chapter is intended to provide a general overview of evolving topics in oncology research in the era of big data analysis and precision medicine, with a specific focus on the use of tumour biomarkers, tumour biomarker tests, targeted drugs and the changing landscape of clinical trial designs.

Keywords

Clinical trials · Omics · Personalised oncology · High-throughput technologies · Next Generation Sequencing · Tumour biomarkers · Multi-gene panels · Biomarkers Guidelines · Umbrella trial · Basket trials

5.1 Introduction

The plethora of medical discoveries and scientific advancements during the second half of the twentieth century as well as the development of the "Human Genome Project" at the turn of the twenty-first century resulted in an exponential increase in the number of technologies and computational tools that allow for the simultaneous measurement and analysis of thousands of biomolecules, giving rise to the "omics" revolution. The word "omics" refers to several molecular disciplines that use high-throughput methods to characterise and quantify very large sets of biological molecules such as DNA (genomics), mRNAs (transcriptomics), proteins (proteomics), and metabolites (metabolomics). Since the appearance of these four "omics" disciplines, several other areas of biomedical research have

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adopted the omics suffix such as 'lipidomics' and 'glycomics', which technically are subsets of metabolomics devoted, respectively, to the comprehensive study of lipids and sugars.

High-throughput 'omics' technologies are among the most rapidly adopted and exploited tools in research, particularly in the field of oncology where the molecular complexity and heterogeneity of cancers have long been a challenge, requiring a systems-level analysis to discover new fundamental insights into its pathophysiology. As a result, the last decade has seen a dramatic increase in the number of published studies referring to a new era of 'personalised medicine' as a paradigm shift in patient care.

Personalised oncology has been broadly defined as 'getting the right treatment to the right patient at the right time, dose and schedule' [1], an aim that is not new to medicine. Indeed, the notion that different pathologies require different interventions is as old as medicine [2]. However, it is only recently that high-throughput technologies have made it possible to characterise tumours in such depth that it has become evident that tumours from the same anatomic site and with similar histology are not necessarily the same disease at a molecular level. In other words, omics technologies have enabled the discovery of a new set of measurable characteristics (or markers) by which similar tumours can be differentiated, thus refining diagnoses and opening up the possibility to rationally design treatments that improve patient care and outcomes. Cancer biomarkers were increasingly linked to specific molecular pathway alterations important for cancer pathogenesis, which in turn formed the rationale for the development of molecularly targeted drugs. This newer approach to cancer diagnosis and treatment has transformed the long-standing goal of personalised medicine into a clinical reality.

Nevertheless, what we call 'personalised medicine' today is perhaps more accurately labelled as 'stratified medicine' because, to date, treatment customisation is restricted to identifying subgroups of patients that are more likely to benefit from a limited set of available treatments. One major challenge is to integrate high-throughput data from different sources in order to model the complex network of interactions between genes, transcripts, proteins and metabolites and thus identify molecular targets for each individual. Personalised medicine will truly materialise when the comprehensive characterization of a patient's tumour can be used to tailor therapies individually. Consequently, the identification of reliable and specific biomarkers as well as the development of new network computing system technologies continue to be fundamental areas of research.

5.2 Onco-omics Approaches

5.2.1 What are High-throughput Technologies

Although it is beyond the scope of this chapter to provide a compendium of accepted definitions, it is necessary at this point to clarify some of the most frequently used terminology in the field of onco-omics research. So far, we have mentioned the emergence of 'high-throughput sequencing' (HTS) technologies, but what exactly are they? Although HTS technologies differ in their details and specific application, they follow essentially the same principle: template preparation, clonal amplification, and cyclical rounds of parallel sequencing (spatially separated) that enables to determine the order of nucleic acid residues of millions of different strands of DNA from a single biological sample.

With reduced costs, several HTS methods have been developed with applications that go beyond the sequencing of genomes. HTS can be used to perform analyses of genome methylation (BS-seq, RRBS-seq), transcripts (RNA-seq), genome-wide mapping of DNA regulatory elements (ChIP-seq), translation (ribo-seq), chromatin 3D conformation, RNA structure (PARS, ATAC-seq), RNA-protein interactions (RNA-MaP, RIP-seq), microRNA target discovery (PARE-seq). Although some of these technologies were initially developed using DNA microarrays, they are enabled by sequencing [3], primarily next-generation sequencing (NGS).

The significant cost reduction of NGS in the last decade, has opened up the possibility of using gene panels to simultaneously sequence multiple genes, not only in academic institutions and research centres but also in the clinic to guide therapeutic decisions. NGS can be used to determine either the nucleotide sequence of DNA coding regions alone (whole-exome sequencing; WES) or the entire genome, which includes the exome as well as non-coding regions, or introns (whole-genome sequencing; WGS). NGS has the advantage of detecting other potential gene alterations not found in hotspots. Although WGS would provide the most complete genomic analysis of a tumour, its clinical applicability is constricted by its high cost, turnaround time as well as by the vast amount of information, which is not always actionable and which requires complex bioinformatic and computational tools to be analysed. In contrast, WES represents a more viable technique that could be incorporated as a routine clinical test. Nevertheless, to date, targeted exome sequencing of a panel of preselected genes continues to be the most commonly used tool to molecularly profile tumours [4].

5.2.2 Omics

Genomics is concerned with the study of the genome: the entire DNA sequence, organization of genetic material, and functional consequences of sequence variations through the mapping of somatic mutations, gene copy number variations, and profiling gene expression alterations using genomic technologies, primarily NGS. However, genomics tools cannot identify changes in the expression of DNA that are not associated with variations in the nucleotide sequence, such as those caused by DNA methylation and chromatin remodelling, which are studied using epigenomics tools [5].

Results from the Human Genome Project revealed that only a small percentage of the genome is expressed in each cell, and that a portion of the RNA synthesised in the cell, is specific for that cell type. Transcriptomics refers to the study of the transcriptome: the entire genome expressed as RNA of a specific tissue cell type, at a particular stage of development, under physiological or pathological conditions [6]. Genes lack any catalytic or signalling capabilities; they may not reflect key dynamic changes occurring in the cell. Instead they exert their effects through translation into active proteins.

Moreover, key natural biological processes such as RNA alternative splicing and post translational modification of proteins (e.g. phosphorylation, glycosylation, ubiquitination, acetylation, nitrosylation, methylation, proteolysis) lead to inherent limitations in genomic and transcriptomic studies, opening up the field for proteomic approaches.

Clinical proteomics refers to the application of proteomics in clinical practice, which involves the use of data obtained from proteomic approaches to determine and improve diagnostic, predictive and prognostic values and to elucidate underlying molecular pathways [7]. In the study of cancer, clinical proteomics may be able to reveal novel diagnostic biomarkers that can be used to help monitor prognosis, disease progression and response to treatment. The accessibility of cancer-related proteins in tissues and body fluids (serum, plasma, tissue, urine, cerebrospinal fluid, saliva, nipple fluid, ascites, pleural fluid etc) has triggered extensive protein-focused research to detect biomarkers [8], which will be discussed in further detail below.

5.2.3 Tumour Biomarkers vs Tumour Biomarker Tests

The definition of biomarkers has evolved over the last decade to encompass the growing range of analytes and their applications. The term ‘biomarker’ refers to a defined characteristic, factor or process that can be measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or interventions, including therapeutic interventions [9].

Tumour biomarkers were initially defined as ‘factors or processes found in malignant tissues but not in normal tissues or biospecimens’ [2]. Unfortunately, this definition is no longer valid as

circulating tumour cells (CTCs), cell-free circulating tumour DNA (cfDNA) and RNA (cfRNA) can be found in blood samples from cancer patients [10]. Perhaps, a tumour biomarker ought to be more generically defined as an identifiable biologic factor or a process originating in cancer cells [11].

In contrast, a 'tumour biomarker test' refers to a specific assay that is used to detect or quantify a tumour biomarker. There can be more than one 'tumour biomarker test' to detect a single 'tumour biomarker'. Similarly, biomarkers and their tests can be classified according to their application as: (a) susceptibility/risk biomarkers, which are used to assess the probability of developing a disease or a medical condition independently or after a particular exposure; (b) diagnostic biomarkers, which are used to confirm whether a disease or condition is actually present or to define a subset of the disease; (c) monitoring biomarkers, which are used to detect changes in the degree or extent of a disease; (d) prognostic biomarkers, which are used to determine the risk of clinical event or outcome such as cancer recurrence or disease progression; (e) predictive biomarkers, which can identify individuals more likely to benefit from a specific intervention, due to increased responses or reduced adverse effects; (f) response biomarkers, which can establish if a biological response has occurred following a given intervention; (g) safety biomarkers, which are used to assess the toxicity related to an intervention, as well as its grade.

5.2.4 Omics-based Tests

Furthermore, a tumour biomarker test is not always an omics-based test, the latter is defined as 'an assay composed or derived from multiple molecular measurements and interpreted by a fully specified computational model to produce a clinically actionable result'. Breast and lung cancer biomarkers and their tests will be used to exemplify these concepts, with a particular focus on the human epidermal growth factor receptor 1 (EGFR/HER1/erbB) and 2 (HER2/erbB-2 and neu in rodents).

Since the 1970s the oestrogen receptor (ER) has been used as a biomarker for the response to anti-oestrogen or 'endocrine' therapy in patients with breast cancer. Consequently, ER testing is routinely performed on diagnostic biopsies, with the ER/PR pharmDx being the most commonly used immunohistochemical (IHC) assay [12]. Similarly, gene amplification and protein overexpression of the 'human epidermal growth factor receptor 2' (HER2) occurs in approximately 25% of all breast cancers and is associated with reduced disease-free survival and overall survival. In September 1998 Trastuzumab (Herceptin®), a humanised anti-HER2 monoclonal antibody (mAb), was approved by the US Food and Drug Administration (FDA) for the treatment of female patients with HER2-positive metastatic breast cancer [13, 14]. On the same day, the FDA approved HercepTest® (DAKO), a semi-quantitative IHC assay to determine HER2 protein overexpression, as an aid in the assessment of patients eligible to receive treatment with Herceptin. Subsequently, DNA-based methodologies such as fluorescence in situ hybridization (FISH) were developed into FDA-approved tests to quantitatively determine *HER2* gene amplification, like the HER2 IQFISH pharmDx™ test and the PathVysion®HER-2 DNA Probe Kit (ABBOTT) [15]. ER and HER2 are actionable molecular targets as well as tumour biomarkers with more than one test designed to detect their expression. Although the ER/PR pharmDx and the HercepTest® are tumour biomarker tests, they are not omics-based tests.

Turning now to lung cancer biomarkers and their tests, it has been shown that mutations in the epidermal growth factor receptor 1 (EGFR/HER1/erbB) result in the constitutive or sustained activation of signal transduction pathways involved in malignant transformation [16]. The most common oncogenic EGFR mutations are short in-frame deletions in exon 19 (Del19) and a specific point mutation in exon 21 at codon 858 (L858R) [17]. These mutations are found in approximately 10–40% of lung adenocarcinoma patients [18, 19] and it makes them eligible to receive treatment with tyrosine kinase inhibitors (TKIs). Several tests can be used for the sensitive and specific

detection of EGFR mutations such as the Cobas® (Roche) EGFR Mutation Test, and the Therascreen® (Qiagen) EGFR amplification refractory mutation system assay [20]. The Cobas® EGFR Mutation Test V1 was approved in May 14, 2013 for detecting sensitizing EGFR mutations, by qRT-PCR, in DNA derived from formalin-fixed paraffin-embedded (FFPE) human NSCLC tumour tissue samples. Subsequently, in June 2016 the Roche Cobas® EGFR Mutation Test v2. became the first and currently the only FDA-approved companion diagnostic test for the detection of tumour EGFR mutations in cfDNA isolated from blood plasma of NSCLC patients. In this example, EGFR mutations are actionable molecular targets and tumour biomarkers with different tests to identify them. However, the Cobas® and the Therascreen® EGFR mutation tests are omics-based test, given the scale of the information that they process for each patient's sample.

As innovative as these DNA-based tumour biomarker tests are (HER2 IQFISH pharmDx™, PathVysion®HER-2 DNA Probe Kit, the Therascreen® and the Cobas® EGFR Mutation Test), they determine mutations and expression changes in single genes or proteins. This type of "hotspot" testing focuses on identifying well established genetic alterations that have already been associated with an effective targeted therapy. As previously mentioned, cancer is a complex disease involving the interaction of multiple genes and proteins. Until recently, the only way to overcome this limitation was to increase the number of single-marker tests performed on individual cancer tissues, with some laboratories testing for multiple individual markers at the same time (EGFR, KRAS, ALK, MET, BRAF, KRAS, NRAS, PIK3CA). However, this approach requires more tumour tissue and it significantly increases the cost of testing, which makes it an unsuitable approach in clinical practice.

Recently, several companies have commercially launched testing panels as integrated solutions, processing tumour samples to establish the particular genetic profile of a patient's tumour and providing a complete report along with treatment and/or clinical trial recommendations, specific to the patient. In June 2017, the Ion Torrent Oncomine Dx Target Test, which delivers multiple biomarker results in as little as 4 days, became the first NGS-based *in vitro* diagnostic test, approved by the FDA as an aid to select lung cancer patients eligible to receive treatment with specific FDA-approved drugs for this indication.

Then, in November 2017, FoundationOne CDx™ became the first comprehensive genomic profiling (CGP) assay approved by the FDA as a companion diagnostic test for all solid tumours, or pan-cancer. It analyses 324 genes known to drive cancer growth, reporting novel genomic biomarkers such as microsatellite instability (MSI) and tumour mutational burden (TMB), as well as providing actionable information regarding available on-label targeted therapies and relevant clinical trial information [21]. The feasibility of routinely using multi-gene panels in a clinical setting is hindered by the fact that: (a) they can deliver an unmanageable amount of information, which is not always actionable and for or which there are no clear management guidelines; (b) cancer cells are constantly evolving and thus the molecular profile of a tumour changes over time; (c) genes do not act in isolation, in many cases it is the summed contribution of all genes that will lead to a particular phenotype.

Thus, it comes as no surprise that currently, the most commonly used tumour biomarkers in clinical practice are HER2, ER, PR, EGFR, ALK, BRAF, and PD-L1 (which will be discussed in further detail later on), since there are various well-established tests to identify them as well as FDA-approved drugs to target them.

5.3 Application Of Omics-based Technologies in Next Generation Clinical Trials

5.3.1 General Guidelines

In the era of "omics" research, multidisciplinary research groups are no longer sufficient to ade-

quately integrate the vast and complex high-dimensional data that are continuously being generated. High-quality "omics" research requires the establishment of truly interdisciplinary collaborations between research groups specializing in complementary and often non-overlapping fields of science and technology (e.g. oncologists, statisticians and bioinformatics experts).

"Omics" research generates large and complex datasets that require even more sophisticated computations to be meaningfully analysed, making the independent replication and verification of results more difficult than for single analyte results. Furthermore, the lack of standardised and unbiased bioinformatic pipelines, as well as their growing intricacy, makes "omics" data particularly prone to errors in design, analysis and reporting [22]. Consequently, the need for statistical rigor and reproducibility is higher for "omics" research than for other types of research with well-established methodologies. Several journals have introduced policies that require authors of large-scale "omics" studies to provide the raw data and the software code used for analysis so that other researchers can execute the same analysis and either verify the findings and build upon them, if the same results are obtained, or reassess their validity if inconsistencies are found. Indeed, this should be the norm for all "omics" publications [23].

Several reporting guidelines have been published to improve the quality of biomedical research and indeed some of them are particularly relevant to "omics" research. One of the main issues in clinical proteomics studies, such as biomarker discovery studies, is that the quality of the study depends on the conditions of the starting material. A first step towards obtaining high-quality samples is the set-up of biobanks to collect and process biological materials, and their associated genetic, epidemiological and clinical information. The method of transport and storage of samples is also of great relevance as biological materials or protein biomarkers may degrade if samples are not processed or stored immediately after their collection. Therefore, international standard operating procedures for biological samples are necessary with short processing times and specific storage guidelines. The 'Biospecimen Reporting for Improved Study Quality' criteria calls for a more detailed reporting about the pre-analytical information of biospecimens such as collection, processing, and storage, which could influence experimental outcomes, their validity and reproducibility [24–26].

Some investigators have raised concerns that the use of biomarkers in early clinical trials is subject to imprecise assays, excessive cost, ethical issues surrounding tumour biopsies and, most importantly, the potential to abandon effective drugs due to incorrect patient selection. A series of considerations were also compiled in the 'Reporting Recommendations for Tumour Marker Prognostic Studies' (REMARK), a pivotal initiative led by the Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics, highlighting study design and analytical issues that need to be addressed to develop high-quality studies in cancer biomarker research [27]. Similarly, a proposed general pre-phase III clinical development plan for antineoplastic agents suggests taking into account the following points [28]: (a) to explore dose range up to maximum tolerated dose (unless limited by formulation, bioavailability, or cost); (b) to include as heterogeneous a patient population as can be ethically justified; (c) to define the relationship of dose to toxicity and pharmacokinetics; and (d) to look carefully for mechanism-related toxicity as a readily observable biomarker.

Similarly, it is important to consider the inclusion of biomarker analysis on readily accessible tissue to assess the action mechanisms and, potentially, the minimal effective dose as well as the use of tumour biopsies at highest dose if results are to be used to make a practice decision [28, 29]. Overall, it is important that early clinical trials consider incorporating validated predictive biomarkers to question and answer key biological issues in cancer biology.

In 2012, the U.S. Institute of Medicine appointed an expert committee charged with the task of issuing a report with recommendations regarding the criteria for the appropriate development and evaluation of clinically applicable omics-based biomarker tests (from discovery, validation, up to their first use in a clinical trial),

with a special focus on tests having the potential to guide therapeutic decisions and improve patient outcomes [30]. Based largely on this report, McShane and collaborators published a checklist of fundamental criteria that a prospective clinical trial (using an omics-based test) should meet in order to generate the body of evidence necessary to justify its clinical use (prove its clinical utility) [31]. Although these guidelines were created with biomarker development in mind, they can, and should, be applied to other areas of onco-omics research.

The following are some of the main applications of "omics" research:

1. Discovery of new and actionable molecular targets.
2. Discovery of tumour biomarkers
   (a) To identify the probability of a clinical event, disease recurrence, progression or death (prognostic).
   (b) To identify patients that are more likely to benefit from already approved therapies than similar patients without the biomarker (predictive), either for the same cancer type or for a completely different type.
   (c) To identify patients that are more likely to benefit from experimental therapies being tested in phase I/II clinical trials.
3. Development of tumour biomarker tests.

5.3.2 Clinical Trials, an ABC

Traditionally, clinical trials have been divided into three different phases according to their scale and primary objectives. Phase I trials are designed to assess the safety and tolerability of a new drug in order to determine the appropriate dosage to use in a Phase II trial. However, due to their relatively small sample size, Phase I trials may not detect rare adverse effects and very often novel drugs are tested in a group of healthy volunteers that may not respond in the same way as the patient population for whom the drug is intended. In Phase II and III clinical trials patients are randomly assigned to two or more groups, each receiving either a new or an already approved drug, in order to establish which is more effective for a particular disease. Phase III trials enrol a very large number of patients to establish the superiority or non-inferiority of a novel drug compared to a standard of care intervention. Phase III trials strive to provide robust experimental evidence of whether a new drug should be approved for a specific indication in a well-defined group of patients. This three-phase process has worked relatively well for the last 6 years. However, the therapeutic responses of unselected patients recruited in Phase I-II trials are generally poor, and in many cases the trial does not meet its primary end-point for efficacy, halting the advancement of novel agents into further stages of clinical development [32].

An interesting example of molecularly targeted anti-tumour drugs, showing lower than expected responses due to the lack of a molecular biomarker, comes from tyrosine kinase inhibitors (TKIs), such as gefitinib, which resulted in a response rate of 10–20% when used in unselected patients [33, 34]. Similarly, erlotinib administration elicited better responses in subgroups of patients such as those with an adenocarcinoma histology, female gender, non-smoking status, and Asian ethnicity [35]. These findings led to molecular characterization of EGFR in lung cancer patients [36, 37] and ultimately to the selection of EGFR mutated patients to receive TKI treatment [38].

More recently, two clinical trials exploring the efficacy of an IDO inhibitor (epacadostat) in combination with pembrolizumab in patients with metastatic melanoma (ECHO301/KEYNOTE-252) or in combination with durvalumab in patients with pancreatic cancer (ECHO-203 trial) found no improvement in the therapeutic responses in comparison to the monotherapy arm. However, the leading researchers from these trials have suggested that the disappointing results obtained were likely due to the lack of biomarkers to select patients likely to benefit from this treatment [39].

Conducting Phase 1-2-3 clinical trials for every new targeted agent for a specific "cancer type", while accruing patients likely to show therapeutic responses, is becoming increasingly difficult, time consuming and expensive, which in turn significantly delays the delivery of novel and better therapeutics to the patients that need them [40]. To overcome this limitation, scientists and researchers are exploring novel clinical trial designs, that enable the stratification of patients to multiple treatment arms in a single clinical trial. These trials, termed "umbrella" and "basket" trials, allocate patients into the trial that best fits their cancer and where they are more likely to derive a benefit [41].

5.3.3 Next Generation Clinical Trials

5.3.3.1 Umbrella Trial

Umbrella trials enrol patients with a specific tumour histology that is, under the 'umbrella' of that histology. However, patients are assigned to multiple arms (testing different investigational drugs) based on the genetic mutations that are most prominent in their tumour type. This trial is designed to simultaneously assess the effect of different targeted drugs on specific mutations found in a particular type of cancer, which further personalises the choice of treatment and increases the probability of achieving better therapeutic responses. Umbrella studies can be complicated, but they allow researchers to test several different medicines in patients with a similar disease, reducing the time necessary to identify patient subgroups who are more likely to benefit from the experimental drugs tested. Notable examples are:

1. The UK National Lung Matrix Trial that will explore the efficacy of multiple molecularly targeted treatments in patients with different subtypes of NSCLC, profiled using a hotspot panel and then assigned to the arm of the trial that best matches their specific driver mutations
2. The lung-MAP trial for the assessment of multiple second line interventions in patients with squamous cell lung cancer, profiled with FoundationOne CDx™. This trial combines both a 'test-the-drug' as well as a 'test-the-test' analyses, in which not only the efficacy of the drug is evaluated but also the strategy to allocate patients according to their mutational profile [11, 42].
3. The SAFIR-01 and 02 in breast cancer, randomizing patients into two arms, a standard of care maintenance therapy arm versus maintenance therapy using a targeted agent (NCT02299999).

5.3.3.2 Basket Trials

Basket trials also allocate patients to different treatment arms according to the molecular profile of their tumours, but patients are enrolled independently of tumour histology [43]. This design is based on the idea that similar driver mutations can occur across a variety of cancer types (they are histology independent), which suggests that if the same molecular alterations are found in different tumour types, they can potentially be targeted with the same biological agent and similar responses will be achieved across tumour types. Testing the efficacy of a specific therapeutic agent(s) on a defined molecular target allows to identify its effect on several cancer types at the same time, effectively obviating the need for multiple Phase 2 trials and expediting the extended approval of a drug for different indications. Two recent studies reported that the actionability rate of genetic alterations, detected in the plasma of patients, was similar across patients with different cancer types [43, 44]. Notable basket trial examples are:

1. The EORTC-CREATE trial testing crizotinib, an inhibitor of MET and ALK, in anaplastic large cell lymphoma, inflammatory myofibroblastic tumour, papillary renal cell carcinoma type I, alveolar soft part sarcoma, clear cell sarcoma and alveolar rhabdomyosarcoma (NCT01524926) [45].
2. In the vemurafenib basket trial, patients with tumours harbouring BRAF V600 hotspot mutations were treated with vemurafenib (BRAF inhibitor) regardless of primary his-

tology. Preliminary results showed efficacy in BRAF V600-mutated NSCLC, Erdheim–Chester disease, and Langerhans' cell histiocytosis. However, no efficacy was found in patients with colorectal cancer harbouring the same mutation [4], which highlighted that tumour context mattered more than what was initially expected.

We will briefly discuss the case of the first biomarker-driven approval of an anti-cancer agent across all solid tumours (tumour-agnostic), whose approval further supports the concept behind basket trials. Immunotherapies are directed towards increasing the ability of the immune system to recognise and attack cancer cells thus controlling tumour growth. The most successful strategy has been the use of immune checkpoint inhibitors (ICIs), antibodies targeting co-inhibitory molecules, to promote an immune-mediated anti-tumour effect [46]. Antibodies targeting PD-1 (pembrolizumab, nivolumab) and PD-L1 (atezolizumab, durvalumab, and avelumab) have been approved by the FDA for the treatment of malignancies [47]. For instance, anti-PD-1 ICIs have become standard-of-care drugs used in the first-line [48] and second-line [49–51] treatment of NSCLC patients whose tumours express PD-L1. However, tumour PD-L1 expression has not been the most reliable predictive biomarker, which has led to an intensified search and testing of better predictive biomarkers such as number of tumour infiltrating lymphocytes, tumour microsatellite instability and mutational burden. Indeed, pembrolizumab received accelerated and simultaneous approval for microsatellite instability-high (MSI-H) or mismatch repair (MMR)–deficient colorectal cancer (in patients who have progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan) as well as for all unresectable or metastatic MSI-H or MMR-deficient solid tumours (paediatric and adult) that have progressed on previous treatment and with no satisfactory alternative treatment options. This ground-breaking approval is the first tissue-agnostic indication for an anticancer drug based solely on a tumour biomarker [52].

5.4 Conclusion

We have now entered the era of omics-based stratified medicine. However, the integration of different "omics" data into biologically meaningful interpretations continues to be hindered by the complex mathematical and computational models required to analyse them as well as by the limited computational power that is accessible to most laboratories. We envision that future progress in these two key areas will further speed the development of omics-based discoveries with clinical utility. Newer clinical trial designs will further accelerate the delivery of increasingly better and more specific antitumor agents. Several recent trials have shown that combination therapies may elicit higher responses in a subset of patients. However, it is currently not known which combinations, which doses or which schedules are more effective for each patient. The trial designs discussed above can evaluate the efficacy of novel targeted agents, either as a monotherapy or in combination in a fraction of the time that it would take for traditional trials to yield this information.

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Issues and Ethical Considerations in Pharmaco-oncogenomics

6

Gilberto Morgan

Abstract

The rapid advancements of treatment modalities and vast amounts of information being generated through novel technologies, paint the picture of a very promising future, one that will allow for a more efficient and precise DNA sequencing and potentially more tailored cancer therapies for patients. However, with all these advances we must address the ethical and legal considerations each one of these technologies will raise. This is a necessity in order for advancement, not to stand in the way of science and development, but as a safeguard in protecting humanity and our personal genetic information.

Keywords

Oncology · Ethics · Bioethics · Data · Trial · Good clinical practices · Consent · Genetics · Research · Cancer

6.1 Introduction

Throughout history, oncology has revolved around three pillars of cancer treatment: Surgery, radiation therapy and chemotherapy. The classification of cancers for a long time have been based on type, stage and tumor subtype. However the last two decades have seen some changes and modern day oncology is quickly altering the future of cancer treatment and doing so with great speed. The rapid advancements of treatment modalities and vast amounts of information being generated through novel technologies such as Next Generation Sequencing (NGS), paint the picture of a very promising future, one that will allow for a more efficient and precise DNA sequencing and potentially more tailored cancer therapies for patients.

One key event happened happend a little longer than two decades ago, when the BRCA1 and BRCA2 genes were cloned. This development gave us a new found appreciation for germline genetic analysis in high risk individuals and in term has evolved genetic risk assessment for Breast, ovarian, CRC and Endometrial cancer. Due to the BRCA work, we were able to introduce general genetic risk assessment for women who are at risk for breast and ovarian cancer, however in the beginning, testing was only being done with the patients that had the highest risks.

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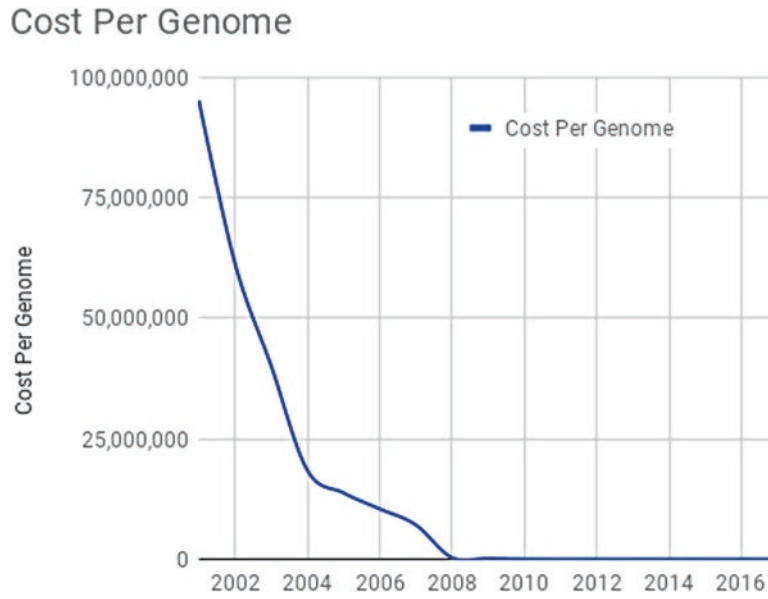
However, this also lead the way to prophylactic surgeries, as more women with BRCA1 or 2 mutation were being offered risk reducing salpingo oophorectomy after the completion of childbearing. Another key moment was in April 2003 when the human genome project was completed with the pricetag for the first human genome sequence being between 500 million and 1 billion dollars.

Flashforward around two decades from these events and we find ourselves with new molecular markers, novel treatments that are part of a new era of precision medicine and along with this, technologies which constantly discover new information about human genetics. The cost of sequencing a human genome has dropped dramatically as now anyone can have their genome sequence for a little over a thousand dollars and this being done under a few hours. These new technologies have advanced to such a level, that the copious genetic information that is being produced, leaves us with the question: how we are to use all this data? There is a large concern that the more information we generate, the more it seems to leave unanswered queries and uncertainties, this in term leads to patients that want answer as to the significance of their data and physicians that are unaware of how to implement this information. Throughout the evolution of precision oncology, the use of biomarkers have given us a way to detect many at risk populations and through the use of targeted therapies give the most pertinent treatment for a specific cancer and avoid giving toxic therapies to those who we know would not benefit because of their genetics. There will no doubt be many pitfalls in the path to newer ways of testing and identifying treatment, such as the need for: resources and an infrastructure in order to store and handle the amount of data that is being generated, physician education on genomics in a way that it is easy to implement and easy to relay the information to the patient in an understandable way. The European Society for Medical Oncology came up with a position paper in 2014 entitled "delivering precision medicine in oncology today and in the future" in this paper, it states that "A new era of personalized cancer medicine will touch every aspect of cancer diagnosis, tumor classification, treatment and outcome, this will demand a new level of in depth education and collaboration between researcher, cancer specialists, patients and others stakeholders. Due to the importance of personalized medicine in cancer, it is of paramount importance that oncologists and caregivers are at the vanguard of all of these developments and evolve alongside the technologies, however this cannot be possible unless there is the right infrastructure available to provide support in making these decision. In this chapter we will discuss the different ethical aspects of precision medicine in oncology, from the side of the patient (Patient confidentiality, ownership of information), the side of the doctor (physician education on genomics, patient doctor communication), the improvements that are needed to support the infrastructure (Storage of data, easy interpretation and availability of a database, the need for guidelines), the potential impact it can have on a specific society (financial toxicity, inequalities based on race, sex, education and income) and the possible ethical issues that may arise with the newer directions of pharmaco-oncogenomics (Graph 6.1).

6.2 Modern Day Pharmaco-Oncogenomics

Gene research has become a necessity in our day to day medical practice, as many of our decisions on treatments depend on pathology reports, genetic and molecular markers. This modern day way of practicing oncology would not be possible had it not been for our ability to use diagnostic tests which are both predictive and prognostic to the disease and to monitor its progression. One does not need to look very far to see how we use these markers in our everyday decisions, some example of these personalized treatments are trastuzumab in HER2 positive breast cancer, vemurafenib in malignant melanoma and imatinib in CML and there are many others. Due to their growing role in oncology, there is a dire need to develop new molecular and genetic cancer markers, to develop new therapies that target those specific types of cancers and to have frequent



Graph 6.1 Cost of Genome Sequencing in USD throughout the years from 2002 to 2016

evaluation and constant regulation from health organizations on these. The continous growth of data that is coming from NGS is adding to the complexicty of the situation as patients may often be faced with the dilema where they may receive news that they carry a gene mutation for which there are not current expert recommendations or very limited at best, in terms generating high amounts of anxiety and stress for the patient.

One way which genetic and molecular markers are making way to our daily clinical practice is through the use of commercial panels which test certain selected genes often found in a specific tumor population and which also determine the risk across different cancer genes. Some of these panels have made their way to the patient population and have completely bypassed the physician. In 2018, the American company 23andme was the first to be awarded with FDA authorization for a genetic test that is available straight to the consumer. This test gives information on 3 genetic variants that are commonly found in BRCA1 and BRCA2 which are associated with a higher risk of breast, ovarian and prostate cancer. Although there is a level of comfort in being able to get geneting testing in the comfort of home, the fact that these kind of panels are able to give patients information without a physician consultation leading the way to the possibility of patients becoming more confused or anxious by the results and their interpretation. The other large question looming with these private panels is the subject of their accuracy. There is always a chance that a patient is given a result of being a non carrier for a BRCA mutation and that this might be a false negative or that our patients will think that there is a very low lifetime risk of developing breast cancer, but as we know there are many other factors and genes that can contribute to the development of breast cancer and it can give a false sense if security to many and would lead to less check ups.

6.3 The Patients

6.3.1 Understanding

The technologies that we use have changed and with them, so have our patients, with many adapting to the role of members of the oncology team and being highly involved in the decision making of finding the best treatment for that person's cancer. Throughout the years medicine has seen the rise of the citizen scientist, the informed patient who is not hesitant to get to

know the disease, research treatments and explore all treatment possibilities. This issue has led to much stress in the medical community, mainly because people fear that having a patient so involved will not only require an extensive amount of time, but also because in most cases the patient will not fully understand the disease in a way that the medical, surgical and radiation oncologist do. However, many feel that a level of awareness on behalf of patients is something that should not be feared and instead it should be endorsed because if we have the complete acceptance and understanding of a patient, there will be less problems with treatment compliance and they will take a deeper role in their contribution and help achieve the best possible result. Just as every doctor is in need of a formal medical education, if patients are going to adopt this role, they need to have a framework that can help in the education of their disease so when they speak with their oncology team, they can play a bigger role in the process. These new technologies have generated more complexed information and it is not hard to see as to how any patient would be confused, hence the right education must be offered to them in a simple and effective way. In all fairness, the current situation has shown us that the problem is not just with the patient but also with the doctor, the level of understanding of genetic information is very low, this starts in primary care and stretches to many practicing oncologists. The information is not always straightforward and could be very tricky to interpret. The sequencing of genes of more individuals and the sharing of information will help in finding new variants and will advance the field, however because every person is different, every person has many different variants that are unique to that individual, hence there will always be variants that will cause their fair share of confusion. This is why in order for these technologies to be effective, we need to provide further education on precision medicine, clinical pharmacogenetics and the interventions that are involved not only to the oncologists but also to primary care physicians and also to the patients.

There is great debate as to the level of detail the physician should discuss with patients in terms of targeted treatments and genetic tests results. The information that would need to be relayed to the patient would require long in depth discussions about the genes that are undergoing testing, many of them which are not completely understood yet and this could possibly overload the patient in making a decision. The other problem is resources, as the practicing oncologists with a full workload will no doubt find it hard to have time to explain the details of test results, instead of just recommending the best therapy. In theory this level of patient involvement will be virtually impossible to accomplish, without the influx of new resources.

There is no one size fits all answer as to what information to divulge to patients and the level of details we should go into. Just as there are patients that would like to know every detail of every test, there are also those that would like the doctor to give the best recommendations and they will decide if to follow them or not. So as a physician, how do you know when to go into the intricacies of their cancer and is that patient ready and able to understand them? Sometimes the choices are not so simple and given the intricacies of the genes that are currently being studies, the explanations will no doubt be just as complex. As mentioned before, just knowing the results of a variant might not lead to an action, there are many genetic variants where we are uncertain on what the right course of action is if there is a present mutation. Such type of situations could affect the patients psychosocial well being and that of their family, by giving them extra stress, hearing "there is a mutation in a gene, but we don't know what it means or what to do with it" might just create more anxiety and affect that person's quality of life.

One can not forget that genes are something that are common in a specific family and that we also need to support family communication and outreach. New ways of delivering the information have to be developed in order for our patients and their families to fully understand the risks and benefits that are at stake with testing of their genes and they have realistic expectations of what a positive result could mean. Knowing one's genetic predisposition could definitely give a person a certain sense of empowerment and one

can see the allure of having genetic tests done. Knowing this information can lead to a decrease in anxiety, for example if a person comes from a family that has a certain predisposition for a gene mutation and upon testing one finds out that one is a non carrier for that familial gene, this could lead to a drastic decrease in the level of anxiety a person could have and improve that person's quality of life or in the case of BRCA, knowing that one carries a mutation could help make the choice of a prophylactic surgical procedure a lot easier for the patient.

However, what about the possibility for false positives or false negatives? would a false negative in a BRCA patient be the difference between not getting a hysterectomy and decreasing the chances for a relapse or in the case of breast cancer would a false positive lead to a patient receiving a double mastectomy when it was not necessary. The problem is that we rely quite heavily on these factors and the importance of genetic mutations are sometimes enough to make these hard decisions, that's why as physicians it is detrimental that their level of precision is something we can fully trust and is the reason that the link between physician and the patient is always open.

6.3.2 Privacy

Ownership is a topic that has taken center stage in regards to genomic data, with the big question being: who owns a copy of your genome? Even if you own your genome and you give a company access to your DNA, does the company doing the testing have any right to the information that is being analyzed? Can these groups doing the testing use the information for other purposes or save it for a later use? If a genetic variant is found and a therapy is derived from this, does the patient have any right or claims since it was that person's genome that lead to a therapy?

In 2008 the Genetic Information Nondiscrimination Act (GINA) was passed in the United States in order to protect people and their genetic information against employers and insurance companies who could try to use the information from genetic screening in order to discriminate. Although this was a big step in protecting genetic ownership, there are many loopholes that nowaday threaten this information. As one might imagine there are many ethical and legal questions that have arisen regarding this and around the question of informed consent especially in genetic research. People that are giving consent to use their data for genetic research need to understand the potential implications that are involved such as incidental findings. They also need to know that their data is protected and that it will not be stored for future use and most importantly which part of their health history and genomic data will be stored.

Those people who do undergo genomic research and disclose the results of genomic testing, could benefit by receiving insight and the necessary tools to make the decisions to avoid future problems, change behaviours or take prophylactic actions in order to decrease the risk of cancer in the future. However even with all this information, not everyone who undergoes genetic testing or involved in genetic research will gain, as there are many individuals who have very aggressive kinds of cancer that normally come with a bad prognosis and for these patients the possibility for a "last chance therapy" is detrimental, however most of these may include therapies that have very little gains in survival, even if they are targeted therapies. That is why the medical community has to take on the responsibility to make patients understand the goal of the treatments they are undergoing and the difference between, curing, prolonging life and palliation.

The incorporation of NGS data in daily clinical practice will make it virtually impossible that every bit of information will be returned to the patient, not because of the physician's desire to withhold information, but because of the cheer volume of the information. This leaves the question as to what should the patient be notified on, what findings in a genetic test would mean that a patient needs to be notified and most importantly, who decides this and why?

Let say that there is a group of people that are carriers for a specific gene that leaves them at a higher risk for a specific type of cancer and they

know this because they underwent genetic testing, once they are notified and this goes into their medical record, who is to keep the information? Will there be a database of people with specific mutations? If that information was derived by a test that was payed by an insurance company, does that information partly belong to that insurance company? And does that insurance company have a right to discriminate on the coverage they give a patient all pending on the kind of mutation they carry. Although thanks to GINA, in the US insurance companies can't discriminate based on this information, what about this person's family members. The chances that there are some family members carrying the same mutation are a lot higher than in the general population, so one would assume that it would be easy for an insurance company to pinpoint members of the family if they know that there is a carrier in the family, of course in reality this would be harder to detect, but the risk still remains.

6.3.3 The Family

The information that is generated does not just affect one person, genetic testing can affect all family members and provide the perfect setting for psychosocial strain. Another area where there are ethical issues is in family dissemination and the physician's role in reporting a genetic mutation to family members. For Example, when a patient tests positive for HIV, that doctor has a medical responsibility to notify the state health department which can contact that person's partners and suggest HIV testing. Much in the same way, would knowing that a specific person has a genetic mutation that could lead to a specific cancer render that physician or health department responsible of contacting all possibly affected individuals members of that family and could the physician do so, even against the persons wishes who was originally screened. It is still very unclear as to the level of responsibility the health care professional has to ensure that the relatives are informed of genetic mutations that might run in the family. Recent guidelines take the confidentiality of the patient as first priority, but as this become increasingly more common we might start to see a shift and the disclosure of genetic information could be a right all family members will have.

In the settings of genetic trials, the patient is in an extreme need for protection, especially when thinking about confidentiality. Patients that are taking part of a specific trial must be allowed to access the cohorts data so that they will be able to use their own data in order to make future decisions about their cancer treatment.

6.3.4 Issues in the Future

Although full disclosure towards our patients regarding genetic information seems to be the way we are moving forward, it is hard to determine if a patient is ready to be a partner in the decision making as there is still a portion of the population that want the doctors to make the decisions. There is a certain truth in that access to one's own information (genetic or otherwise) is empowering, however will our patients understand of are we going to produce a very confused and eventually frustrated patient. The modern world makes it easier for physicians and patients to be connected through the use of smartphones and social media and this will make it easier for this information to be shared, but how much of this data that is generated could be shared. How do we plan to incorporate the connectivity that has been generated in a way that would protect the patient and not make such personal information public. One of the companies that is producing a panel available to the patient (23andme) has also develop a social network feature where their customers are able to connect with others who share certain parts of their genomes. Through the power of social media, it is only a matter of time before other social media platforms start using this kind of information to link people. As this kind of developments continues, the only thing we can hope for is that the security of the people and their data will also be a matter of priority for these companies.

6.4 The Doctor

To many of us that practice as physicians, medicine is an artform and at the cornerstone of every patient visit for risk assessment is a thorough review of that person's family history, however new molecular methods are helping make treatment decisions in a simpler way and based on molecular markers. Not to take away the importance of a family history, but as technologies like NGS share and improve the field of cancer genetics, they have also given oncologists more options to quantify the risk of cancer in a specific population and the chance of success of specific treatments based on a person's genetics. There are now many multigene panels for risk assessment available today and this has been due to the fact that NGS is becoming increasingly quicker, cheaper and more accessible. However as physicians, what does this mean for us, what is our understanding of all this information, is there an established framework for physicians to make decisions based on this information? Having the right biomarkers can help us take decisions more easily, we can increase or decrease the intensity of our treatments depending on the need. Many of these targeted therapies have allowed us to not only keep clinical toxicity low, but have the potential to keep financial toxicity low as well, since it will allow us to give a specific treatment only to those who we know will respond.

6.4.1 Having the Right Support

We need to realize that many of the practicing physicians finished medical school way before the human genome project was completed and their knowledge and grasp of cancer genetics may not necessarily be up to date. In order to advance, we need to have the following (1) a very supportive framework in which to put this new information into clinical practice (2) we need further education for physicians (3) the availability of evidence based guidelines which will also serve to identify patients that are at high risk for certain cancer, lead to specific screening procedures, chemoprevention and even options for prophylactic surgeries.

Our roles as physicians puts us in situations where as professionals we must be able to recommend the most appropriate treatment for a given situation, we welcome tools that will make these decisions faster, however can we fully trust them and can we be sure that the decision is always the right one.

Take the issue of oral contraceptives, new research seems to support that their long continuous use can decrease a woman's risk of developing ovarian cancer, certain published reports have shown a 50% decrease in ovarian cancer in those with long term use. Decisions like this seem obvious, all signs point towards the notion that it's a good thing to take oral contraceptives, however there is recent research showing that there might be an increase risk for the development of breast cancer in those with long duration oral contraceptive pills, so in the face of uncertainty what do we recommend? What about situations where a young woman has an estrogen receptor positive breast cancer and lymph node metastasis and after adjuvant chemotherapy and treatments, is recommended tamoxifen treatment under many years and she does not want to wait to get pregnant as she is already 34 years of age. Of Course we can recommend RRSO, embryo or oocyte cryopreservation, but at the end of the day that choice will always be hers, even if it comes at a risk of increasing the chances of a relapse by getting off of Tamoxifen.

In the case of NGS one example is if a cancer patient is analyzed by NGS and then an incidental mutation is found, however the type of cancer that person has does not normally have that mutation and is not known to be a driver mutation. In this particular case there is a targeted agent that one could use against this specific mutation, but there is no previous evidence that this agent can treat this type of cancer, the question is, should the patient receive this agent? Is this enough evidence in order to treat?

There is also great potential in genetic testing, specially when thinking about cancer prevention

and what efforts here can lead to. Through the testing of hereditary cancer risk through deep screening, prophylactic surgery and even chemoprevention it is possible to make cancer prevention a reality. More study needs to be invested in this field, but if we were to successfully identify the causation for certain mutations, we could also be able to give a more accurate assessment of a lifetime risk for specific cancer and thus aid in making decisions such as having children.

6.5 Infrastructure

NGS has given us the availability to get comprehensive genetic information at lightning speeds and how we can sequence an entire human genome in a matter of hours, this in turn has generating and continues to generate information. However there are some limitations to these advancements, such as the question of where will this data be stored, who will keep up the necessary infrastructure to keep this data and who will keep this data safe. Such a feat will require a collective global effort with plenty of computational power and an ability for large data storage. This data will have to be available to many and there needs to be a place where this data can be pooled and shared and as this data mounts, it needs to be readily and easily accessed by researchers and physicians.

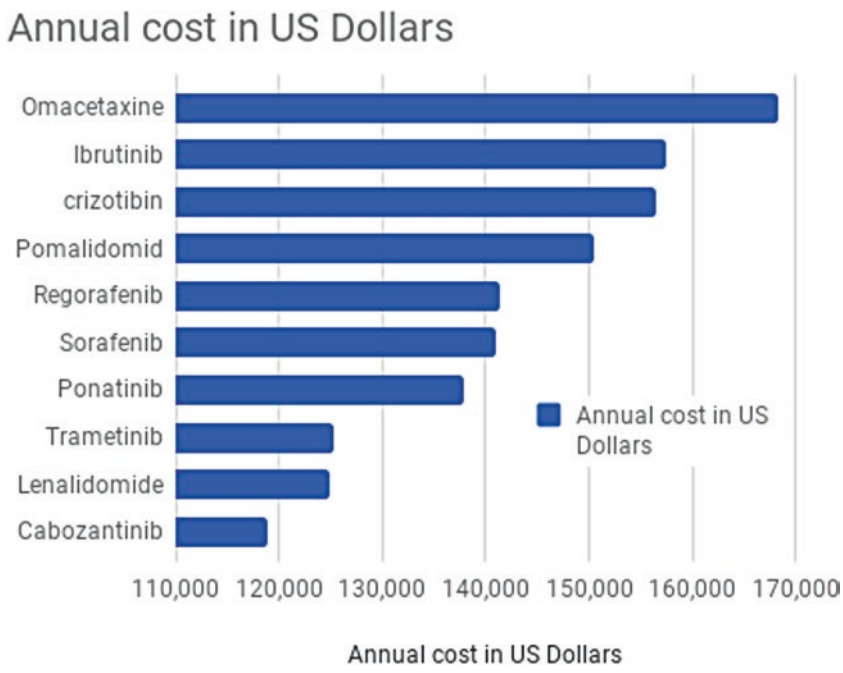
6.6 Society

Newer technologies, for the most part always come at a cost and although there are many new therapies that are coming as a result of these newer technologies and information, these targeted therapies make up around 40% of the total price of modern cancer care. Although they provide often the best choice for treatment for specific cancer, the choice is often based on the economics of a specific individual or that of the society for which that individual lives in as someone must shoulder the cost. Even before one can even be offered a specific targeted therapy for a cancer, there are predictive tests that are needed to determine if one is even a candidate for such therapies and these may not be available to all individuals due to the financial cost. It is problems like this that end up leaving this new technology only available to the specific few who have the financial means to afford it and irrelevant to those who can not (I). What about the morality aspect of cancer care? Does everyone have a right to the best kind of cancer care, regardless of the financial strain that paying for this care might put on a society? What about societies that are not able to afford such tests or therapies, is it unethical that these treatments and technologies exists only to benefit those with the financial wealth to afford them. There is also the case of people with terminal cancer, mortgaging their houses in order to pay for a cancer therapy that is not covered in their insurance, but that will give them an extra month of life. Even in a well functioning wealthy society, there is no agreement as to how to manage the cost of new technologies within a healthcare system and who is responsible to pay for them. Although there is a moral obligation to give the best, how can we justify such a financial expenditure when many of these newer treatments in specific situations show gains that are marginally beneficial for the patient in terms of prolonging life, often doing so by weeks to months. In societies with limited social resources it seems that investing in expensive therapies is not a fair allocation of their resources, when the rest of the society members may be facing other hardships or there is a dire need in general healthcare (Graph 6.2).

6.6.1 Inequalities

6.6.1.1 The Argument for Racial Bias

There are many ways that these new technologies can lead to inequalities that are based on being a member of a minority group, age, sex, having a low level of education or income. For example in the case of race, most of the modern day testing is being done in rich countries and among those



Graph 6.2 Annual cost in USD of certain novel cancer drugs in 2015 per patient

with resources to participate, this leaves that the information that is derived comes from mostly whites of European descent and findings would not completely apply wholly to the global population. These ethnic differences play a big role in interindividual variation in anticancer drug sensitivity. Because of this fact There needs to be more investment in ethnic specific biomarkers for drug response. There have been recent studies that have shown that African American women have a higher tendency to develop breast cancer and these are often diagnosed at later stages of the disease. Although there are range of issues affecting this, specially socioeconomic there are genetic differences between the races that could lead to susceptibility to specific types of cancer. Another form of inequality is the access to healthcare among the races, specially between black and white americans. In certain studies, its has been shown that there is the lack of health access for black men, which has led to higher prostate cancer mortality. Another example can be native american women, who could benefit very little of genomic testing to detect a cancer risk, when there are hurdles impeding them to get regular mammograms and in general get less thorough follow ups than most caucasian women with the financial means.

6.6.1.2 The Argument for Bias against Low Education and Income

The level of education and income also have a large role to play as well, as normally these two factors are linked to each other. There are many living in rural areas or in low income inner city areas that do not have access to hospitals with the best level of healthcare and for these people access to the newest technologies, therapies and tests will not be part of their reality, mainly because of their resources. These kind of issues could leave us with one population that does not have the access to personalized treatments and yet another population with means that will have extra tests, without real significance. When thinking of the people that are participating in NGS experiments, without the required

infrastructure the only people participating will be the ones living in the cities, leaving those living in rural communities underrepresented.

In order for this to become a success in clinical practice we need to be able to give healthcare providers the right infrastructure to help the underserved and to target them correctly. Through the development of therapeutic alliances, doctors and patients could be able to work to erase these inequities and together be able to implement the best level of healthcare. This will not be an easy task as the access to healthcare depends much on geography, socioeconomic status, the availability of insurance and the type of healthcare structure there is in a specific country, hence there are big ethical concerns in regards the fair distribution of global healthcare resources.

There are many hurdles to be faced, will all patients have the availability to genomic tests and their corresponding targeted therapies and will their physicians have the access to the genomic data and the tools to translate what it means. Even in the cases where these genomic tests show a genotype that points towards a targeted therapy, how do we deal with the issue of cost, what if the patient can't pay for it and neither will their insurance, what if it's covered but falls outside the standard of care for their specific type of cancer? Hence the issue of health disparities will continue to be present as long as the cost for treatments are high and there is an inability for all to pay, it seems that the trend will continue, those able to pay for treatment out of pocket, will get the most out of new technologies.

Another way of thinking is that the development of targeted therapies, although expensive, could save money because the right therapy will be given to patients, at the right dose and at the precise time, but who will pay for the testing, who will pay for the therapy and who will educate the physicians who will implement the information. Will the financial burden fall on the taxpayers, will they be left to shoulder the financial burden for a particular population or will it come back to the insurance companies and if so, will insurance companies have the right to use this information in the future when it comes to decision on that person or their family members.

6.7 The Problems

Many of these new technologies and new personalized treatments have been blamed for disrupting innovation and doing it at a high price, as they are criticised for drawing money and resources away from much needed research. In the case of NGS, there is an enormous amount of information that is being generated and many critics think that there simply is not enough resources available yet to to, store and implement it. One of the biggest obstacles is the analyzing and the interpretation of all the data that is being generated and in order to do so we would require access to a genomic and health information database that could be easily used to identify and integrate the information generated. In order for this to work, these new technologies can not use the bulk of our resources as not enough being done to advocate cancer prevention and many cancer prevention advocates seem to think that instead of finding cures where not all will benefit, it is better to invest in life changing behaviors and influencing environmental factors.

6.8 Conclusion

New technologies and a shift to personalized medicine are improving the field of oncology. Through the use of biomarkers we are able to identify patient subgroups in order to give the best possible treatment for that individual and in terms elicit the best possible response. Although there is no denying that personalized oncology will undoubtedly improve health outcomes in the future, it will come at a price, putting a heavy strain on the healthcare system, the payers and the physicians. There are those that argue that by finding a better therapeutic fit for a patient, these new technologies will not only provide a better health outcome, but also a better financial outcome as we will be able to avoid ineffective treatments. This is why we have to be careful about the very real fact that there are alot of inequalities in the access to health care and mindful that not everyone has the same kind of coverage, if this is not addressed

then any benefit that could be expected with genomic testing will be very limited. That is why societies like the European Society for Medical oncology have made it a point to say that the specialty of medical oncology has to play a key role and carries the responsibility to raise awareness of new technologies and achievements of targeted therapies and in this way help our patients find clinical trials in order to have equal access to the most innovative treatments.

7 Pharma-Oncogenomics in the Era of Personal Genomics: A Quick Guide to Online Resources and Tools

Rohan P. Joshi, David F. Steiner, Eric Q. Konnick, and Carlos J. Suarez

Abstract

The past two decades have seen unprecedented advances in the field of oncogenomics. The ongoing characterization of neoplastic tissues through genomic techniques has transformed many aspects of cancer research, diagnosis, and treatment. However, identifying sequence variants with biological and clinical significance is a challenging endeavor. In order to accomplish this task, variants must be annotated and interpreted using various online resources. Data on protein structure, functional prediction, variant frequency in relevant populations, and multiple other factors have been compiled in useful databases for this purpose. Thus, understanding the available online resources for the annotation and interpretation of sequence variants is critical to aid molecular pathologists and researchers working in this space.

Keywords

Oncogenome · Next generation sequencing · Benign or suspected passenger variant · Pathogenic or driver variants · Diagnostic biomarker · Prognostic biomarker · Germline variants · Somatic variants · Human genome variation society · Online tools

7.1 Introduction

The field of oncogenomics began in the early 1990s with laborious sequencing of a relatively small number of genes from the human genome. Advancements in next generation sequencing (NGS) and other technologies over the past two decades have exponentially lowered cost and increased sequencing bandwidth, to the point where routine sequencing of selected genomic targets, or even whole tumor genomes is now possible. For research, these changes have helped reveal an unprecedented level of detail of the underlying gene mutations, epigenetic alterations, and changes in gene expression. In the clinical realm, a new era of personalized medicine has emerged, in which treatments and clinical decisions may be informed by the genetic alterations in the patient's tumor.

This detailed view of the "oncogenome" has also increased the complexity of interpretation required for genomic data. Raw data from NGS is

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typically processed using a computational pipeline that identifies genetic variants by comparing DNA sequencing reads from a patient's tumor to a reference genome. However, many of the variants identified are suspected to be passenger mutations with no clear functional consequence. Thus, annotating somatic variants for their functional, biological, and clinical implications is essential for the selection of targeted therapies and for informing translational research.

The annotation process entails the classification of variants into different categories, and various schemes can be used for that purpose. One classification strategy is to evaluate the alteration's functional effect by differentiating those having no effect (likely benign or suspected passenger variant) from those having an oncogenic effect (pathogenic or driver variants). A further useful framework is to classify variants into those used to establish the tumor's diagnosis (diagnostic biomarker), the patient's prognosis (prognostic biomarker), or the tumor's response to targeted therapies (predictive biomarker). These classifications often require review of data from decades of scientific studies, with the knowledge that there is a constant stream of new scientific information that may be relevant to the classification. Given the breadth of evidence that needs to be investigated for each variant, online knowledgebases and databases have been created to store and provide rapid access to known clinical and functional effects of variants. However, this stored and curated data still pales in comparison to the scope of possible genetic variation in any given tumor. Therefore, our current understanding of specific genetic variants in cancer is constantly growing, and these online resources need to be constantly updated to reflect the latest discoveries.

Although evidentiary criteria for the classification of germline variants is relatively well established, the criteria for evaluating somatic variants is more variable across institutions and laboratories. Readers are referred to recent guidelines for clinical reporting of somatic variants, but are cautioned that guidelines will likely continue to evolve and be updated in the years to come [1, 2]; however, a non-exhaustive list of potentially important questions to consider when querying online resources to annotate somatic variants include the following:

1. Has the variant been established as a biomarker of a therapeutic target? Data supporting the use of variant-specific biomarkers for targeted therapy selection may be one of the most exciting aspects of tumor genomic profiling, but only a small number of alterations have a clinically established therapeutic target.
2. Is there evidence in primary literature that the variant is a driver mutation? Strong evidence in experimental models that indicate the variant provides a growth advantage suggests a mutation is more likely to be oncogenic in a patient. This information also may suggest an existing or experimental targeted therapy or be relevant to translational research into the prognostic or predictive potential of these disease-driving genetic alterations.
3. Has the variant been previously reported as a somatic mutation in cancer, or has it been previously classified by other laboratories? While recurrent mutations are not necessarily oncogenic, such mutations may warrant a higher level of suspicion of pathogenicity in both clinical and research settings. If previous annotations exist, consensus classifications from multiple sources may reduce the need for exhaustive examination of primary literature or other databases for variant effects.
4. Is there evidence that the variant may actually be a germline variant rather than a somatic mutation? Variants that are found at high allele frequency across populations or subpopulations are more likely to represent normal human genetic variation. These should be interpreted with caution as certain pathogenic germline variants can be relatively common in subpopulations. Special consideration must be given to variants identified in patients that belong to under sampled populations, as rare, population-specific variants can be incorrectly interpreted as somatic mutations. Additionally, some mutations can be observed as both somatic and germline mutations. If a true somatic variant is also a pathogenic germline variant, this evidence can aid in the interpretation of the somatic alteration.

5. Is the variant present in a known tumor suppressor or oncogene and/or does it affect known functional domains? If the variant is a frameshift, nonsense, or splice site mutation in a tumor suppressor gene and disrupts functional domains or is predicted to cause nonsense mediated decay, the variant most likely can be characterized as oncogenic. In contrast, these same loss of function variants in an oncogene would likely be characterized as variants of unknown significance, unless specific evidence has been documented to support oncogenicity. In addition, recurrent hotspot mutations in oncogenes often confer oncogenic gain of function effects while recurrent hotspots in tumor suppressors can raise suspicion for a dominant negative functional effect or for disruption of a functional domain. When considering such alterations, it is important to note if the gene under investigation has known pseudogenes that may contain variants that are incorrectly mapping to the true gene and giving the impression of a deleterious mutation.

7.2 Online Resources and Tools

The most useful online tools and resources may provide answers to one or more of the above questions and one often must examine multiple different sources to gain sufficient knowledge of a variant for effective interpretation. Some general considerations for the use of online resources when interpreting somatic variants include the following:

1. Understand the limitations of the data source, what data are included, and what data may be missing. For example, frequency rates for alterations can differ depending on whether the data are from whole exome sequencing versus aggregated data from targeted panels which may cover different exons or genes from one another. Additionally, there are instances of somatic mutations that have found their way into germline databases as well as germline mutations that are present in somatic databases. Thoughtful consideration of these issues and consideration of the data source, as well as any biases from processing and filtering, or lack thereof. Also, be thoughtful about the underlying curation process for any functional interpretations provided, as some resources may involve more rigorous criteria than others or simply different criteria. Review of primary literature may be necessary if information is incomplete or inconsistent among resources.
2. Confirm that nucleotide changes observed in the sequencing data represent the amino acid change being evaluated. Many databases provide only amino acid level information, but amino acid position is dependent on the reference transcript used. Be aware that some well-studied mutations were described initially using slightly different coordinate systems (e.g. *BRAF* p.V600E was previously denoted as p.V599E), so double-checking the reference amino acid at a specific position denoted in databases is wise. When possible, check the genomic coordinates of the variant and the reference transcript for consistency. For hotspot mutations, this may be less of an issue since consistent reference transcripts have often been established, but even for well-characterized variants, this can be important, especially if reviewing primary literature.
3. The therapeutic implications for somatic alterations are often categorized by "level of evidence" which can range from FDA approved or standard of care therapies from large trials to pre-clinical evidence. Understanding how different knowledgebases organize and attribute biomarker significance is important for accurate interpretation, clinical curation, and reporting of such alterations. Additionally, this knowledge can be important for the design of targeted sequencing panels to ensure inclusion of standard of care biomarkers and to inform decisions regarding whether to incorporate experimental or emerging biomarkers.
4. The best way to learn how these resources may be used in oncogenomic studies is to explore them directly. This will allow the user to understand the style and organization of the most up to date information available and to

develop personal preferences. For some alterations, one somatic knowledgebase may be sufficient as much of the information is overlapping; yet, for a more extensive annotation or for users with more limited background knowledge, multiple resources may provide a more complete understanding.

7.2.1 Nomenclature

As alluded to above, one important aspect of utilizing genomic data is a system to ensure researchers and clinicians alike are referring to the same findings, despite the various detection methods, reference transcripts, and variant descriptions. To this end, the Human Genome Variation Society (HGVS) has put forth nomenclature guidelines to help harmonize variant descriptions (https://varnomen.hgvs.org/) [3]. Mutalyzer (https://mutalyzer.nl/) is a specific, web-based tool to provide users with an easy way to generate variant descriptions, change reference transcripts, or check that a variant description is consistent with HGVS guidelines at both the nucleotide and amino acid level [4, 5]. This tool also provides a useful visualization of the variant in the context of the reference genome and can be especially useful for harmonizing the description of insertion or deletion variants.

7.3 Specific Databases and Resources

Below, we describe several reliable resources and how they may be most useful in the curation and annotation of somatic variants (Table 7.1). While this is not a comprehensive list, it is intended to highlight the range of online resources that may be used for oncogenomic efforts.

7.3.1 OncoKB (http://oncokb.org/#/)

OncoKB is a knowledgebase of somatic mutations in cancer with an emphasis on treatment implications of specific gene mutations and alterations [6]. Curated by a team at Memorial Sloan Kettering Cancer Center (MSKCC), OncoKB was launched as an online resource in 2016 and continues to be updated with new genes and references as new information becomes available. The OncoKB curation process involves a multitiered level of evidence system that is notable for considering cancer-type specific evidence in its searchable knowledgebase of variant-specific treatment implications. As a tool for the interpretation of somatic mutations, OncoKB is easily searchable by gene (477 genes at the time of writing) and provides a useful, expert annotated summary of each gene's relevance in cancer including easy to find information about tumor suppressor versus oncogene classification. Even this simple distinction can be very helpful for the interpretation of somatic mutations when evaluating the implications of specific mutation. For example, the significance of a loss of function mutation in a well characterized oncogene is likely quite different than loss of function mutation in a tumor suppressor gene. Additionally, the OncoKb knowledgebase provides variant-level literature curation of experimental evidence (with links to manually curated primary literature) as it pertains to the predicted or demonstrated functional impact of specific mutations. For each gene, OncoKB also provides mutation lollipop plots to quickly evaluate for hotspots or recurrent mutations and also provides a bar chart of gene mutation frequency in different cancer types generated from data obtained using the MSK-IMPACT Clinical Sequencing Cohort [7]. One notable aspect to be aware of regarding these plots is that they only include annotated mutations, and thus the data and values will differ from databases that include all reported mutations. OncoKB is a very useful tool to quickly understand the role and current understanding of specific genes in cancer as well as to find mutation level information and primary literature references. As with most current online resources though, it may not always be entirely comprehensive and only limited information is provided for many genes. As such, all conclusions reached from this database may still benefit from review of the primary literature or other databases to fully interpret less well characterized mutations.

Table 7.1 Overview of Online Resources for Pharma-oncogenomics

Resources Name	Source or Sponsoring Organization	Summary
OncoKB	Memorial Sloan Kettering Cancer Center (MSKCC)	Curated gene level summaries and variant level annotations with literature references for extensive list of known tumor suppressors and oncogenes.
My Cancer Genome	Vanderbilt University and GenomOncology	Thorough summaries and discussion of genes and variants with cancer-specific context.
PCT Knowledge Base	MD Anderson Cancer Center	Well-organized and extensive curation for a focused set of genes in regards to summary information, therapeutic implications, and clinical trials.
CanDL	Ohio State University	Tiered annotation of specific variants with particular attention to level of evidence and functional data
CIViC	Washington University	Open-access, community driven database curated by domain experts with focus on evidence, biomarker category, and literature references when available.
Precision medicine knowledgebase (PMKB)	Institute of Precision Medicine (IPM) at Weill Cornell	Collaborative database of variant interpretations used for reporting results from actual clinical cases on the genomic profiling panel used at the IPM at Weill Cornell.
JAX-CKB	The Jackson Laboratory	Variant level annotation with useful literature summaries that include functional and preclinical data references as well as case reports or clinical trials if available.
PeCan	St. Jude Children's Research Hospital	Unique and high-quality data visualization resource that aggregates mutation frequencies, locations, patterns and more.
Cbioportal	Memorial Sloan Kettering Cancer Center (MSKCC)	Extensive database drawing from TCGA and other tumor profiling studies. It can be a great resource for finding previously identified mutations by cancer type as well as co-mutation patterns, survival data and more.
COSMIC	Wellcome Sanger Institute	Combines informatic and manual curation to assemble one of the original and most extensive databases of somatic mutations observed in cancer sequencing studies.
ClinVar	National Center for Biotechnology Information (NCBI)-NIH	A public repositary of variant annotations with well-defined submission guidelines. Currently this resource is mostly germline variants, but there are a growing number of somatic variants as well.
gnomAD	Broad Institute	An extensive aggregation of genome sequencing data from over 120,000 individuals. Harmonized analysis and variant calling help make for an exceptionally useful database of germline variants and their population frequencies, including many rare variants not well identified or quantified elsewhere.
VarSome	Saphetor	An impressive aggregation of information from other databases combined with visualization tools for variant positions, functional predications, and classifications. It can be a little overwhelming at first glance, but one of the most comprehensive and well-organized source of specific data from other databases.

7.3.2 My Cancer Genome (https://www.mycancergenome.org)

My Cancer Genome was the first public database for somatic variant information, launched in 2011 out of Vanderbilt University [8]. An exclusive commercial partnership with GenomOncology may limit the ability to easily integrate the available data with automated interpretation workflows, but the resource continues to be a useful source of expert curated information. My Cancer Genome provides regularly updated information on specific mutations organized in a cancer-type manner and is searchable primarily by cancer with optional search fields for specific genes and/or variants. This resource

is particularly useful for the well-curated descriptions and summaries of characterized genetic alterations across cancer types, and includes alteration-specific functional annotation, frequency, and primary literature references. Additionally, My Cancer Genome provides an extensive amount of well-organized information for many topics in molecular medicine, including testing methods, cancer treatments, immunotherapy, and pathways in cancer, along with many useful figures and schematics. Although the paragraph-style descriptions can be harder to review for specific information than the structured tables of some other databases, My Cancer Genome continues to be a very useful resource for updated, thorough descriptions of the functional and clinical implications for specific somatic mutations in cancer.

7.3.3 Personalized Cancer Therapy Knowledge Base for Precision Oncology (https://pct.mdanderson.org)

The Personalized Cancer Therapy Knowledge Base for Precision Oncology is a resource from the MD Anderson Cancer Center [9]. This useful resource requires registration and provides well-curated information across a limited number of characterized genes (approximately 30 as of June 2018). The information for each gene includes overview descriptions, high quality pathway schematics and images, and expert annotation of mutation frequency and clinical outcome data. Similar to My Cancer Genome, much of the information is provided in paragraph form rather than data tables, and provides extensive literature annotation with links to primary sources. While the number of genes is not extensive, for the well-studied genes that are included, the PCT resource includes a significant amount of information regarding (1) therapeutic implications by level of evidence, (2) drug-gene interactions based on data from several other databases, and (3) clinical trials selected via an informatics pipeline along with manual curation. The high-quality organization, user-interface, and documentation of the annotation process also add to the value of this useful resource.

7.3.4 The Cancer Driver Log – CanDL (https://candl.osu.edu)

The Cancer Driver Log (CanDL) database was originally curated and developed at Ohio State University and provides mutation-level information, specifically regarding predictive biomarker potential [10]. The data are classified into four tiers including (1) FDA or NCCN recommended therapies associated with specific mutations (2) Support for targeted therapy based on evidence from clinical trials or case reports, (3) Alteration may predict theraputic response in pre-clinical data, or (4) Putative driver mutation based on functional data. Users can browse by gene (62 genes as of June 2018) and the available data for specific alterations include a level of evidence label, links to primary literature, and relevant cancers. Although searching by specific mutation is not immediately intuitive, users can search by amino acid position by including this information in the search field (ie. *EGFR* L858R). However, as with many resources, the mutations are not searchable or annotated by the coding nucleotide position, only by amino acid. Advanced options allow selection of fields to display and export a CSV version of the information. Additionally, users can suggest and submit data for review and inclusion in the database.

7.3.5 Clinical Interpretations of Variants in Cancer (https://civicdb.org/home)

Clinical Interpretations of Variants in Cancer (CIViC) was developed at Washington University in St. Louis and launched in 2016 [11]. CIViC is somewhat unique from many of the other resources in that it was specifically established as an open-access, community-driven resource, relying on active sharing of knowledge regarding somatic mutations along with curation and approval by a team of domain experts (typically MD or PhD level). This infrastructure allows for frequent updating that includes new genes and variants as well as revisions to existing interpretations. For curated alterations, the annotation includes a short summary of the gene, including

genomic coordinates, and variant-level biomarker evidence including predictive, prognostic, and diagnostic categories as well as a "pre-disposing" category for germline variants. Levels of evidence include validated, clinical, case study, preclinical, and inferential. Expert provided summaries of clinical evidence are provided as well as links to primary literature. Additionally, coding nucleotide changes (with reference transcript) and genomic coordinates are annotated and there are also links to view variants on other databases such as COSMIC and ClinVar when available. Given the open access nature of CIViC and the clear goal of data sharing, advanced users may also be able to incorporate information from this knowledgebase directly into a curation pipeline via the well documented application programming interface (API) that is available. Early partnerships with ASCO and Illumina in 2017 provided an initial boost to the scope of information in this crowd-sourced database. Although the database will only be as comprehensive as the data contributed, with a steadily growing database of variants, drugs, evidence items, and contributors since its initial launch in 2017, CIViC will likely become increasing useful as an online tool for the annotation of somatic and germline mutations.

7.3.6 Precision Medicine Knowledgebase (PMKB) (https://pmkb.weill.cornell.edu)

While many databases provide information on gene variants and possible biological impact, annotations used for actual clinical reporting and their supporting sources themselves are usually maintained as private at clinical institutions. In contrast to this trend, the PMKB was created by the Institute of Precision Medicine (IPM) at Weill Cornell Medicine and serves as a collaborative database for clinical variant interpretations [12]. Over 1500 variant interpretations for over 600 genes are currently available. Each variant can be examined for its clinical interpretation and associated references as well as the tumor types and tissues in which it has been observed. Historical interpretations are also available, allowing the user to observe if any recent changes in variant interpretation have been made. Pending changes to variant interpretation that are under review can also be seen. All data is accessible through an application programming interface (API), allowing integration into third-party variant interpretation pipelines. Currently, the database is updatable only by clinicians at Weill Cornell Medicine; however, this list of variant interpretations continues to grow. As one of the few freely available and actively used databases for clinical-grade variant annotations, the PMKB is a very promising development in clinical oncogenomics and is an example for other institutions to follow.

7.3.7 JAX Clinical Knowledgebase (https://ckb.jax.org)

The JAX Clinical Knowledgebase (JAX-CKB) from the Jackson Laboratory for Genomic medicine is another online resource geared towards predictive annotation with the goal of linking tumor genomic profiling data to therapeutic strategies and/or clinical trials [13]. Built in part to support clinical reporting for the Jackson Laboratory's own targeted sequencing panel and launched in 2016, this resource combines bioinformatics and manual curation of literature and clinical trials to provide a searchable database of genes, mutations, and drugs in cancer. As of June 2018, this knowledgebase contained information for 86 genes and allows users to search by gene, variant, drug class, or drug. For the purposes of annotating somatic mutations, the variant-level descriptions provide functional domain information as well as experimental functional effects based on primary literature review. This resource also provides a "Gene Level Evidence" tab with information regarding predictive or prognostic implications associated with specific mutations (or types of mutations) in a given gene. This evidence includes pre-clinical findings as well as case reports and clinical trials. In summary, although this knowledgebase has fewer genes than many other resources, it can provide high level information on recurrence and functional annotation for specific mutations and also pro-

vides useful details and references regarding prognostic or predictive significance associated with some mutations.

7.3.8 St. Jude PeCan Data Portal (https://pecan.stjude.cloud/home)

The Pediatric Cancer (PeCan) data portal was created by St. Jude Children's Research Hospital as part of an initiative to characterize mutations in pediatric cancers [14]. The main goal of the resource is to provide a pediatric cancer-focused variant visualization tool that focuses on ease of exploration of pathogenic germline mutations, gene fusions, and variant stratification by cancer subtype. This resource is also particularly useful for visualization of recurrent mutations and cancer subtype stratification even in adult cancers, as there is an option to view data pulled from the COSMIC database. The primary visualization tool, ProteinPaint, overlays reported variants, including gene fusions, onto protein domain structure along with exon boundaries and reference transcript information. This allows quick annotation of hotspot regions by mutation type (missense vs. nonsense), as well as visualization of reported fusion partners. Zooming in on regions reveals the relative number of mutations at each position; the most frequent variant at each position; and optionally for each amino acid variant, a list of all variants, their associated cancer subtype, and links to PubMed articles reporting the variants. Pediatric cancer data is sourced from the St. Jude – WashU Pediatric Cancer Genome Project (PCGP), the Therapeutically Applicable Research To Generate Effective Treatments (TARGET) study, the Shanghai Children's Medical Center pediatric ALL project (SCMC), the UT Southwestern Medical Center Wilms' tumor study (UTSMC), and the German Cancer Research Center Wilms' tumor study (DKFZ) [15]. Apart from these pediatric cancer sources, ProteinPaint also pulls data from COSMIC and ClinVar, two resources essential for variant annotations of adult samples.

7.3.9 cBioportal (http://www.cbioportal.org)

cBioPortal was developed by Memorial Sloan Kettering Cancer Center as a repository for genomic data and tool for exploratory analysis [16]. Large datasets of genomic data from published studies, including whole exome sequencing, targeted sequencing, and RNA sequencing, are passed through a variant re-annotation pipeline to harmonize variant interpretation; these data are available for download and visualization. Information from a wide variety of sources and tissues is included, such as data from TCGA and other sources. Mutations and copy number variations from individual studies can be visualized, along with clinical data including survival plots if available. The common variant annotation also allows examination of multiple gene mutations and copy number variations across multiple studies at a time. In this mode, cancer subtype frequencies, co-occurrences between mutations including volcano plots, mutation types, survival plots, and gene network analysis can be obtained. The massive amount of data, ease of use, and power of the available visualization tools makes cBioPortal a premier site for oncogenomic data exploration.

7.3.10 COSMIC (https://cancer.sanger.ac.uk/cosmic)

The Catalogue of Somatic Mutations in Cancer (COSMIC) is a manually curated database of somatic variant information hosted by the Welcome Trust Sanger Institute [18]. Genes that are recurrently mutated in cancer are selected and a team of curators performs an extensive literature review to find samples with variants in the gene of interest. Literature curation includes the following features: affected individuals' age, gender, ethnicity, family information, environmental exposures, and previous therapies; the source of the tumor along with grade, stage, drug response, and cytogenetic data; and mutation loss of heterozygosity, microsatellite stability status, and if

paired-normal tissue was tested. This wealth of information is presented to users in easily interpretable table and graph form. Each curated variant is given a COSMIC mutation ID, and users are presented with a bar graph of tissue distribution of the variant across cancers as well as a table of each sample where the variant has been found and its associated curated information, including the PubMed ID of the publication where the sample was reported. Users can therefore use COSMIC to very quickly access primary literature. It is important to note that because COSMIC is manually curated, genes that are rarely observed in cancer or variants that are less commonly seen may not yet have received curation and may be missing from the database. Additionally, one should use caution when considering the frequency of a particular mutation as well-known genes are sequenced much more frequently in targeted studies and thus the data will be enriched for these genes. Nevertheless, a very large number of curated genes and variants are present, making COSMIC an essential tool for variant annotation.

7.3.11 ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)

ClinVar is run by the National Center for Biotechnology Information (NCBI) and seeks to serve as a public repository for variant annotations and information relating gene variants to phenotypes [17]. For many variants, ClinVar also includes the specific evidence provided by the submitting laboratory supporting the relationships. Currently, the majority of variants in ClinVar are germline, but somatic mutations are also included. As such, users should pay specific attention to the source to distinguish between germline and somatic annotations. As a repository, ClinVar depends on submissions from the laboratory community, including companies, researchers, clinics, and expert panels. Required information for a variant submission includes submitter information, the disease or condition associated for the individual, an interpretation of the variant's pathogenicity, how the data was collected, whether the variant is germline or somatic, and the criteria used to interpret the variant's significance. Users can readily access aggregated and laboratory-specific interpretations from different institutions, making ClinVar a particularly useful tool to observe how other institutions are interpreting variants of interest. While the number of institutions regularly submitting variant annotations appears to be still limited, the hope is that more institutions will participate in the future.

7.3.12 GnomAD (http://gnomad.broadinstitute.org)

The Genome Aggregation Database (gnomAD) was created by a coalition of investigators whose initial goal was to harmonize whole exome data from many datasets [19]. Exome data from disease-specific and population studies were obtained, and the information from individuals with severe pediatric disorders was removed, allowing robust reporting of population frequencies for individual gene variants. The initial dataset was available online as part of the Exome Aggregation Consortium (ExAC), which included exome data from 60,706 individuals. gnomAD expanded on this dataset including 123,136 whole exomes and 15,496 whole genomes. While over 50% of the individuals characterized in gnomAD are of European decent, a significant number of individuals of Latino, African, East Asian, South Asian, Ashkenazi Jewish decent have been included. gnomAD conveniently reports allele frequencies for these individual sub-populations, allowing tailoring of germline variant interpretation to individuals of interest. Further information is also available for each variant, including visualization of read data through an online viewer, quality metrics for read data, and links to dbSNP, UCSC Genome Browser, and ClinVar. In the setting of oncogenomics, the information provided by gnomAD can be used for variant annotation and subsequent variant filtration and prioritization as part of somatic sequencing pipelines. Specifically, benign polymorphisms can be identified and filtered out using the population frequency data from the gnomAD.

7.3.13 VarSome (https://varsome.com)

In addition to the large number of resources coming out of Universities and publicly funded research efforts, there are also some useful resources from the private sector. One example is VarSome, a component of the analysis services provided by the Swiss genomics company, Saphetor. VarSome is a knowledgebase of human genomic variants with a well-designed user interface that aggregates data from several online resources and databases. This allows users to get many different pieces of information about a genetic variant from a single site. However, if the user is seeking to answer a specific question, some of the other databases described here may still be the best place to look first.

7.4 Selected Databases for Individual Genes

7.4.1 International Agency for Research on Cancer *TP53* Database (http://p53.iarc.fr)

The International Agency for Research on Cancer (IARC) *TP53* Database provides a focused resource for many individual *TP53* mutations [20]. The database contains detailed information on specific *TP53* variants including curated frequency in different cancer types as well as detailed functional data covering functional assays in both yeast, mouse, and cell-line models. This level of functional data can be especially useful for characterizing the potential pathogenicity of less common *TP53* variants. A helpful option to search by either cDNA, protein, or genomic description also makes it easy to use this database with different sources of input data. Additional features such as the ability to search and filter by specific mutation features including structural motifs or type of nucleotide changes may also be useful for some users. This database also has a validated set of neutral polymorphisms, a convenient feature for the interpretation potential germline *TP53* variants.

7.4.2 BRCA Databases

Building on the large number of individuals who have been tested for BRCA mutations, many publicly available *BRCA1* and *BRCA2* variant databases exist and can be useful in the interpretation of alterations in these genes. Primarily focused on functional consequences of BRCA alterations, example of these databases include (1) the ARUP BRCA mutation database (http://arup.utah.edu/database/BRCA/) and (2) the Breast Cancer Information Core (BIC) database from the National Human Genome Research Institute (NHGRI) (https://research.nhgri.nih.gov/bic/) [21]. Both of these databases contain thousands of individual variants with information on genomic position, pathogenicity, and primary literature reference when it is available. The BIC database also provides information on ethnicity to help further inform the distribution of variants within populations.

7.5 Future Advances

The overarching goal of oncogenomics in clinical and translational research settings is to connect annotated genotypic information with phenotypes and inform patient care. Currently, online databases and tools are mostly focused on the biological interpretation of gene variants, but tools and databases that connect gene variants to patient outcomes and available treatments are increasingly emerging. Much of this progress is attributable to the availability of public NGS and clinical data from large cohorts, such as The Cancer Genome Atlas (TCGA), and increasing ability to utilize unstructured data from clinical charts using electronic health records (EHR). Two notable examples of emerging tools are presented below.

1. Extending on the idea behind cBioPortal, Oncoscape (oncoscape.sttrcancer.org) is a newly developing tool to visualize and connect genetic and clinical data [22]. Clinical data is sourced from the Genomic Data Commons (GDC) TCGA project. Molecular

data is sourced from a variety of sources including TCGA and various GEO datasets. Users can create cohorts of patients defined by both molecular and clinical characteristics and then visualize survival curves, gene expression principal component analysis, timelines of patient histories and events, and raw clinical data as a spreadsheet. Currently, only spreadsheet data is exportable, but this may change in the future. Through these visualizations, Oncoscape hopes to become a resource for clinicians to determine gene-personalized treatments for their patients and for researchers to investigate molecular and clinical characteristics of patient subpopulations.

2. FlatIron Health, recently acquired by Roche Group, created a tool to efficiently source clinical data from both structured and unstructured electronic health record (EHR) data. This outcome data can be paired with genomic data to allow discovery of genotype-phenotype interactions. For partnered healthcare institutions, FlatIron Health provides an interface to investigate and analyze these interactions easily, using the institutions own data rather than a publicly available database. While the added value of routine investigation of genotype-phenotype interactions on an institutional level remains to be proven, this service has the potential to offer entities with an easy way to investigate personalized medicine at their institution. FlatIron Health also aggregates these data from their partnered institutions and sells the collected data to interested pharmaceutical companies for research and development of new drugs. FlatIron Health is therefore positioned to offer large scale clinical and research services.

Today, clinical curation of somatic variants is a painstakingly manual process, requiring PhD or MD level education, specific training in molecular pathology, deep knowledge of the NGS pipeline used, and the ability to provide quick but robust review of primary literature. Simplification of this bottleneck task during variant interpretation would greatly decrease cost and turnaround times. Advances in artificial intelligence and natural language processing techniques in other fields have simplified similar complex tasks, and investigators are now exploring the use of these tools for clinical variant annotation. In this broad vision, variants called by an NGS computation pipeline would simply enter another computational pipeline that not only automatically retrieves information from online databases including primary literature, but also assembles evidence of variant pathogenicity and actionable therapeutic trials.

A data science competition organized by Kaggle for the 2017 Neural Information Processing Systems (NIPS) conference tasked competitors to classify variants into 9 classes indicating passenger and driver mutations and their level of evidence. A small dataset was provided to contestants by MSKCC and included the gene, amino acid change, and text of literature associated with the gene and/or variant. Interestingly, contestants performed relatively poorly in this challenge, with large differences in scores between the training and test datasets. While the small size of the training dataset may have contributed to the poor performance, an additional hurdle is deficiencies in current algorithms used in machine learning. For example, pathologists bring a great deal of a priori knowledge gained through significant training when interpreting scientific literature – knowledge that today's algorithms are not able to take advantage of during machine interpretation. Another recently published case report compared IBM Watson Genome Analytics (WGA) variant actionability for to that of the New York Genome Center (NYGC) [23]. In processing of paired whole genome sequencing and RNA-seq data from a glioblastoma multiforme tumor, NYGC identified 13 variants which had an actionable clinical trial, while WGA identified only 7 variants which had an actionable clinical trial. The results from the data science competition and IBM Watson suggest that much larger variant annotation datasets and greater advances in predictive models are necessary to make clinical grade decision support tools for variant annotation.

In the immediate future, more simple support tools could be helpful for continued manual curation. The variety of databases and tools described above each have their own strengths and weaknesses, requiring curators to often visit multiple websites and enter duplicative information to retrieve necessary data. A single commercially or publicly available informatics tool to pull or show data from databases and tools of interest could greatly streamline workflow and prevent errors in user queries. These tools could also be paired with a public variant interpretation database such as Cornell's Precision Medicine Knowledgebase, enabling ease of retrieval of past interpretations and information for new interpretations. The creation of such tools could help smaller institutions with less NGS case volume continue to provide high-quality variant annotations and provide an easy to use resource for research applications.

7.6 Conclusion

The wealth of oncogenomic data generated by cancer sequencing with current technologies requires a thorough curation of variants to properly interpret the functional, biological, and clinical implications of findings. Numerous resources and tools are available to assist in this curation process and more are expected to emerge in the future. In particular, as more institutions share their internal knowledge databases publicly and more clinical-oriented online tools are developed, the availability of well-annotated online resources will increase. While the complexity and challenges of variant interpretation may someday be addressed by machine-learning-based decision support systems, it will take some time before those technologies are sufficiently mature. In the interim, support tools enabling the integration of data from different online resources may be developed and will be important in the advancement of pharma-oncogenomics.

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Immuno-Oncology in the Era of Personalized Medicine

8

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Abstract

Personalized medicine in oncology utilizes evidence derived from genetic, immune, and proteomic profiling to inform therapeutic options as well as provide prognostic information for each unique individual and their tumor. Our ability to biologically and immunologically define each patient's tumor has been driven by the development of assays characterizing the genomic and proteomic profiles of tumors that in turn have led to the development of large biologic databases and computational tools for the analysis of these large data sets. In Immuno-oncology, the introduction of checkpoint inhibitors and their approval across multiple tumor types has led to the recognition that the majority of patients will not clinically respond to these therapies but will remain at risk for the development of significant immunologic side effects. This challenge highlights the need for the development and validation of both predictive biomarkers for response to such therapies as well as biomarkers prognostic of disease course. Despite extensive investigation into predictive biomarkers using these biologic databases and computational methods, only recently has progress been made in this area. This progress is the first step allowing us to identify patients likely to benefit from these therapies and moving our field closer to a truly personalized approach to the use of immune therapies in oncology.

Keywords

Immune monitoring · T-cell · Antibody · Immunologic biomarkers · Predictive biomarkers · Prognostic biomarkers

8.1 Introduction

8.1.1 "One-size Fits All" vs Precision Medicine

The concept of personalized medicine describes therapeutic interventions that are individualized to each patient [1]. In oncology, the genetic, immune, and proteomic profile of the tumor and the individual contribute to the unique characteristics of each patient's cancer. Historically, oncology patients were prescribed a first line therapy for a predefined period of time that was determined by the tissue type of the cancer and not by

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more specific characteristics of the malignancy. In the past two decades, there has been a steady evolution in oncologic management towards personalization of treatment, led by the identification of cancer biomarkers. Early biomarkers were identified by immunohistochemistry while more recent biomarkers are identified by mutational analysis and expression patterns. In particular the development of large biologic databases (human genome project), assays for characterizing the genomic and proteomic profiles of tumors, and computational tools that allow the analysis of large data sets have driven significant progress in our ability to biologically and immunologically define each patient's tumor [1]. Such tools are capable of providing molecular signatures that may be strong predictors of benefit from immune therapies.

The early use of immune therapies (Interleukin-2 (IL-2), etc.) were likewise non-personalized but were given with the intent of stimulating T cells and Type 1 anti-tumor immune responses. Immune therapies such as IL-2 and interferon gamma (IFN-γ) cytokines were administered to patients with renal cell carcinoma and melanoma. Yet response rates to these toxic treatments are limited, with partial response rates of 13–17% and complete response rates of 4–9% in treated patients [2, 3]. Unfortunately, the majority of treated patients suffered through significant side effects from these cytokine therapies with no clinical benefit. For these treatments no biomarker was discovered to allow the identification of patients who would derive benefit from these otherwise toxic treatments.

As immunotherapy has come of age, the roles of immune linked biomarkers are being recognized as either prognostic or predictive. Figure 8.1 illustrates some of the altered immune interactions between tumor cells and immune cells that can serve as biomarkers in immuno-oncology.

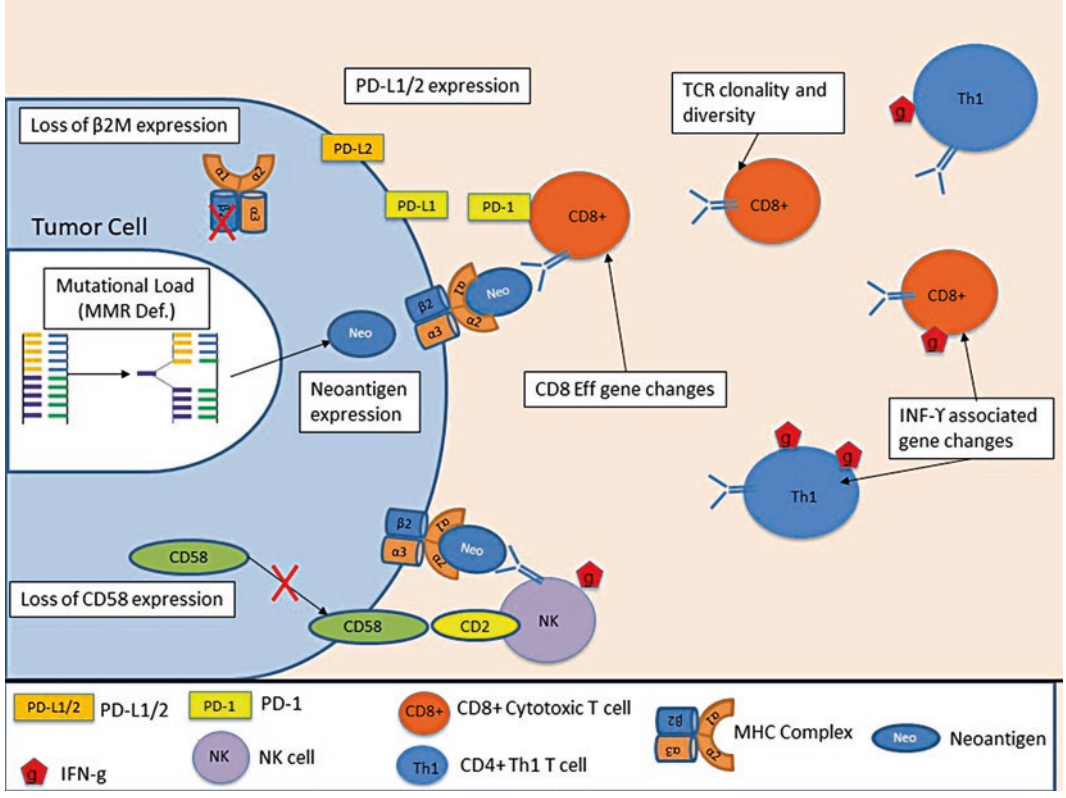

Fig. 8.1 Illustrates some of the altered immune interactions between tumor cells and immune cells that can serve as biomarkers in immuno-oncology

Prognostic immune biomarkers provide information on the likely course of the cancer in an untreated individual, while predictive biomarkers are used to identify subpopulations of patients most likely to respond to a given therapy. The same tumor types that immunologically and clinically responded to IL-2 and similar therapies are the tumor types that were the first to demonstrate clinical benefit from the introduction of immune checkpoint inhibitor therapy leading to FDA approval of these therapies across multiple tumor types. Yet, the clinical benefits of immune checkpoint blockade again have been limited to a minority of oncology patients. Despite extensive investigations into predictive biomarkers, only recently has progress been made in this area with the association of response to PD-1/PD-L1 targeted therapies and PD-L1 expression of >50% in NSCLC [4] as well as the discovery of the presence of MSI high and responsiveness to PD-1/PD-L1 blockade [5]. These predictive biomarkers are the first step in allowing us to identify patients more likely to respond to these therapies.

These currently approved immune checkpoint molecules seek to induce tumor antigen specific T cells that will drive Type I anti-tumor immune responses. Type 1 immunity represents a tumor destructive environment consisting of $CD4^+$ T cells that secrete cytokines (IFN-γ) that stimulate $CD8^+$ cytotoxic T cells (CTL). Conversely, a Type II immune environment consists of $CD4^+$ T cells that secrete cytokines (IL-10) that in turn limit the activation of CTL [6].

8.2 Prognostic Immune Biomarkers

Prognostic immune biomarkers include immune linked gene signatures, tumor immune cell content and location, and circulating immune cell composition, Table 8.1.

8.2.1 Interferon Genetic Signatures

As IFN-γ is a major driver of Type I anti-tumor immunity, studies have evaluated the presence of IFN-γ related gene signatures in regards to prognosis and response to immune therapy. One study in metastatic melanoma evaluated the expression level at diagnosis of type I interferon-stimulated genes (ISGs) and whether their expression could be related to clinical outcomes. This investigation

Table 8.1 Immune monitoring prognostic methods

Analysis Method	Tumor Types Evaluated	Methods	Limitations	Key Examples	Future Directions
Prognostic Biomarkers					
Interferon gene signature	Melanoma	RNA sequencing of genes associated with CD4+ T cells, CD8+ T cells, NK cells, B cells, neutrophils, and macrophages from peripheral blood	Not prospectively evaluated Assoc with prognosis may be limited to tumor types with loss of 9p21.3	Linsley et al. Plos One 2014	Prospectively evaluate in melanoma
Immunoscore	Colon cancer	CD45RO+ and CD8+ T cell density at tumor center and invasive margin Points assigned per cell type and location then added for sum	Cannot perform with core biopsy Cannot perform post-Chemoradiation	Mlecnik et al. Immunity 2016	Application to multiple tumor types Multi-country validation study
Neutrophil-to-lymphocyte ratio	Melanoma, Gastric cancer	Absolute neutrophil count and absolute lymphocyte count used to calculate NLR ratio	Different cutoffs for (+) NLR Coexisting conditions can affect NLR	Ferruci et al. BJC 2015	NLR as a immunotherapy predictive marker

revealed that expression of ISGs (presented in this study as ISG hi vs ISG low) at the time of diagnosis significantly predicted patient survival (p = 3.4 e-5) [7]. In addition, the investigators observed a graded increase in survival according to ISG expression with a median survival of 5106 days, 2184 days, and 813 days for ISG hi, ISG med, and ISG lo, respectively. The overall difference between the survival curves was significant for each ISG expression group (p value = 5.7×10^{-3}) [7].

In breast cancer, gene expression analysis of early stage node-negative breast cancers have identified a relationship between the interferon-induced genes and risk of distant metastatic disease in an analysis of >600 patients with early stage breast cancer. Interestingly this association was dichotomous between breast cancer subtypes. In hormone receptor positive (HR+) HER2 negative (HER2-) breast cancers, the expression of the INF inducible genes was associated with an increased risk of distant metastases (p = 0.0424). Interestingly, in HER2+ (overexpressing) breast cancer cases this gene signature was associated with a decreased risk of distant metastases (p = 0.0099) while there was no such association found between this INF gene signature and HR-/HER2- (triple negative) breast cancers (p = 0.2235) [8].

Another study in gliobastoma multiforme (GBM) illustrated that the over-expression of eight genes linked to INF signaling predicted a worse survival. Specifically, upregulation of downstream targets of INF signaling were associated with significantly worse clinical survival. These INF molecular targets included: MXI (HR: 1.86 (1.37, 2.52) p value < .0001), IFI44 (HR: 1.55 (1.16, 2.06) p value 0.003), ISG15 (HR 1.50 (1.14, 1.96) p value 0.004), OAS1 (HR: 1.70 (1.19, 2.41) p value 0.003), and STAT1 (HR: 1.62 (1.11, 2.35) p value 0.011). As noted in the above breast cancer study, the association of INF signaling and survival was dependent on GBM subtype, with the Proneural subtype of GBM revealing a significant association with survival while no association was found in the Classical and Mesenchymal GBM subtypes [9].

8.2.2 Immunoscore

The "Immunoscore" is an example of a prognostic immune biomarker that quantitates the immune cell composition of a cancer. The development of the Immunoscore for colorectal cancer was based on the observation that the density and location of CD8+ and CD45RO+ T cells in the tumor microenvironment proved to be prognostic of patient survival [10, 11]. The concept of Immunoscore has been evaluated in other tumor types using the evaluation of other aspects of the tumor immune composition in predicting survival, these include tumor infiltrating lymphocytes (TIL) in breast cancer and CD8+ T cells in NSCLC.

In patients with locally advanced colon cancer, Galon et al. have reported the use of Immunoscore, using CD8+ and CD45RO+ T cells, as a superior prognostic indicator for time to relapse in 1300 colon cancer patients when compared to histopathological methods currently used to stage colorectal cancer (TNM staging). In this study, Stage I/II/III colon cancer patients were split into a training set and an internal validation set. In the validation set (n = 630) time to relapse was shorter among the 303 patients with Low-Immunoscore colon cancer vs. the 327 patients with High-Immunoscore colon cancer [HR (95% CI), 0.54 (0.34–0.84); P = 0.006]. In both groups, results were independent of patient age, sex, tumor stage, and location of the cancer within the colon [12].

An analysis by Adams et al. studied the relationship between TIL location (stromal – sTIL vs intraepithelial – iTIL) and content with clinical outcome in patients with triple negative breast cancer enrolled in two adjuvant clinical trials [13]. In this analysis of >400 TNBC cases, higher sTIL immune scores were associated with improved prognosis and the level of sTIL was a continuous variable in regards to survival. Specifically, for each 10% increase in sTILs there was an associated decreased risk of recurrence or death (p = 0.02). Multivariate analysis of the relationship between sTILs level and prognosis revealed sTIL level to be an independent prognostic marker for disease free survival and overall

survival. Interestingly, the presence of iTILs and lymphocyte predominant breast (LPBC) cancer was not statistically associated with outcome in this study though this may have been due to small numbers of cases having these specific immune infiltrates [13].

The concept of Immunoscore has also been applied to non-small cell lung cancer (NSCLC). In a study of ~800 NSCLC patients with stages I-IIIA disease, investigators evaluated the density of CD8+ T cells within TIL [14]. Multivariate analysis of this study revealed that stromal CD8+ TIL density was an independent predictor of disease-free survival ($p < 0.001$) and overall survival ($p < 0.001$) independent of pathologic stage. Interestingly, further analysis revealed that stromal CD8+ TIL density had a significant prognostic role within each pathologic stage (Stages I-IIIA) [14].

8.3 Predictive Immune Genomic Signatures

Predictive biomarkers include alterations within T cell populations, T cell linked gene signatures, genetic polymorphisms, neoantigen loads, and IHC evaluation of immune linked molecules (ex. PD-L1) Table 8.2.

8.3.1 T Cell Receptor Sequencing – Responsiveness, Clonality, and Diversity

Though recognized to be the mediators of Type 1 anti-tumor activity, the role of T cells in the anti-tumor response has traditionally been defined through either ELISPOT identification of antigen specificity, cell surface protein expression, or less commonly the use of tetramer analysis. A more comprehensive analysis of changes in T cell population is now possible through DNA or RNA sequencing of the V(D)J region of the T cell receptor (TCR) beta chain. Analysis of TCR through next generation sequencing allows for the interrogation of important aspects of T cell responsiveness including: clonality, diversity, and somatic allelic mutation. The identification of the peptide/MHC target for each T cell clone will be the next major step in this area, but currently the majority of specific T cell targets are not known. Tumeh et al. provided a key example of the use of TCR sequencing in immunotherapy monitoring in metastatic melanoma patients [15]. In this study, tissue samples collected pre- and post-anti-PD-1 therapy (pembrolizumab) demonstrate that patients with a more restricted TCR beta chain usage, reflecting a T-cell population that was less diverse in repertoire and more clonal in nature, significantly correlated with clinical response to pembrolizumab treatment ($p = 0.004$). Conversely, patients who had progressive disease were noted to have total T cell number and clonality below the median for the trial [15].

In addition to providing predictive information, TCR sequencing can function as a pharmacodynamic biomarker. In our previous study in patients with advanced HER2+ cancers infused with HER2 vaccine primed autologous T-cells, we evaluated for the development of TCR clones after T-cell infusion. The median number of clonal TCRs identified in pre-infusion peripheral blood was 4 (range 1–14) while post infusion the median number was 10 (range 3–17). The development of new TCRVβ species demonstrating clonality post autologous T-cell infusion was associated with those patients who demonstrated tumor regression with this therapy ($p < 0.001$, $R2 = 0.967$) [16].

The quantification of the effects of immune therapy on T cell receptor (TCR) populations was also evaluated in a pilot study of intratumoral HSV-GM-CSF oncolytic viral therapy (OVT) followed by autologous DC–CIK cell therapy in advanced cancers. TCR populations were evaluated pre-OVT therapy, post-OVT therapy, and after DC–CIK therapy. In this study the majority of patients had a dynamic response in their TCR repertoires after OVT therapy as well as an expansion of multiple T cell clonal populations following DC–CIK therapy [17]. This highlights how different immune therapy approaches can distinctly affect an individuals T cell repertoire.

Table 8.2 Immune monitoring – predictive methods

Analysis Method	Tumor Types Evaluated	Methods	Limitations	Key Examples	Future Directions
Predictive Biomarkers					
TCR Sequencing	Mutiple tumors types (Melanoma, Breast, Ovarian cancer, etc.)	DNA or RNA isolation V and J gene primers amplify rearranged V(D)J segments Sequence of unique amplified CDR3 segments determined, identifying V, D, and J genes	Limited by sequencing depth and accuracy Depending on the stage of T cell activation activated T cells might or might not synthesize TCRs,	Tumeh et al. Nature. 2014	Linking specific TCR clones and their target epitope
PD-1/PD-L1 expression (IHC)	All major tumor types (Melanoma, NCSCL, RCC, etc.)	Percentage of tumor cells staining for PD-L1 is scored Positivity generally defined as >5% staining	Expression variable within tumors No standard for positivity	Herbst et al. Nature. 2014	In-vivo imaging of PD-L1 expression
Neoantigens (HiSeq)	Melanoma, colon cancer, RCC, NSCLC, gastric, H&N Ca, uterine, bladder, hepatocellular, prostate, breast, GBM	Genomic DNA obtained & Exon regions captured Paired-end sequencing by Hi-Seq Sequence data are then mapped to the reference human genome sequence and mutations identified	Neoatigens do not always overlap between patients Not all Neoantigens elicit T cell responses	Robbins et al. Nat Med. 2013	Identify neoantigen specific T cells by immortal B cell presentation of neoantigens
SNP analysis	Melanoma Bladder cancer	Genomic DNA isolated & sequenced Sequencing reactions performed Bidirectional re-sequencing of regions of interest (CTLA4, etc.) Software identifies genotype callings	Few patients in trials to date Contradictory results from reported studies	Breunis et al. J Immunoth. 2008	New SNP analysis methods such as Network Phenotyping Strategy (NPS)
β2 microglobulin and CD58 expression	Diffuse Large B cell Lymphoma	Whole genome DNA sequencing for B2M and CD58 Flow cytometry analysis of HLA-I Tumor microarrays for B2M, HLA-I, and CD58	Inactivation of CD58 may be limited to DLBCL Additional mechanisms not identified contribute to mislocalized B2M and CD58 expression	Challa-Malladi et al. Cell. 2011	Evaluation in other tumor types

(continued)

Table 8.2 (continued)

Analysis Method	Tumor Types Evaluated	Methods	Limitations	Key Examples	Future Directions
Microsatelite instability (MSI)	Colon cancer and Multiple other tumor types	Exome sequencing of tumor tissue Identification of known microsatellite sequences prone to copying errors	The presence of rare microsatellite sequences are not commonly evaluated for	Le et al. NEJM 2015	Relationship between the number and type of mutations and response to Immune therapy

8.3.2 CD8 Effector T Cell Gene Signature

The CD8+ Effector T cells are T cells that mitigate anti-tumor cytolytic immunity critical to Type 1 anti-tumor immunity. T cells mediate their cytolytic actions through the release of cytotoxins, perforin, granzyme, and the upregulation of the FAS ligand. Studies have begun to evaluate the presence of CD8+ effector T cells in a more comprehensive manor through their associated gene signatures. One study in follicular lymphoma patients evaluated a six-gene effector T-cell (Teff) signature designed to reflect the functional components of cytotoxic effector cells. In this study the Teff gene signature was prognostic of survival (P = .008). In addition the use of this gene signature allowed the separation of follicular lymphoma patients into 2 groups, an "inflamed" subset (Teff-high) and an "uninflamed" subset (Teff-low). The patients with the inflamed (Teff-high) tumors had a longer progression-free survival (PFS HR, 0.39; 95% CI, 0.21–0.70; P = .002) compared to the "uninflammed" subset. This inflamed subset of patients also demonstrated high expression of other T-cell associated genes as well as counter regulatory genes, which also correlated with PFS [18].

In another small study (n = 32) in follicular lymphoma a baseline Teff gene signature was evaluated in patients treated with concurrent Rituximab and Padilizumab (anti-PD-1 IgG-1k monoclonal antibody). In this study 19 (66%) patients achieved an objective response to therapy with 15 (52%) patients achieving a complete response. In the treated patients a low baseline expression of the Teff gene signature was associated with less tumor shrinkage and a shorter median PFS of 12.7 months (95% CI, 6.5–21.6) these treatments while the median PFS has not been reached in patients with a high expression of the Teff gene signature. In using this Teff gene signature in a data set of FL patients who had received primarily cytotoxic chemotherapy, there was not a significant difference in overall survival when comparing high vs low Teff gene signatures, suggesting that the clinical relevance of this Teff gene signature may be specific to PD-1 targeted therapy [19].

The predictive role of Teff gene signature has also been studied in NSCLC. In a study in treatment naïve NSCLC patients the immune effects of combining chemotherapy ±PD-1 therapy ±VEGF directed therapy was evaluated with a Teff gene signature. The combination of all three modalities (chemo, PD-1, and VEGF) induced longer progression-free survival across all tested subgroups of patients as defined by Teff gene signature. High expression of the Teff gene-signature conferred a greater progression-free survival benefit; however, the degree of benefit was similar to that for high PD-L1 expression in this study [20].

8.4 Tumor Specific Proteomic Immune Analysis

8.4.1 PD-L1 and PD-L2 – Expression

The programmed cell death receptor 1 (PD-1) is expressed on activated T cells as well as B and NK cells. When a T cell recognizes specific peptide fragments bound to MHC complexes and binds to the MHC-peptide complex, the T cells become activated and express the inhibitory receptor PD-1 on their cell surface. If the PD-1 engages with its ligands PD-L1 and PD-L2 this leads to the down regulation of the immune

responsiveness of the T cell. PD-L1 molecules are expressed by multiple tumor types as well as by some normal tissues. Inhibition of the interaction of PD-1 with its ligands through anti-PD-1 and anti-PD-L1 antibodies has led to significant clinical responses and subsequent FDA approval across multiple tumor types.

The KEYNOTE 001 study [4] evaluated the level of PD-L1 expression and clinical response to PD-1 therapy in 655 advanced melanoma patients. In this study, there was a strong correlation between PD-L1 expression and the number of patients with an objective response, up to a maximum level of 65% PD-L1 expression. Interestingly above this level the proportion of clinical responses decreased. Yet the correlation of PD-L1 expression and response to PD-1/PD-L1 targeted therapies has not been clear in other melanoma trials of PD-1/PD-L1 blockade. In the Checkmate 037 study [1] in patients with melanoma previously treated with ipilimumab, the proportion of patients who achieved an objective response to PD-1 therapy was 44% in patients positive for PD-L1 expression, but within the PD-L1 negative patients 20% also responded to nivolumab.

In the KEYNOTE-024 randomized Phase III trial, treatment naive advanced NSCLC patients with ≥50% tumor PD-L1 expression were randomized to receive either PD-1 therapy (Pembrolizumab) or chemotherapy. Enrolled patients gained a longer median progression free survival with pembrolizumab (median 10.3 months, 95% CI, 6.7 to not reached) compared to chemotherapy (median 6.0 months, 95% CI, 4.2 to 6.2) as their initial therapy. In addition the objective disease response rate was higher in this population of high PD-L1 expressing patients who received Pembrolizumab compared to chemotherapy alone (44.8% vs. 27.8%).

Another key study in the evaluation of PD-L1 expression in predicting response to anti-PD-1 therapy was reported by Herbst et al. In this study, 277 patients who received the anti-PD-L1 therapy MPDL3280A had pre-treatment tumor tissue specimens stained for PD-L1 expression. This analysis revealed that across multiple tumor types there was a significant association between patients whose tumor-infiltrating immune cells expressed PD-L1 and positive clinical responses (NSCLC, $P = 0.015$; all tumors, $P = 0.007$), while the association with tumor cell PD-L1 expression did not reach statistical significance (NSCLC, $P = 0.920$; all tumors, $P = 0.079$) [21]. This data suggests that tumor associated immune cells may also function to suppress tumor specific immune responses.

A trial of patients with relapsed or refractory Hodgkin's lymphoma who received nivolumab (3 mg/kg) every 2 weeks, revealed that 17% of patients achieved a complete response and 70% of patients achieved a partial response, and three patients (13%) had stable disease. There is evidence that in some tumor types the expression of checkpoint molecules are increased due to genetic changes in the cancer. In the nodular-sclerosing type of Hodgkin's lymphoma, PD-L1 and PD-L2 genes are key genetic targets of a recurrent genetic abnormality, chromosome 9p24.1 amplification. The analysis of these genes specifically in Reed-Steinberg cells revealed copy-number gains in PD-L1 and PD-L2 in all tested patients in this study along with increased expression of these ligands. This trial highlights how some pathologic changes specifically related to a malignancy may drive expression of immune therapeutic targets [22].

8.5 Role of Tumor Specific Mutations in Immune Therapy

8.5.1 Mutated Proteins as Antigens

Tumor antigens that are recognized by effector T cells can be grouped into three broad categories: differentiation antigens (antigens expressed in a single tissue type), germline antigens (antigens not expressed in adult tissues with the exception of testis), and neoantigens (antigens derived from tumor-specific somatically mutated genes). There is increasing interest into whether tumors with a higher mutational load are more susceptible to immune based therapies as these tumors in turn frequently have a higher burden of neoantigens. These clinical responses suggest that an immune

response against tumor-associated antigens is present in some tumors types and only requires augmentation to induce anti-tumor clinical responses. In clinical trials of checkpoint inhibitors, the presence of pre-therapy CD8+ T cell tumor infiltration is associated with clinical responses to PD-1 targeted therapies [15]. The tumor associated antigen targets of this immune response are either non-mutated peptides that have a restricted tissue expression pattern or mutated proteins, i.e. neoantigens, that are created from tumor-specific DNA alterations [23].

8.5.2 Neoantigen Load

In considering mutational load in cancer subtypes, the prevalence of somatic mutations is highly variable between cancer subtypes as well as within cancer subtypes. An analysis of the mutational rate across cancer types demonstrated a range of mutational burden between 0.0001 mutations per megabase (Mb) to 400 per Mb. Cancers such as ALL and thyroid cancer typically have the fewest mutations per Mb while cancers resulting from chronic exposure to mutagens (lung cancer and melanoma) typically have the highest prevalence of somatic mutations [24]. The highest rate of clinical benefit from checkpoint blockade has also been seen in the tumor types with the highest somatic mutational burden, suggesting that these highly mutated cancer subtypes are likely creating neoantigens. If the production of immunologically relevant neoantigens is a frequent event in tumors with mutational loads above 10 somatic mutations per Mb, many tumors with a mutational load of 1–10 per Mb may still carry neoantigens that can be recognized by T cells. However, based on the fact that even for melanomas with a mutational load around 10 mutations per Mb, T cell reactivity is not always observed [25]. Tumor types with a mutational load below 1 mutation per Mb appear less likely to express neoantigens that can be recognized by autologous T cells [25].

Investigations have begun to address whether there is a correlation between the predicted neoantigen load and the level of immune mediated cytolytic activity within such tumor types. In evaluating multiple tumor subtypes (n = 18), Rooney et al. [26] reported that the number of predicted MHC Class 1 associated neoantigens was correlated with the cytolytic activity found within each respective tumor. Yet, immune mediated cytolytic activity was lower than expected in some tumor types including colon cancer. In addition, this study noted that select recurrently mutated genes were associated with increased cytolytic activity across multiple tumor types and included Caspase 8 and HLA-A [26].

8.5.3 Immunologically Relevant and Irrelevant Mutations

It also must be recognized that the vast majority of mutations do not yield neoantigens that are immunologically recognized by T cells [25, 27]. Studies investigating this concept have typically analyzed the neoantigen responsiveness of T cells within TIL. Recent investigations have evaluated if T cells specific for neoantigens can be detected in the peripheral blood of patients that have the same neoantigen specificity as the T cells contained within TIL. In one study, tumor DNA and normal DNA from patients with metastatic melanoma were isolated and candidate mutations leading to neoantigens were identified. Immunogenic epitopes from these neoantigens was identified using a peptide-MHC–binding algorithm. These epitopes in turn synthesized and used to generate panels of MHC tetramers. This strategy identified 9 mutated epitopes from 5 patients that elicited an immune response from T cells within the TIL. Next T cells from the peripheral blood were exposed to these 9 mutated epitopes and revealed that T cells within the peripheral blood were reactive with 8 of the 9 epitopes and were detected at frequencies ranging between 0.4% and 0.002% [28].

In seeking ways to augment the number of neoantigens targeted by T cells in patients with metastatic melanoma, one study sequenced tumors and identified potential neoantigens predicted to bind HLA-A∗2:01. Of the 57 potential neoantigens, only 2 neoantigens elicited an

immune response by T cells from TIL. The investigators then obtained naïve T cells from healthy donor PBMCs and tested these against the panel of 57 neoantigens and revealed 11 immune responses. This suggests that T cell specificity to neoantigens is lacking in melanoma patients and this could be due either to ineffective T cell priming or the development of tolerance [29]. In seeking to augment the T cell activity against additional neoantigens, this study generated autologous T cells with TCRs from neoantigens-specific healthy donor T cells that were in turn able to recognize the patient-derived melanoma cells harboring the relevant neoantigens [29].

8.5.4 Microsatellite Instability/ Mismatch Repair Deficient Tumors

Some tumor DNA alterations that are either inherited or acquired are recognized to induce a significantly higher number of mutations. One example is mismatch-repair (MMR) deficient or microsatellite unstable colorectal cancers, where MMR deficient colorectal cancers contain up to 100× the number of somatic mutations as MMR-proficient tumors. A study of primarily colorectal cancers revealed a median of 1700 somatic mutations and 578 potential neoantigens in MMR deficient cancers compared to a median of 73 mutations and 21 potential neoantigens in MMR proficient colorectal cancers. In this study, 41 patients, primarily with colon cancer, were treated with Pembrolizumab and were stratified as to whether they were MMR deficient or not. In the MMR deficient colorectal cancers, the objective response rate was 40% (95% CI, 12 to 74) and the progression free survival was 78% (95% CI, 40–97%) while in the MMR proficient tumors the objective response rate was 0% (95% CI, 0–20) and the PFS was 11% (95% CI, 1–35%) [30].

As MMR deficiency is recognized to be present in other tumor types, this same group performed a follow-on study of pembrolizumab treatment for all comers with metastatic carcinomas (n = 87) that were MMR deficient. This study reported a significant clinical benefit from Pembrolizumab treatment in this population with an objective response rate of 53% (95% CI, 42–64%) with 21% of patients achieving a complete response [5]. Correlative studies of TCR sequencing noted a rapid expansion of neoantigen specific T cells following Pembrolizumab treatment in the patients with clinical responses [5]. These studies supported the selection of checkpoint blockade for patients solely based on genetic status and led to the FDA approval of Pembrolizumab for patients with mismatch repair deficient malignancies in 2017.

8.5.5 Mutations in Immune Resistance – β-2 Microglobulin and Costimulatory Molecules

Though the expression of PD-L1 is a recognized mechanism of immune escape in the minority of cancers, other immune escape mechanisms are also utilized to prevent tumor cell eradication by either the adaptive or innate immune systems. A study in diffuse large B cell lymphoma (DLBCL) evaluated for genetic mutations or lack of surface expression of two critical immune molecules, β2-microgolobulin (B2M) and CD58. B2M is a critical subunit required for the assembly of the human leukocyte antigen class I molecules (HLA-I), critical for the plasma surface presentation of self and non-self-proteins on most nucleated cells [31]. This study revealed that through B2M mutations and other protein processing mechanisms, 75% of DLBCLs tested lacked B2M surface expression (n = 29/53) or displayed abnormal B2M expression patterns (n = 11/53). In turn, loss of B2M expression resulted in the lack of HLA-I surface expression in 75% of these cases (n = 40/53) [31].

This DLBCL study also investigated changes in CD58 in DLBCL. CD58 is a ligand for the CD2 receptor and is required for cell adhesion and activation of T cells and natural killer cells. In this study, 67% of DLBCL cases lacked CD58 cell surface expression and this was also due to either genetic changes in the CD58 encoding gene or in protein processing. Of note, reintroduction of CD58 into DLBCL cell lines significantly increased

the level of cytolysis (30–50%, $p < 0.001$). Taken together 61% of evaluated DLBCL cases lacked both HLA-I and CD58 on their cell surfaces ($p < 0.042$). These examples highlight another mechanism of immune escape from anti-tumor cytotoxic T cells and NK cells [31].

8.6 Host Genetic Variants as Influences of the Immune Response

8.6.1 Single-Nucleotide Polymorphisms and Response to Immune Therapies

In immuno-oncology the analysis of single nucleotide polymorphisms (SNPs) has focused on polymorphisms found in immune related molecules. SNPs in the CTLA-4 immune checkpoint molecule has been linked to an increased risk of multiple cancer types [32] and as a predictive measure for the clinical benefit of immunotherapies. In melanoma, three studies attempted to correlate CTLA-4 SNPs with clinical responses to anti-CTLA-4 mAb therapy (Ipilimumab). These studies did not demonstrate a consistent association between selected SNPs and clinical responses to Ipilimumab [33, 34]. In a study by Breunis et al. [33], seven common SNPs were selected from the CTLA-4 gene in 152 Caucasian patients who underwent CTLA-4 blockade and were evaluated to determine correlations to treatment responses [33]. The results of this analysis demonstrated that three of the seven selected SNPs were associated with either partial or complete clinical response to Ipilimumab therapy, these SNPs included: the proximal promoter SNP rs4553808 ($P = 0.002$), the proximal promoter SNP rs11571327 ($P = 0.02$), and the nonsynonymous SNP rs231775 ($P = 0.009$) [33].

New technology has been implemented in a recent re-analysis of data from a 2010 study evaluating the relationship between CTLA-4 SNPs and clinical benefit from high dose interferon therapy in high-risk melanoma cases. In the original study no statistically significant difference in relapse-free survival or OS was demonstrated among the melanoma patients with six CTLA-4 SNPs [35]. In the re-analysis a new method called Network Phenotyping Strategy (NPS) was used for the SNP analysis. The NPS analysis method is a graph-theory based method that captures allele relationship patterns. With this method the investigators were able to relate polymorphic SNP patterns (distances between PRP and RRP pairs) differentiating two survival groups (longer and shorter than 5 years) with statistical significance for these pairs ranging from $P = 0.002$ to $P = 0.043$ [36].

8.6.2 CXCR3/CCR5 Polymorphisms

Begdonetti et al. utilized gene expression to investigate the role of polymorphisms and overexpression of CXCR3/CCR5 chemokine ligands in immune-mediated tumor rejection in melanoma and clinical response to adoptive cell therapy. In this study, 142 metastatic melanoma patients enrolled in adoptive therapy trials with tumor-infiltrating lymphocytes (TILs). Enrolled patients were genotyped for *CXCR3* rs2280964 and *CCR5*-Δ32 deletion. This study demonstrated that under-expression of both CXCR3 and CCR5 according to gene expression and polymorphism data (protein prediction model, PPM) was associated with response to adoptive therapy (odds ratio = 6.16 and 2.32, for CR and OR, respectively) [37].

SNPs from immune related genes have also been used to predict recurrence in patients receiving therapy with bacille Calmette-Guérin (BCG) immunotherapy for non-muscle-invasive bladder cancer (NMIBC) [38]. In the context of BCG immunotherapy, SNP evaluations have been retrospective, not prospective [38, 39].

8.7 Conclusion

The concept of personalized medicine describes therapeutic interventions that are individualized to each patient [1]. The introduction of immune checkpoint blockade has revolutionized the field of oncology, yet the clinical benefit from these

therapies has been limited to a minority of oncology patients. This challenge has highlighted the need to personalize the application of immune therapies through the development of predictive immune biomarkers. Despite extensive investigations into predictive biomarkers, only recently has progress been made in this area with the association of response to PD-1/PD-L1 targeted therapies and tumor PD-L1 expression of >50% in NSCLC [4] as well as the discovery of the association between MSI high tumors and responsiveness to PD-1/PD-L1 blockade [5]. These predictive biomarkers are the first step toward the identification of patients more likely to respond to these therapies. Ultimately, the characterization and statistical validation of these immune biomarkers will be critical to the personalization of immuno-oncology.

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CAR-T cell and Personalized Medicine

9

Marlid Cruz-Ramos and Jesús García-Foncillas

Abstract

Adoptive T cell transfer (ACT) is a new era for cancer treatment, involving infusion of autologous lymphocytes. Chimeric antigen receptors (CAR) on the surface of T cells are emerging as a novel therapeutic that is giving other direction to T-cell specificity and precision medicine. T cells are engineered modification to recognize specific target antigen and are co-stimulated with intracellular signal to increase the T cell response. CAR-T cells have impressive involvement in outcome on hematological malignancies; however severe toxicities as cytokine release syndrome or neurotoxicity are a challenge to face. Solid tumors have heterogeneous antigens and tumor microenvironment that hinder CAR-T cell efficacy and increase the risk of on-target/off-tumor. Novel strategies to increase CAR-Ts specificity, safety and efficacy are ongoing in clinical trials to improve clinical outcomes in hematological and solid malignances.

Keywords

Adaptive immune system · Chimeric antigen receptor (CAR) · Hematological malignancies · Solid tumors · Target antigen

9.1 Introduction

Diverse are the efforts to find new therapeutic options to treat malignancies. For long time several immunotherapy approaches have been tested in cancer to strength the patient's immune system against tumor. Now we have improved our understanding about tumor immunosurveillance and molecular biology tools, increasing our capacity to personalize immune therapy options with clinical efficacy and safety. An emerging immunotherapy approach is adoptive cell transfer (ACT) that consists in the collection of autologous or allogenic T cells with high affinity to tumor antigens (TA) to fight against patient's cancer. The most common types of ACT are: tumor infiltrating lymphocytes (TILs) from the tumor microenvironment that are isolated from surgically resected patient's tumor and are *ex vivo* propagated to be re-infused back into the same patient [1]. The others ACT types are genetically engineered T cells expressing high affinity receptors (TCRs) to tumour-specific antigen or chimeric antigen receptors (CAR-Ts) that consist of an extracellular antigen-recognition domain and an intracellular signaling domain [2].

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9.1.1 CARs Structure Designs

Chimeric antigen receptors are formed by an extracellular antigen-recognition domain usually an antibody single chain variable fragment (scFv) specific for a TA, or less frequently a peptide or protein and an intracellular signaling domain, which usually consists in TCR-associated CD3ζ (CD3 zeta) chain.

The external domain of CAR allows the specific antigen recognition by T cell and, posterior stimulation of intracellular domain that stimulates T cell proliferation, cytolysis and cytokine secretion to eliminate target cell. To generate this T modified cells, is necessary to isolate own patients' T cells, to activate and genetically modify them using retroviral or lentiviral vectors or non-viral methods such as transposon, and finally to reinfuse back into the same patient. This strategy carries low risk of graft-versus host disease and enables lipid, protein and carbohydrate antigens to be targeted by T cells in a MHC non dependent fashion [3] (Fig. 9.1).

The antigen-recognition domain is anchored to the cell by a flexible spacer/hinge region and a transmembrane domain. The intracellular domain consists of termed signaling domains necessary for T cell activation [4]. There are three CAR-Ts generation. First generation contains CD3ζ signaling chains, as termed signal 1. This CAR-T has limited efficacy in clinical trials, one possibility could be the activation-induced cell death of the transplanted T cells or lack to maintain long-term T cell expansion [5, 6]. To avoid these difficulties second generation CAR-Ts includes in their structure a first generation backbone and two co-stimulatory signaling domains to provide a second activation signal. For example, second generation CD19-CAR-T cells include a CD3ζ chain and CD28 signaling domain, this structure enhances persistence a proliferation of CAR-Ts compared with first-generation CD19-specific CAR-T. In acute lymphoblastic leukemia B (B-ALL) second generation CAR-Ts includes CD28 or 4-1BB (CD137) co-stimulatory signaling domains to enhance the response. Third generation CAR-Ts contains a CD3ζ domain and two co-stimulatory domains that could include CD28, 4.1BB or OX40 (CD143), showing higher antitumor efficacy than second-generation CAR-T cells [7, 8] (Fig. 9.2).

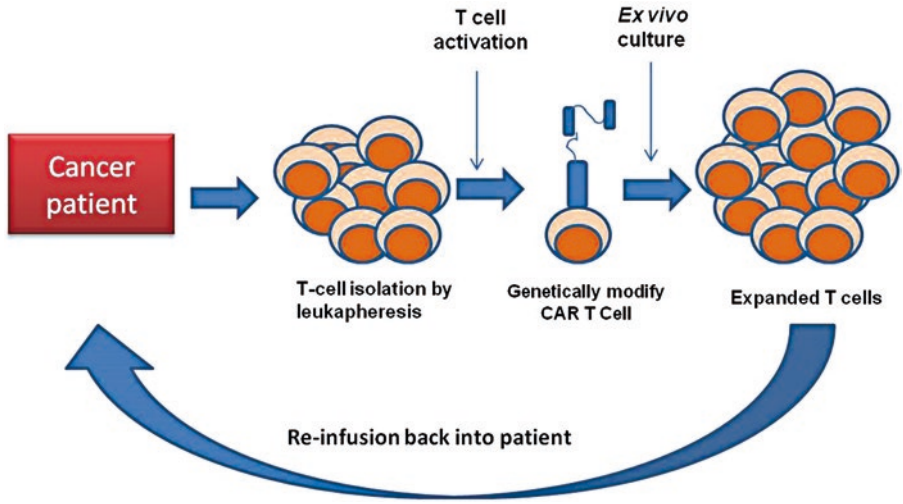

Fig. 9.1 CAR T cell manufacturing and treatment process.
Patient T cells are isolated by leukapheresis, then are *in vitro* activated by stimulation of T cell by magnetic beads or artificial antigen presenting cells. Cells are genetically engineered CARs are delivered by lenti-viral or retroviral and transposon method. The cells are expanded in culture divices. The patient undergo to prior infusion treatment, and then CARs are re-infusion back

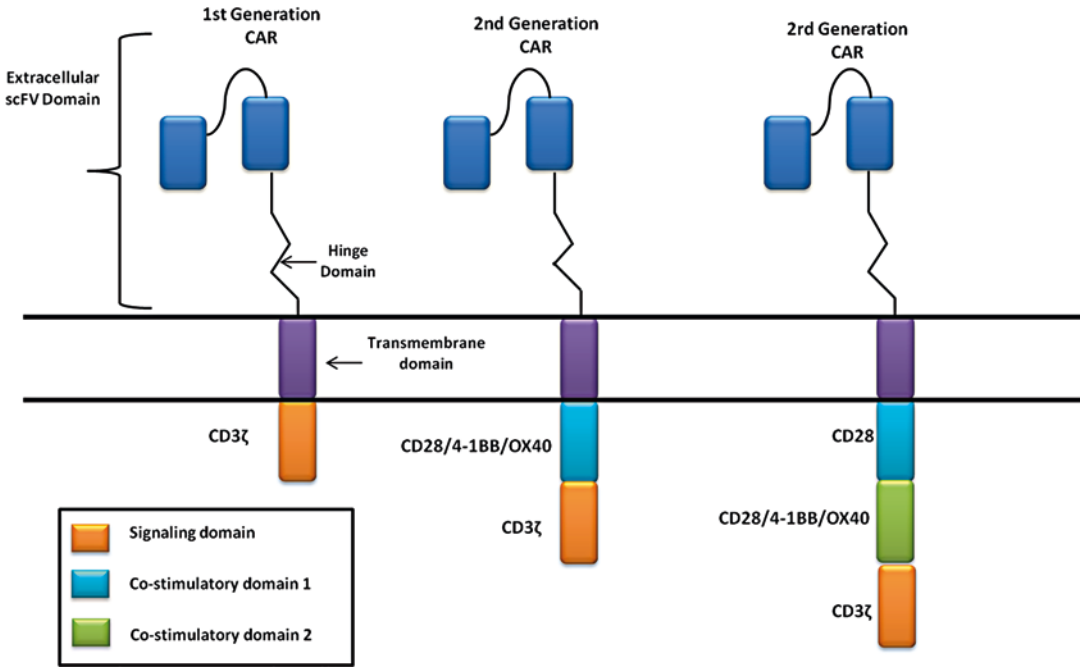

Fig. 9.2 Structure of CAR-T cells generation. First generation CAR consist in extracellular scFV domain with antigen recognition region, the hinge domain and intracellular activation domain. Second and third generation CAR-Ts had one or two co-stimulatory domain including signals such as CD28, 4-1BB or 0X40. *CAR* chemeric antigen receptor, *CD* cluster of differentation, *scFV* single chain variable fragment

9.2 CAR-Ts and Hematological Malignancies

CAR-Ts efficacy have been proved in hematological malignancies. Clinical trials of CARs-T therapy have mostly been conducted in patients with CD19-positive hematological diseases, such as acute and chronic B leukemia (B-ALL or CLL), follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and mantle-cell lymphoma. For example, CD19-targeted-CAR-Ts have achieved 70–90% response rate in B-ALL or CLL resulting in an important tool to treat these malignancies. Below the most important trials in hematological diseases are described. Table 9.1 summarizes CD19-specific published trials. Table 9.2 shows the ongoing clinical trials in hematological malignancies.

9.2.1 B-Cell Malignancies

Memorial Sloan Kettering Cancer Center (MSKCC) published their results of second generation CD19 specific-CAR-T (CD28/CD3ζ), called 19–28 z, in 33 relapsed-refractory adults with B-ALL. All patients received conditioning chemotherapy and then 1–3 × 10^6 19–28 z CAR-T cells/kg. Minimum residual disease (MRD-negative) was achieved in 81% and overall complete response rate (CRR) was 91%. The toxicities reported were cytokine-release syndrome (CRS) and neurological toxicities [9].

The University of Pennsylvania and Children's Hospital of Philadelphia conducted a trial in 57 pediatric and adult patients with B-ALL treated with CD19 transduced second-generation CAR-Ts cells (4-1BB/ CD3ζ) or CTL019 cells. Patients receive doses of 1–10 × 10^6 CAR-T

Table 9.1 Published clinical trials of CD19-specific-CAR-T cells in hematological malignancies

Institution	CAR structure	Patient characteristics	CR rate	Toxicities	Reference
UPenn/ CHOP	CD3ζ and 4-1BB	N = 30 children and young adults with B-ALL	90%	B-cell aplasia CRS	NCT01626495 [11, 21]
MSKCC	CD3ζ and CD28	N = 32 adults relapsed B-ALL	91%	B-cell aplasia CRS	NCT01044069 [22]
NCI	CD3ζ and CD28	N = 20 children and young adults B-LL	70%	B-cell aplasia CRS	NCT01593696 [13]
FHCRC	CD3ζ and 4-1BB	N = 32 adults B-LL	93%	CRS Neurotoxicity B-cell aplasia	NCT01865617 [17]
SCRI	CD3ζ and 4-1BB	N = 45 children and young adults	93%	CRS Neurotoxicity	NCT02028455 [23]
CHOP	CD3ζ and 4-1BB	N = 30 children and young adults ALL, DLCL	87%	CRS	NCT02374333 [24]
UK/ German	CD3ζ	N = 5	25%		[25]

ALL acute lymphoblastic lymphoma, *B-ALL* B cell-acute lymphoblastic leukaemia, *B-LL* B Lymphoma/leukaemia, *CHOP* Children's Hospital of Philadelphia, *CR* complete response, *CRS* cytokine-release syndrome, DLCL diffuse large cell lymphoma, *FHCRC* Fred Hutchinson Cancer Research Center, *MSKCC* Memorial Sloan Kettering Cancer Center, *NCI* National Cancer Institute, *SCRI* Seattle Children's Hospital, *UK* United Kindom, *UPenn* University of Penssylvania

Table 9.2 Ongoing Trials of CARs-T in Hematological Malignancies

Target	CAR Structure	Malignancy	Reference
CD19	KIR2DS2/ DAP12-	Lymphoma, leukemia	NCT02685670 [26]
CD20	CD3ζ; CD3ζ /CD28	CD20+ malignancies	NCT01735604 [27]
CD19 and CD20	CD3ζ/4-1BB	Leukemia, lymphoma	NCT03097770 [28]
CD22	CD3ζ/CD28	FL, NHL, DLBCL, B-ALL	NCT02315612 [29]
CD30	CD3ζ/CD28	HL, NHL	NCT01316146 [30]
CD33	CD3ζ/CD28	AML	NCT01864902 [31]
CD123	CD3ζ/CD28	AML	NCT02159495 [32]
CD138	CD3ζ/4-1BB	MM	NCT01886976 [33]
ROR1	CD3ζ/4-1BB	CLL, SLL	NCT02194374 [34]
Igκ	CD3ζ/CD28	CLL	NCT00881920 [35]
LeY	CD3ζ/CD28	AML	NCT01716364 [36]
BCMA	CD3ζ/4-1BB	MM	NCT02215967 [37]

AML acute myeloid leukaemia, *B-ALL* B cell-acute lymphoblastic leukaemia, *BCMA* B cell maturation antigen, *CD* cluster of differentiation, *CLL* chronic lymphocytic leukaemia, *DLBCL* diffuse large B-cell lymphoma, *FL* follicular lymphoma, *HL* Hodgkin lymphoma, *Igκ* immunoglobulin kappa cahin, *KIR2D2* simulatory killer immunoglobulin-like receptor 2DS2, *LeY* Lewis Y antigen, *MM* multiple myeloma, *NCI* National Cancer Institute, *ROR1* inactive tyrosine protein kinase transmembrane receptor ROR1, *SLL* small lymphocytic lymphoma

cells/kg. This group reported 93% of CRR, 55% had recurrence free survival (RFS) and 79% overall survival at 1 year. Twenty patients relapsed, 13 with CD19 disease. Patients CTL019 persistence had B-cell aplasia, which continued up last assessment (1–39 months) in 24/34 patients with ongoing CR. Cytokine release was seen in 88% of patients [10–12].

National Cancer Institute (NCI) performed an "intent-to-treat" clinical trial in 21 children and young adults with relapsed or relapsed B-ALL or NHL. They were treated with CD19-CAR-Ts (CD28/CD3ζ), CRR of 60.8% with 90% of responders negative for minimal residual disease (MDR-) was observed. The median leukemia free survival (mLFS) of MDR-CR responders was 18.7 months; the median disease survival of

MDR- CR responders was 49.5%. Severe CRS occurs in 13.5% [13, 14].

The Fred Hutchinson Cancer Research Cancer (FHCRC) group used central memory-enriched CD8 cells for starting material of 29 adults to be treated with CD19-targeted CAR-Ts (CD3ζ/41BB) and defined composition of CD4:CD8 T cells. This treatment approach with a defined subset composition achieved 83% complete response rate. The peak level and duration of persistence of both CD4+ and CD8+ CAR-Ts were associated with clinical response [15, 16]. Posterior update reports 93% of bone marrow remission, these investigators identify as risk factors for severe toxicity CAR-T cell dose and tumor burden [17].

Dr. Porter et al. reported their results of CTL019 in CLL. The overall response rate in heavily pretreated patients CLL was 57% (8/14 patients). The in vivo expansion of CAR-Ts correlated with clinical response. CAR-Ts persisted and remained functional beyond 4 years in the two patients whose achieving CR. All responding patients developed B-cell aplasia and experienced cytokine release syndrome that correspond with T cell proliferation [18].

CD-19 specific CAR-Ts (CD28/CD3ζ) have been studied in DLBCL, indolent lymphoma or CLL. In a multicenter phase 2 trial that enrolled 11 previously treated patients with large B-cell lymphoma, including diffuse large B-cell lymphoma and primary mediastinal B-cell lymphoma. Those patients receiving a conditioning therapy and after CD19-specific CAR-Ts showed 82% objective response, 54% complete response and 28% partial response. With a median follow-up of 15.4 months, 42% of these patients had still response, 40% of them with complete response. The most common adverse events were pyrexia (85%), neutropenia (84%) and anemia 66% [19].

9.3 Multiple Myeloma

CD19-specific CARs-T cells were evaluated in 10 patients with multiple myeloma (MM). Patients received pre-conditioning treatment with autoHSCT and melphalan followed by an infusion of second generation CD19-targeted CARs-T cells (4-1BB/CD3ζ). One patient experienced a complete response for 12 months following treatment and six patients remained progression free [20].

9.3.1 CAR-Ts and Solid Tumors

There are diverse research efforts to evaluate efficacy and safety of CAR-Ts in solid tumors, but results are less exciting than findings in hematological malignances. Prior identification of new possible target antigen and posterior preclinical models of solid tumors it is necessary to evaluate efficacy and animal safety of these therapies based on these antigens. The response to CAR-Ts depends on diverse parameters: (1) A good choice of the target epitope, (2) Specific target antigen, (3) CARs structure, CAR-T cell dose, frequency or administration way, (4) Tumor environment, (5) Patient's lymphodepletion and pre-condition treatment previous to CARs-T administration, (6) CAR-Ts engraftment and trafficking capacity [38].

The ideal target antigen is one that could be found specifically in epithelial cancer cells; however solid tumors have over-expression of proteins that are also expressed in normal cells, making difficult the work of find a specific antigen. Despite of this, there are several efforts to test CAR-T in solid tumors.

9.3.2 EGFR and EGFRvIII

The alternately splice variant of EGFR (EGFRvIII) is commonly associated with glioma cells and is necessary for their survival. A phase 1 trial has been done in 10 recurrent glioblastoma (GBM) patients, whose has been previously treated. After EGFRvIII determination, patients were treated by an infusion of EGFRvIII-CAR-T cells. One patient showed residual stable disease for over 18 months of follow-up. All patients had demonstrable detected transient expansion of EGFRvIII-CAR-T in peripheral blood. Seven patients had posterior surgical intervention, which allowed tissue-specific analysis of

EGFRvIII-CAR-T trafficking into the tumor; patients had a patchy pattern of lymphocyte infiltrate com-posed of CD8 T cells after CARs-T infusion compared with pre-infusion brain tumor specimens from the same patient [39].

9.3.3 HER2

The tyrosine-protein kinase receptor erbB2 (HER2) is commonly expressed in diverse epithelial cells such as gastrointestinal, respiratory, urinary and reproductive systems. However, HER2 overexpression has been detected in tumors cells of gastric, breast, colon and ovarian cancer.

A third generation HER2-CAR-T cell (CD28/4-1BB and CD3ζ) was tested in metastatic cancer (NCT00924287 trial). This trial stopped ahead of time because one colon cancer patient with lung and liver metastases died for acute respiratory failure. Post mortem analysis exhibited signs of systemic ischemia and hemorrhagic microangiopathic injury. The lungs had a diffuse alveolar damage with an immediate accumulation of T lymphocyte demonstrated in the patient's lung. The patient had a marked increase of IFN-ϒ, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), IL and IL-10 after HER-2- CAR-T cell infusion. This trial included in the CAR-T cell structure a scFV based on trastuzumab. One explanation for this severe toxicity could be the recognition and depletion of low levels of ERBB-2 in lung epithelium, triggering pulmonary failure and massive cytokine release [40].

Despite of this severe toxicity there are several ongoing trials with HER2-CAR-T in others tumors such as sarcoma where investigators are using a scFv with lower affinity than trastuzumab-based CAR, therefore with better results in safety setting. Nineteen patients with advanced-stage sarcoma have been treated with these second-generation HER2-specific CAR-T cells (CD28/CD3ζ). HER2-CAR T cells persisted for at least 6 weeks in seven out of nine evaluable patients. HER-CAR-T cells were detected at tumor site in 2 patients. Four patients had stable disease for 12–14 weeks. Three patients with surgery after HER-CAR-T cells infusion had ≥90% necrosis. Median overall survival was 10.3 months [41].

9.3.4 Mesothelin

Mesothelin (MSN) is a novel attractive target for cancer immunotherapy. This protein is low expressed in normal mesothelial cells, but has a high expression in many solid tumors. Physiologically, MSN is expressed on mesothelial cells of pericardium and peritoneal and pleural cavities.It has been found to be overexpressed in mesothelioma and ovarian cancer, and in other tumors such as lung, pancreatic, gastric, endometrial, colon and breast cancer [42, 43].

A clinical trial tested CAR-T cells with mRNA encoding for second-generation MSN-CAR-T (SS1-4-1BB) in advanced mesothelioma or pancreatic tumors by intravenous or intratumor MSN-CAR-T. Cell infusions were well-tolerated and no off-target toxicities were observed (pleuritis, pericarditis or pericarditis). A severe anaphylaxis and cardiac arrest were reported with the third infusion of MSN-CAR-T, secondary to a high production of IgE antibodies targeted against MSN-CAR-T, probably associated with the murine SS1 scFV. Despite of this severe toxic event, the treatment in general was well- tolerated. Antitumor activity was demonstrated by a decrease in tumor-cell numbers in ascitis and a decrease of peritoneal lesions [44].

9.3.5 Disialogangloside GD2

Disialongangloside GD2 is a glycosphingolipid with low-level expression in neural tissues; however tumors as neuroblastoma overexpress this protein. GD2-CAR-T had been investigated in patients with neroblastoma. They used autologous activated T cells (ATCs) and autologus Epstein barr-virus specific T cells (EV-CTLs), each modified with a distinguishable GD2-specific CAR (GD2-CAR-T). Three patients out of 11 with active disease achieved complete remission and persistence of CAR-ATCs or

CAR-CTL-S beyond 6 weeks associated with superior clinical outcome [45]. Third generation GD2-CAR-T cells (OX40/CD28/CD3ζ) in patients with neuroblastoma, osteosarcoma and melanoma are under investigation. To increase the safety of this CAR-T, the investigators modified the GD2-CAR-T cells to express inducible caspase 9 (icaspas9) suicide gene [46].

9.3.6 Prostate Specific Membrane Antigen

Prostate specific membrane antigen (PSMA) is a type II membrane protein expressed in most of prostate-cancer cells and tumor –associated neovasculature of many solid tumors [47]. A second generation PMSA-CAR-T cell (CD28/CD3ζ) has been tested in prostate cancer patients. No toxicity was reported, two out of three patients included in this study had stable disease at 6 months of follow up [48]. Similar results are reported in other study that treated prostate cancer patients with second generation PMSA-CAR-T cell (CD28/CD3ζ). Patients had decreased PSA levels, and disease progression was delayed in two out of five patients [49].

9.3.7 Other Tumor Antigens

Several tumor antigens in solid tumors are investigated on clinical trial, they are summarized in Table 9.3. Those antigens include glycoproteins as carcinoembryonic antigen (CEA) expressed on many epithelial tumorsfrequently located atthe gastrointestinal tract. Other protein is the neural cell adhesion molecule L1 (CD171) that is expressed in ovarian cancer, neuroblastoma and melanoma. This protein is also expressed on normal tissues as peripheral nerve and kidney, but with a different glycosylation pattern than CD171 expressed in malignant cells, making it a suitable target for CAR T therapy. Other proteins under investigation are the glypican-3, a surface proteoglycan overexpressed on hepatocellular carcinoma, and the IL-13R, a high affinity monomer receptor overexpressed in 50% of glioblastoma, with low expression in normal brain tissues.

9.3.8 Future: Strategies to Improve the Safety and Efficacy of CARs-T

CAR-Ts have impressive results in clinical trials for B-cells malignancies, however, there are still concerns about inability to control CAR-Ts after patient's re-infusion back. CAR-Ts have the capacity to attack normal tissue (off-tumor-cross reaction), being the major limiting factor in the clinical setting.

Future challenges to improve CAR-T cells therapy [67].

1. Antigen loss
2. On-Target/off tumor toxicity (CAR-T cell recognize normal tissues and can cause severe and life-threatening toxicities, especially in solid tumors).
3. Tumor Microenvironment (Function as a barrier to CAR-T cells penetration).
4. Production difficulties (Autologous T cells manufacturing)

There are several approaches to improve safety and efficacy of CAR-Ts (Fig. 9.3). New strategy to face antigen loss relapse is for example the modification of CAR-Ts with two distinct CAR molecules with two different binding domains called dual-signaling CAR. Tandem CARs (TanCAR) is other approach, with one CAR molecule containing two different binding domains in tandem that simultaneously targets different antigens, for example HER2 and IL13Rα2 to mitigate tumor antigen escape, showing superior antitumor activity compared with pooled CAR-Ts or co-transduced T cells in mouse glioblastoma model [68].

The inhibitory CAR (iCAR) is a fusion of an antigen recognition domain, usually an antigen expressed on normal tissue, with an inhibitory intracellular domain, which could be a programmed cell death protein 1 (PD-1) or a cytotoxic T-lymphocyte-associated protein 4

Table 9.3 Ongoing clinical trials in solid tumors

Target	CAR-T structure	Tumor	Reference
EGFRvIII	CD3ζ and 4-1BB	Glioma	NCT02209376 [50]
	CD3ζ, CD28 and 4-1BB	Glioma	NCT01454596 [51]
HER2	CD3ζ and CD28	Sarcoma	NCT00902044 [52]
	CD3ζ and CD28	Glioblastoma	NCT02442297 [53]
		Glioblastoma multiforme	NCT01109095 [54]
Mesothelin	CD3ζ and 4-1BB	Malignant pleural	NCT01355965 [55]
		Mesothelioma	
		Pancreatic cancer	NCT02465983 [56]
		Pancreatic and ovarian cancer and malignant mesothelioma	NCT01583686 [57]
GD2	CD3ζ, OX40, CD28	Neruoblastoma, osteosarcoma and melanoma	NCT02107963 [46]
		Neuroblastoma	NCT01822652 [58]
PSMA	CD3ζ and CD28	Prostate cancer	NCT01140373 [59]
			NCT00664196 [60]
CEA	CD3ζ and CD28	Liver metastasis	NCT02331693 [61]
FAP	CD3ζ and CD28	Mesothelioma	NCT01722149 [62]
MUC1	CD3ζ and 4-1BB	MUC1 positive solid tumors	NCT02587689 [63]
CD171	CD3ζ and 4-1BB	Neuroblastoma	NCT02311621 [64]
	CD3ζ, CD28 and 4-1BB		
Glypican-3	CD3ζ, CD28 and 4-1BB	Hepatocellular carcinoma	NCT02395250 [65]
IL-13Rα2	CD3ζ and 4-1BB	Glioma	NCT02208362 [66]

CEA carcinoembrionic antigen, *EGFRvIII* epidermal growth factor receptor variant III, *FAP* prolyl endopeptidase FAP/fibroblast activation protein alpha, *HER2* human epidermal growth factor 2 receptor, *IL-13Rα2* interleukin 13 receptor α2, *MUC1* mucin 1, *NCI* National Cancer Insitute (USA), *PSMA* prostate-specific membrane antigen

(CTLA-4). This fusion leads the inhibition of CARs activation and limits its undesired activation [69]. To improve CARs safety there are also another approach such as suicide genes as inducible caspase-9 (iCaspase-9) switch, included in 19-CAR-T cell encoding vector to remove inappropriately active CARs. This "safety switch" approach modulates the effects of CARTs-19 cells and could reduce severe toxicities related to this therapy [70, 71]. Switchable CARs (sCAR-T) are based on engineering bifunctional switched that consists of a tumor antigen-specific Fab molecule engrafted with a peptide neo-epitope, which is bound exclusively by a peptide-specific switchable CAR-T cell. This switch redirects sCAR-T cells activity through the formation of selective immunological synapses. On this way sCAR-T cell switches

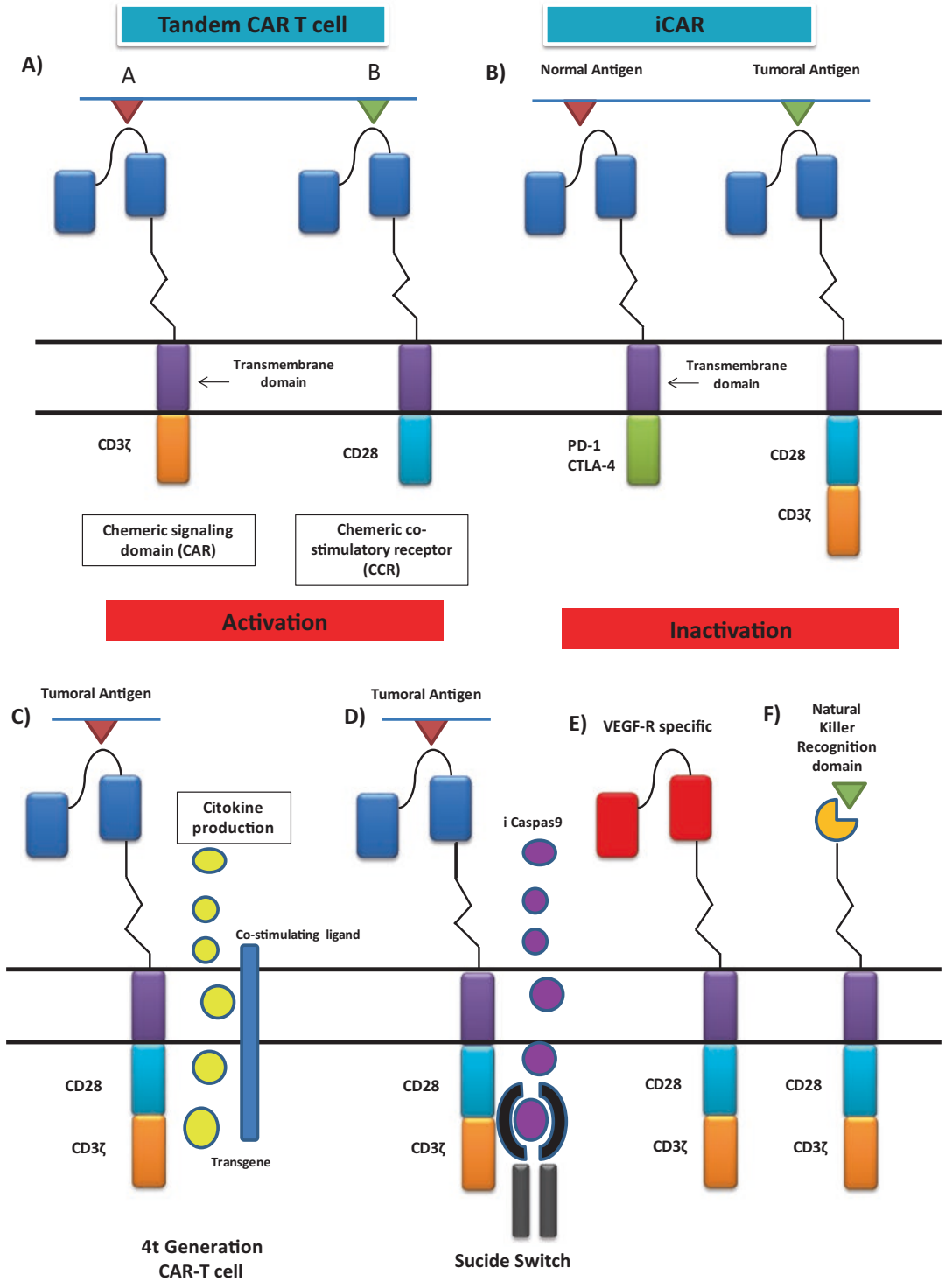

Fig. 9.3 CAR-T: Novel approaches to improve safety and efficacy.
(**a**) Tandem CAR-T cell had two different tumoral antigen (TA) specific scFvs domains to have a synergic activation of both scFV simultaneously. (**b**) iCAR- is a combination of inhibitory receptor specific for the antigen present on normal cell but not on tumor cells, to protect normal cells from a CAR-T cell mediated attract. (**c**) 4th Generation CAR-T cell are associated to a transgene and have the capacity to produce cytokine as IL-12 to increase immune response, (**d**) Switch Suicide CAR-T structure, (**e**) target tumor stroma like VEGFR CAR-T, (**f**) Natural Killer Recognition domain to recognize own antigens and foreign

and targets cells in a structurally defined and temporally control manner [72].

Natural Killer (NK) cells have receptors capable to discriminate between normal and tumor cells. Recently, NK receptors are being used as antigen recognition domains in CAR-T, to improve tumor recognition by T cells. The receptor NKG2-D links to intracellular T-cell signaling domains and enables this receptor to activate T cells. It is under investigation in diseases like AML, MM and myelodysplastic syndrome [73, 74].

The phenotypic heterogeneity of solid tumors hinders CAR-T cell efficacy in these tumors. After initial tumor reduction by CAR-T cell, the antigen-negative tumor cells that are still alive, not recognized by CAR-T, are probably implicated in tumor relapse. TRUCK T cells or fourth Generation CAR-T cells are modified CAR- T cells to secrete pro-inflammatory cytokines (usually cytokine like IL-12). This TRUCK T cell can release this transgenic protein to regulate T-cell response and active innate immune response cells that can kill negative-antigen cancer cells. The transgenic IL-12 is stored into the CAR-T and only is released when is induced [75].

Tumor microenvironment like tumor-vasculature is important for tumor cell survival. VEGF ligand and their receptors are implicated in cancer. VEGFR-2 is overexpressed on tumor stroma cells and some tumor cells. VEGFR-2-specific-CAR-T cells target tumor stroma cells, without harm normal tissue. This is another possible application of CAR-T cells in solid tumors [76].

Genome editing tools as zinc finger nucleases, meganucleases, transcription activator-like effectors nuclease (TALEN), homing endonuceases, and clustered regularly interspaced short palindromic repeats (CRISPR-Cas) system are successfully applied to engineer T cells. Human genome editing led the opportunity generate "universal" CAR-T cells without a functional endogenous TCR or eliminate immunosuppressive signals such as PDL-1 and CTL-4, improving T cells function [77] (Fig. 9.4).

9.4 Toxicities

The most important toxicities related with CAR-T cell therapies are cytokine release syndrome and neurotoxicity and both have very interesting physiopathology. Similar than happens with other immune treatment such as monoclonal antibodies (MoAB), CAR-T cells administration are associated with an immune response mediated by cytokines. Cytokine release syndrome (CRS) has been observed with CD19-specific, CD22-specific and BCMA-specific CAR-T cells therapy, rates of severe CRS are around 25% among various trials. Symptoms occur any time in the first 2 weeks after CAR-T cell infusion and are related with increase cytokine levels. Tumor necrosis factor

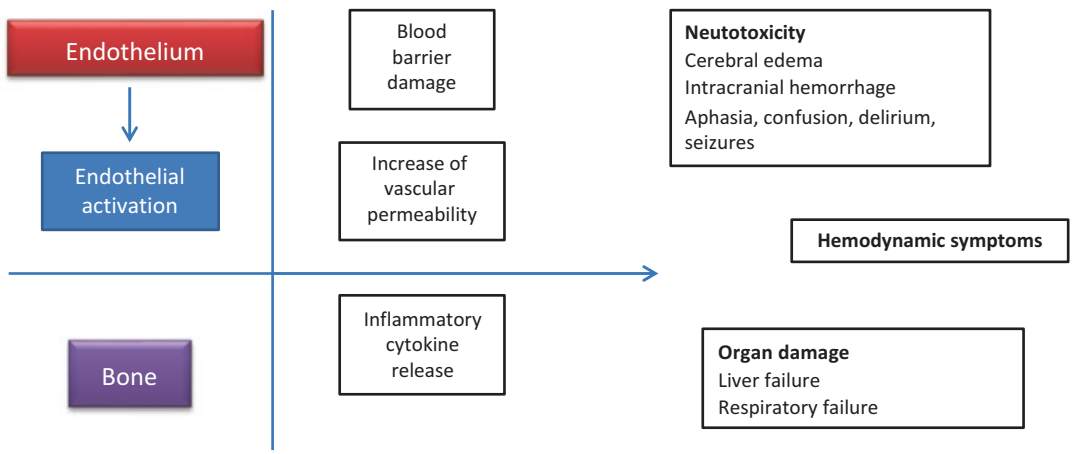

Fig. 9.4 Physiopathology of CAR-T cell toxicity

(TNF) α increases first and is followed by IFNϒ, IL-1ß, IL-1, IL-6; 1L-8 and IL-10 [78]. Others biochemical abnormalities include elevated C-reactive protein and ferritin levels. The CRS severity is related with tumor burden and the anti-tumor responses. The clinical symptoms include fever, hypotension and less common respiratory failure. Consumptive coagulopathy has been also described and is related with severe CRS in children [79]. Lee et al. suggested a CRS grading scale that it is beginning to be used in clinical trials trying to unify outcomes reports (Table 9.4). Most of the CRS related symptoms are manageable with antipyretic, steroid and intravenous fluids. Despite of, some patients will require supportive care as high doses of vasopressors and ventilatory support. Interleukine- 6 (IL-6) is predominantly elevated in these patients and is related with severe CSR. The monoclonal antibody anti-IL6R, tocilizumab, has been used in B-ALL leukemia treated with CD19-CAR-T with good outcomes. Based on these results, tocilizumab is indicated to treat severe CRS [80, 81]. Investigators are working on developing strategies for mitigate CRS occurrence. The group of Seattle Children's Hospital (SCRI) has proposed a strategy which aimed to decrease the rates of severe CRS based on tocilizumab or dexamethasone administration, when patients demonstrate persistent symptoms of mild CRS (Table 9.5). This strategy reduces severe CRS rate in approximately 50%, without impact on efficacy or long-term persistence of the CAR-T cells therapy [49]. When the prevention fails or CRS symptoms remain, MKSCC group have proposed a CRS management algorithm (Fig. 9.5) [82].

Table 9.4 Grading System for Cytokine Release Syndrome (CRS)[a]

Grade	Toxicity related Symptoms[b]	Treatment
1	Fever, nausea, fatigue, headache, myalgias, malaise	Symptomatic treatment only
2	Oxygen requirement of <40%, hypotension responsive to fluids or low dose of one vasopresor. Grade 2 organ toxicity or grade 3 transaminase elevation.	Symptoms require and respond to moderate intervention
3	Oxygen requirement of ≥40%, hypotension high dose or multiple vasopressor. Grade 3 organ toxicity or grade 4 transaminase elevation.	Symptoms require and respond to aggressive intervention
4	Hypotension refractory to high dose vasopressors. Requirement for ventilator support or grade 4 organ toxicity	Life-threatening symptoms
5	Death	

[a]Adapted from Lee et al. [81]
[b]Severe Neurological complications such as dysphasia, confusion, delirium, visual hallucination, seizure-like activity are related with CRS

Table 9.5 CRS management (early intervention)[a]

Symptoms related to CRS	Suggested intervention
Fever ≥38.3 °C	Acetaminophen (12.5 mg/kg) PO/IV up to every 4 h
Persistent fever ≥39 °C for 10 h that is unresponsive to acetaminophen	Tocilizumab (8–12 mg/kg) IV
Persistent fever ≥39 °C after tocilizumab	Dexamethasone 5–10 mg IV/PO up to every 6–12 h with continued fevers
Hypotension	Fluid bolus, target hematocrit >24%
Persistent/recurrent hypotension for longer than 12 h	Tocilizumab (8–12 mg/kg) IV
Use of low-dose vasopressor for hypotension for longer than 12 h	Dexamethasone 5–10 mg IV/PO up to every 6 h with continued use of pressors
Initiation of higher dose vasopressor or second vasopressor	Dexamethasone 5–10 mg IV/PO up to every 6 h with continued use of pressors
Initiation of oxygen supplementation	Tocilizumab (8–12 mg/kg) IV
Increasing of respiratory support	Dexamethasone 5–10 mg IV/PO up to every 6 h with continued use of pressors
Recurrence/persistence of symptoms after ≥48 h of initial dose of tocilizumab	Tocilizumab (8–12 mg/kg) IV

[a]Adapted from Annesley et al. [84]
Tocilizumab is a humanized, immunoglobulin $G1_K$ ($IgG1_k$) anti-human interleukina 6 (IL-6) receptor monoclonal antibody. Suggested doses is 8–12 mg/kg) IV

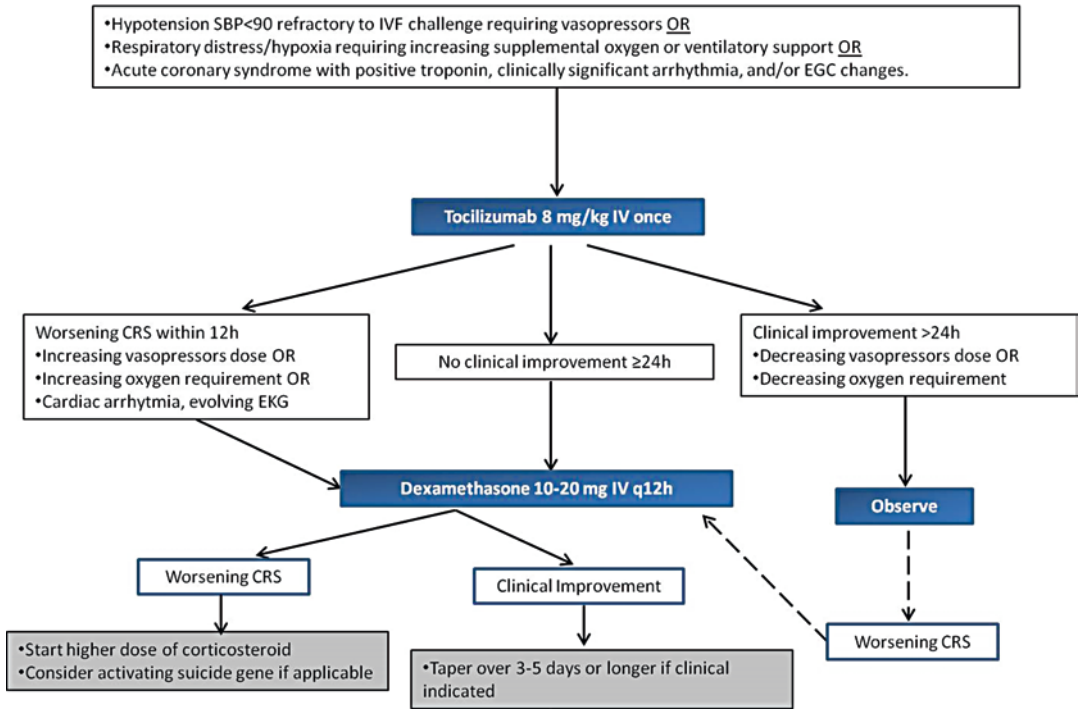

Fig. 9.5 MSKCC for CRS Management Algorithm (for Grade≥2)

Neurotoxicity has been observed associated with CAR-T cell therapy, with a wide spectrum of symptoms since mild confusion and aphasia to life-threatening encephalopathy and intractable seizures. Apparently these symptoms are related with cerebral edema secondary to immune activation. It is not known if cerebral edema is a consequence of CAR-T cell therapy as an extreme manifestation of CRS or it is an independent symptom. The major explanation for this pathology is endothelial injury related to cytokine release, contributing to the onset of neurotoxicity, but the exact mechanism of action is still poorly understood [83]. The Fig. 9.4 summarizes the physiopathology of CRS and Neurotoxicity.

Grades 2–4 refer to CTCAE v.4.0 grading.

9.5 Take Home Messages

1. CAR-Ts are a novel precision immunotherapy strategy specifically designed to attack a tumor antigen, using patient's T cells engineered modification.
2. It is important to select the best target antigen to generate CAR-T cell effective against a specific tumor.
3. Next generation CAR-T cells will be available to improve immune response, decrease off target/on tumor risk, to be more capable to penetrate tumor microenvironment, and to program death or apoptosis.
4. Cytokine release syndrome management is a new challenge in the clinical oncology practice.

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10 Oncobiome at the Forefront of a Novel Molecular Mechanism to Understand the Microbiome and Cancer

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Abstract

The microbiome comprises all the genetic material within a microbiota, that represents tenfold higher than that of our cells. The microbiota it includes a wide variety of microorganisms such as bacteria, viruses, protozoans, fungi, and archaea, and this ecosystem is personalized in any body space of every individual. Balanced microbial communities can positively contribute to training the immune system and maintaining immune homeostasis. Dysbiosis is a change in the normal microbiome composition that can initiate chronic inflammation, epithelial barrier breaches, and overgrowth of harmful bacteria. The next-generation sequencing methods have revolutionized the study of the microbiome. Bioinformatic tools to manage large volumes of new information, it became possible to assess species diversity and measure dynamic fluctuations in microbial communities. The burden of infections that are associated to human cancer is increasing but is underappreciated by the cancer research community. The rich content in microbes of normal and tumoral tissue reflect could be defining diverse physiological or pathological states. Genomic research has emerged a new focus on the interplay between the human microbiome and carcinogenesis and has been termed the 'oncobiome'. The interactions among the microbiota in all epithelium, induce changes in the host immune interactions and can be a cause of cancer. Microbes have been shown to have systemic effects on the host that influence the efficacy of anticancer drugs. Metagenomics allows to investigate the composition of microbial community. Metatranscriptome analysis applies RNA sequencing to microbial samples to determine which species are present. Cancer can be caused by changes in the microbiome. The roles of individual microbial species in cancer progression have been identified long ago for various tissue types. The identification of microbiomes of drug resistance in the

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treatment of cancer patients has been the subject of numerous microbiome studies. The complexity of cancer genetic alterations becomes irrelevant in certain cancers to explain the origin, the cause or the oncogenic maintenance by the oncogene addiction theory.

Keywords
Microbiome · Oncobiome · Cancer · Dysbiosis · NGS · Metagenomics · Metatranscriptomics

10.1 Understanding the Microbiome

Humans have a symbiotic relationship with trillions of microbes residing within the gastrointestinal tract. [1] Thousands of species forming this community are collectively known as the microbiome. The microbiome comprises all the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the human gut) that represents tenfold higher than that of our cells. [2].

There is an important consideration that we must assume, in order to understand the microbiome protective and dysfunctional state, these mean that microbes exhibit a different phenotypic and metabolomic profiles because of exist as single cells in a free-floating environment where are fast-growing and they are susceptible to environmental influences/drugs, in opposition to microbes living in a biofilm which are slower-growing communities of adherent bacteria that are more tolerant of environmental influences, that is the definition of a planktronic state.

The microbiota it includes a wide variety of microorganisms such as bacteria, viruses, protozoans, fungi, and archaea, and this ecosystem is personalized in any body space of every individual, creating a commensal, symbiotic, and pathobiont relationship that has garnered increasing attention regarding its role in pathogenesis. Gastrointestinal tract harbors the highest number and diversity of micro-organism in the human body, just about bacteria representing the bulk of the microbiota (10^{12} bacteria/gm feces).

Interactions between host and microbiome can have dramatic effects on health by aiding digestion, regulating metabolism, conferring resistance against pathogens, and modulating host immunity. [1, 3] Balanced microbial communities can positively contribute to training the immune system and maintaining immune homeostasis. [3, 4] Dysbiosis is a change in the normal microbiome composition that can initiate chronic inflammation, epithelial barrier breaches, and overgrowth of harmful bacteria. All these factors have been associated with carcinogenesis. [3] Historically, members of microbial communities were identified by culture and subsequent staining, which precluded the investigation of nonculturable species and strains. However, next-generation sequencing (NGS) methods have revolutionized the study of the microbiome. [5] Without the need to culture or clone individual organisms, NGS enables simultaneous analysis of thousands of species within a microbial community. With the development of bioinformatic tools to manage large volumes of new information, it became possible to assess species diversity and measure dynamic fluctuations in microbial communities. As institutions began to recognize the important role microbes play in health and disease, new initiatives were launched to aid the research community. [6] The NIH funded Human Microbiome Project [HMP: www.hmpdacc.org] and the European Metagenomics of the Human Intestinal Tract [MetaHIT: www.metahit.eu] both use NGS-based data to establish valuable reference genome databases for the human microbiome.

10.2 Oncobiome: The Link Between Microbiome and Cancer

The burden of infections that are associated to human cancer is increasing but is underappreciated by the cancer research community. The International Agency for Research on Cancer estimates that one in five cancer cases worldwide are caused by infection. [Fig. 10.1].

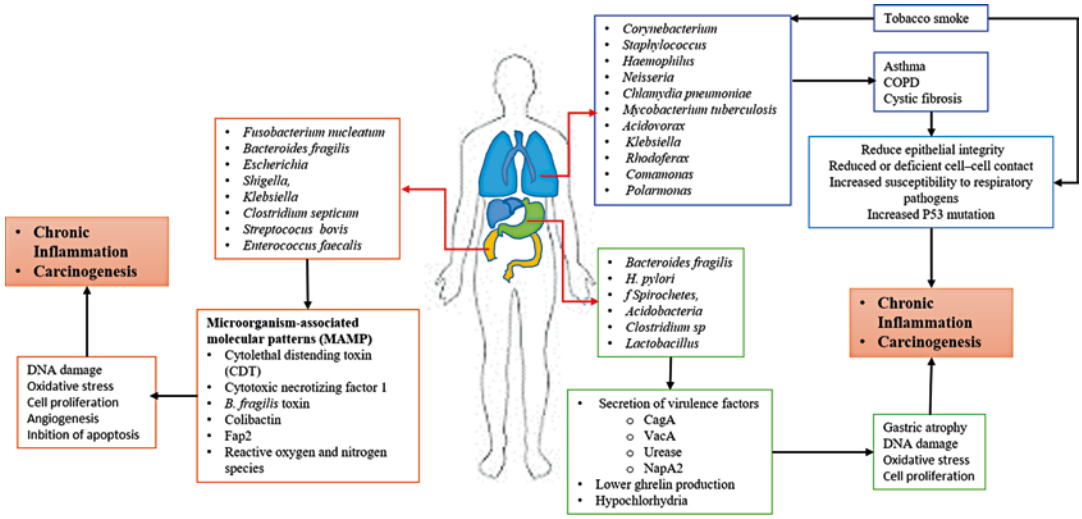

Fig. 10.1 Pathogenic bacterium related to carcinogenesis. The diagram shows the bacterial species that are closely related to the process of carcinogenesis and cancer progression in lung, stomach and colon. The relationship between cancer and the host microbiota, to be termed the "oncobiome"

Since Francis Peyton Rous's landmark experiments on an avian cancer virus in 1911, only seven human viruses have been found to cause 10–15% of human cancers worldwide. Infective agents such as viruses have been part of the modern cancer research and expect that will provide insights into both infectious and non-infectious cancer causes. The focus research of infections that because human cancer is revealing unexpected connections between innate immunity, immune tolerance and tumor suppressor signaling that control infections, microbiome and cancer.

The cheap cost and the high accuracy of sequencing technologies (NGS) has created the opportunity for most research groups to search for infections cause of human cancer. Only a snippet of unique nucleic acid sequence is needed to discover a new human tumor infective agent and to begin characterizing it, so the pool of cancer-causing candidates is almost certain to grow in the coming decade. Equally importantly, the reliability of human sequence databases has matured to a level at which certain classes of cancer agents might be excluded when none is found. Identifying a new infective agent, however, is only the beginning in determining whether it causes human cancer. Cancer causation theories work well for uncommon infective agents that are uniformly present in a cancer. For some human cancers, infection is only one component in their ultimate cause. But failure to recognize the importance of infections in human cancer has led to overlooked opportunities in cancer control and treatment.

The rich content in microbes of normal and tumoral tissue that reflect diverse physiological or pathological states, and the ease with which this material can be sampled, make it a choice for biomarker applications. Changes in the interactions among the microbiota, epithelium, and host immune system are associated with many diseases, including cancer.

Because of the complexity of microbiome and the substantial dynamic range in abundance of its microbiome constituents, indirect approaches can be used to search for cancer associated microbiome that might be transforming or maintaining these pathological states. One approach is to develop a catalogue of microbes that are present or associated to cancer cells.

Nowadays in genomic research has emerge a new focus on the interplay between the human microbiome and carcinogenesis and has been termed the 'oncobiome' (defined as the intricate interplay and study of the human microbiome

and its influence on carcinogenesis) [7]. Recent studies have demonstrated associative relationships rather than causative ones. But the question of whether this emerging field of research is a 'landscape' without a clear picture yet or it represents a new paradigm for cancer research [8]. Bacteria represent the chief member of the microbiota, one of the most recognized links between bacteria and cancer is the case of Helicobacter pylori in gastric carcinoma. However, there is many bacteria which can change the microenvironment and promote the process of carcinogenesis like Chlamydia pneumoniae,

Mycobacterium tuberculosis, Acidovorax in lung, Fusobacterium nucleatum and Bacteroides fragilis in colon. This bacterium has been shown to secrete several virulence factors such as CagA (cytotoxin-associated gene A), VacA (vacuolating cytotoxin A), urease, and NapA2 (neutrophil-activating protein A) that result in oxidative stress, chronic inflammation and host DNA damage that can promote carcinogenesis, progression and poor prognosis in patients colonized with these bacteria. in other pathogens and commensal bacteria are likely to contribute to carcinogenesis. [Fig. 10.1].

The interactions among the microbiota in all epithelium, induce changes in the host immune interactions and can be a cause of cancer. Environmental factors influence tumorigenesis and tumor progression locally and systemically, so manipulation of microbiota could be a key process to improve the effectiveness of anticancer agents. It is well known that inflammatory and metabolism are mechanisms non-characterized yet, that are promoters of carcinogenesis by stablish a transition from eubiosis to dysbiosis in any epithelia. The scientific evidence to answer the question and to propose a new paradigm forward to bring a new perspective to understand and treat cancer by microbiota interaction and manipulation. Numerous microbial species have been implicated in promoting tumor growth associated with local inflammation. [9–11] More recently, microbes have been shown to have systemic effects on the host that influence the efficacy of anticancer drugs. [12–15] Though the mechanisms of these effects are not fully characterized, modulation of the immune system is frequently a factor. The ability of NGS to analyze complex microbial communities may help to uncover new mechanisms of host–microbe interactions that promote cancer or promote drug efficacy. This application note highlights several key discoveries regarding the influence of the microbiome on cancer development and reviews the technologies that can be used to help further investigate host–microbe interactions.

10.3 Eubiosis, Dysbiosis and Oncogenesis

There are about one hundred trillion microorganisms (mainly bacteria) just in the gut microbiota colonizing the human intestinal tract. These colony of micro-organism has be organized and modified by co-evolution between human epithelia and microenvironment, the gut microbiota for example preserve the equilibrium into the host and maintaining the eubiosis, by protecting the pathological colonization of micro-organism and cooperating in metabolic process such as digestion of complex carbohydrates, by a symbiotic agreement, by other side human gut epithelia provides a nutrient-rich, a protective microenvironment and an immune system that tolerates them but immune surveillance against invasion of pathogens. When there is a disequilibrium between the relationship of host epithelia and gut microbiota the dysbiosis appears. Exist many conditions and factors that promote dysbiosis such as pathogenic micro-organisms, passenger commensals, antibiotics, xenobiotics, smoking, hormones, and dietary cues; and of course, natural conditions as aging or non-natural conditions as genetic defects cause of Crohn Disease that affect epithelia and lymphoid components of the gut immune system. All these well-established risk factors promote inflammatory states that increase the risk for oncogenesis.

Nowadays, the Helicobacter pylori is the most recognized link between bacteria and cancer and non-cardia gastric carcinoma. H. pylori has been designated a type I carcinogen by the World Health Organization, The mechanisms of

action of this bacterium is secreting virulence factors such as CagA (cytotoxin-associated gene A), VacA (vacuolating cytotoxin A), urease, and NapA (neutrophil-activating protein A) producing oxidative stress, chronic inflammation, and host DNA damage, all necessaries process that road to dysplasia and cancer. But nevertheless, it has been demonstrated that H. pylori elimination just offer a minimal reduction of gastric cancer, so the evidence that a single pathogenic organism is cause of cancer remains unclear and open the door to enforce the evidence symbiotic microbiota in presence of H. pylori modulates carcinogenesis.

Such as Thomas and Jobin said, there is much of oncobiome research focused on colorectal cancer (CRC), because of is harboring a complex and many micro-organisms in the human body, so is considered the ideal human cancer to explain and study the role of the host–microbe and its relationship with carcinogenesis. The use of advanced genomic approaches is trying to explain the pathogenic relationship, but however, the focus research must correlate the whole microorganism collection of gut microbiota in healthy patients and those with CRC more than to try to correlate a single pathogen to explain a complex process as colorectal carcinogenesis.

10.4 Oncobiome Sequencing Methods in Cancer Samples

Unlike capillary sequencing or PCR-based approaches, high-throughput sequencing technologies have improved the knowledge of human microbiota and how the microbiome impacts human health and disease. [16] Recent developments in microbial sequencing techniques (and bioinformatics pipelines required of analysis of these data sets) have allowed much more in-depth profiling of the structure of microbial communities than was previously possible. [17] NGS-based microbiome sequencing can detect low-abundance members of the microbial community that may be missed or are too expensive to identify using other labor-intensive methods (i.e. cloning steps or bacterial culture).

In fact, with these sequencing approaches, the older epidemiological observations have been supplemented with highly sensitive methods for exanimating the microbiome more completely in tissues. [18].

The NGS technology provides streamlined workflows for several methods that can provide critical genetic insight into cataloging microbiome species and monitoring their dynamics. Three basic NGS applications are available for microbiome studies: shotgun metagenomic sequencing, metatranscriptome analysis, and 16S rRNA sequencing. The choice of application depends on the research question being asked.

10.5 Omics Technologies for Understand the Oncobiome

10.5.1 Shotgun Approaches

Shotgun metagenomic sequencing is used to sequence all genomic content in a microbial sample for species identification and functional analysis. With high sequence coverage, shotgun metagenomic sequencing can detect rare and low-abundance members of the microbial community. The method enables to evaluate microbial diversity and detect the abundance of microbes in various environments.

- Metagenomics (DNA)

Metagenomics allows to investigate the composition of microbial community and discovery novel enzymatic functions, microorganisms and genes in order to understand host-pathogens interactions and novel therapeutic strategies in human disease. [19, 20]

- Metatranscriptomics (RNA)

Metatranscriptomes encompass all RNAs encoded by a group of organisms in a complex sample. Metatranscriptome analysis applies RNA sequencing (RNA-Seq) to microbial samples to determine which species are there, what they are

expressing, and how they respond to changes in the environment (active pathways). Unlike hybridization-based methods, microbial RNA-Seq enables unbiased strand-specific identification of common and novel transcripts. Metatranscriptome information can be used to quantify gene expression changes, predict antibiotic resistance, understand host-pathogen interactions, and track disease progression. [21, 22].

10.5.2 16S rRNA Gene Profiling

16S gene (16s rRNA) is used as housekeeping genetic marker to study bacterial phylogeny and taxonomy in order to characterize complex bacterial communities (bacteria and archaea). 16S rRNA profiling relies on using PCR "universal" primers targeted at the conserved regions and designed to amplify a range of different microorganism as wide as possible. The amplified fragments of the gene correspond to selected short-hypervariable regions ranging from V1-V9, making it cheaper and faster than other high-throughput sequencing technologies. [22, 23].

10.6 Oncobiome Applications in Cancer Research

Cancer can be caused by changes in the microbiome. The roles of individual microbial species in cancer progression have been identified long ago for various tissue types. Localized inflammation has frequently been found in tissues directly exposed to the microbes [NIH: www.cancer.gov/aboutcancer/causes-prevention/risk/infectious-agents; [9–11]] [Fig. 10.2].

Alternatively, some tissues have been identified where the microbial population had a localized protective effect. [10, 24] Furthermore, some immune-modulatory therapies and conventional therapies rely upon the inflammatory response, which is suppressed in the absence of microbial components. [12].

More recently, studies have emerged describing microbiomes acting at a distance to influence sterile tumor environments, [12–15] and can affect both natural autoimmunity and immune-modulating anticancer therapies. Response to CpG-oligonucleotide immunotherapy and platinum chemotherapy was impaired in sterile or antibiotic-treated mice that exhibited poor tumor infiltration by myeloid-derived cells and low cytokine production. [15] Systemic effects influencing the T-cell repertoire were possibly mediated by cross-reactivity between microbial antigens and tumor antigens. Other studies in mice have demonstrated that hematopoietic differentiation in bone can be impacted by gut microbiome perturbations caused by high-fat diet. [25] This study underscores the possibility of incorporating specific dietary instructions into future models of disease treatment. An improved understanding of microbial influences on the immune system as it relates to cancer could impact both conventional cancer therapies and immune-modulating therapies in the future. [Fig. 10.3].

10.7 Oncobiome Effects on Immunotherapy Against Cancer

During the past decade, there was an increasing evidence indicating that the evasion of innate immunity plays a fundamental role in tumorigenesis. Humans are chimeric for numerous infective agents. In some cases, mammals may even exploit latent infections to beneficially regulate their own innate immune systems. So, it is the reason that the eukaryote cell is devoted to protecting the host genome from foreign viral sequences. Innate immune signaling shares many similarities to tumor suppressor signaling, as both processes initiate cell cycle arrest and prime apoptotic pathways.

Recent studies in mouse models have demonstrated the benefit of assessing microbiomes together with immunotherapy approaches, discovering that key species in the gut microbiome exert systemic influences on the efficacy

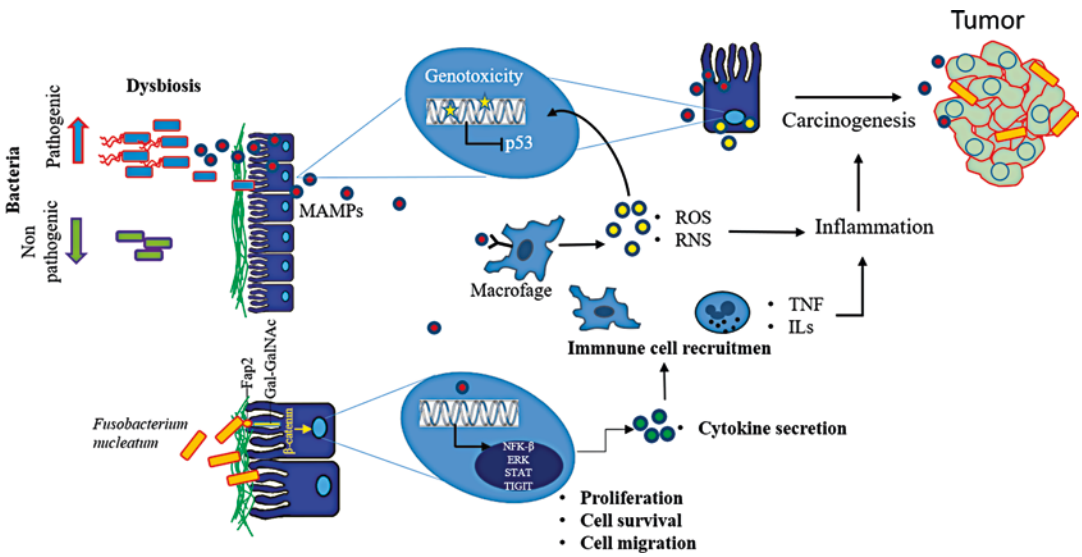

Fig. 10.2 Mechanisms by which the bacterial microbiome modulates carcinogenesis. The bacterial "oncobiome" can promotes carcinogenesis through different mechanisms. Dysbiosis and disequilibrium in the microbiota can induce carcinogenesis in colon due to increase of pathogenic bacterial translocation and immune dysregulation. Microorganism-associated molecular patterns (MAMP) are recognized by TLRs in several cell types which can promote the release of reactive oxygen and nitrogen species (ROS and RNS) which could cause DNA damage and mutations (mutations in p53) likewise MAMPs can initiate inflammasomes-associated immune response and TLR-activated autophagy. On the other hand, *F. nucleatum* uses the virulence factor Fap2 to adhere and invade cells through hots polysaccharide (Gal-GalNAc) and interact with E-cadherin to activate β-catenin signaling to promote NFK-β, ERK, STAT over-expression and modulate immune cells recruitment and proliferation, migration and cell survival and CRC development

of immune-modulatory drugs. Two studies have implicated specific bacteria that impact the ability of checkpoint inhibitor drugs to strengthen the immune response. In one study, a negative outcome using CTLA-4 blockade therapy was associated with the absence of a specific gut bacterium. [14] However, the outcome improved with several combinatorial approaches, such as gavage with the bacteria, using bacterial antigens for immunization, or adoptive transfer of antigen-specific T-cells. An independent study used 16S rRNA sequencing to identify another microbe that mediated the effects of anti-PD-L1 treatment. [13] Similarly, a combinatorial approach significantly reduced tumor growth associated with accumulation of T cells in the tumor microenvironment. Together these studies highlight the potential of identifying beneficial species and using combinatorial approaches involving microbiota manipulation.

10.8 Conclusions

Collectively these studies have revealed that both progression of cancer and the efficacy of cancer treatments can be significantly influenced by microbes living in the host. The diversity of the microbiome can be perturbed by diet and drugs, while key species in the microbiome can cause local or systemic influences on host immunity. There is hope that new methods of treatment will be developed that combine existing cancer therapies with methods to encourage growth of beneficial microbes or eliminate harmful ones. However, the variation between individual hosts will likely require profiling of many more microbial communities and their properties to decipher new mechanisms and methods of treatment. As NGS-based research continues to explore host–microbiome interactions, in order to align trends in microbiology with the evolution of genomic technologies that compliment

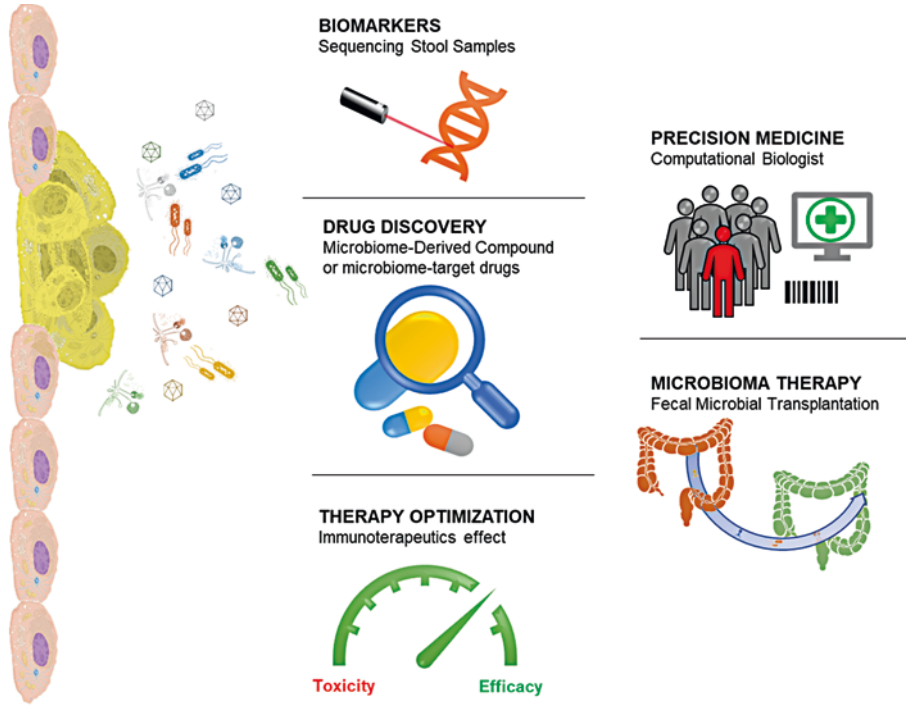

Fig. 10.3 Microbiome and therapeutic tools in cancer. Microbial intervention could generate a diagnostics tools for dysbiosis, develop new drugs or novel therapeutic for cancer patients, using prebiotics, probiotics or live bacteria. The interaction with microbiome could be a personalized therapy adapted to the patient's life style, to comorbidities, comedications, and to genetic

and enable the promise of this field. The scale of data delivered by NGS sequencing systems supports a broad range of cancer research goals. With various NGS applications available, it is possible provides flexible, accurate, and reliable options for analyzing the microbiome. There is a necessity of informatic tools for data storage, analysis, and sharing.

The identification of microbiomes of drug resistance in the treatment of cancer patients has been the subject of numerous microbiome studies using either comparative analysis of drug-sensitive and drug-resistant clinical samples. Comparative analysis of tumoral tissue microbiome profiles using NGS analysis will result in a set of microbiomes that could distinguish responders to cancer drug treatments from non-responders. An algorithm will be developed to predict outcome after the treatment of cancer patients using a specific inhibitor based on pharmacogenomic analysis of tumor samples. The algorithm will be tested with data from several independent cohorts and will be demonstrated to reliably classify patients according to their outcome. This predictor will be also associated with survival after treatment with specific therapies in patients undergoing first-line therapy. A predictive algorithm based on the microbiome profile could predict overall survival and progression-free survival in patients treated. The elucidation of the identity of related microbiome profiles and the basis for their correlation with outcome would add more importance to the microbiome findings.

Given the multitude of approaches to profile the oncobiome in its various dimensions, as presented in this chapter, there is a compelling need for large-scale, integrative and collaborative efforts to elucidate the oncobiome profile, like the current efforts to define the range of genomic alterations in cancer. Although current technologies allow unprecedented depths of analyses, it is

impractical to conceptualize an all-encompassing single human oncobiome project, given the inherent complexity of the microbiome. An alternative would be to conceptualize several oncobiome projects with different cancer types and initiatives with clearly defined objectives and milestones. The aim of such 'oncobiome project' would address the need for cancer biomarkers that have diagnostic relevance on the one hand and the need to define altered signaling pathways in cancer that have therapeutic relevance on the other hand [Table 10.1].

The new development of anti-latent viral drugs and the amazing progress in immunomodulation therapies against cancer are achievable goals that have not yet been pursued and exploited in a new focus cancer therapy control and prevention. The real measure of success for the present century of tumor microbiome research will be the future exploitation of existing research to effectively diagnose, treat and prevent cancers that are caused by microbiome deregulation.

Future progress made so far in microbiome studies of cancers related to microbial infection and epithelial tumors, which have encompassed premalignant and tumor tissues, immune and stromal cells and biological fluids in all cancer stages that include to decipher signaling pathways, in order to identify microbiome signatures related to tumor initiation, promotion, invasion, immune scape, immune tolerance and metastasis, and the discovery of diagnostic, predictive and prognostic biomarkers.

All these efforts that we can implement in order to correlate and evaluate the therapeutically and prevention potential that we can gain with the discovery of microbiome role in oncogenesis, it will be guided by the general principle that it is easier to correct the microbiome function than to restore a loss of microbiome function, disrupting the abnormal cross-talk between pathogenic microbiota and oncogenesis; saying in technical words: the manipulation of the gut microbiota might improve not only the therapeutic effect of anticancer agents, also prevention and an anticipatory cancer diagnostic. The grand complexity of cancer genetic alterations becomes irrelevant in certain cancers to explain the origin, the cause or the oncogenic maintenance by the oncogene addiction theory, so that could explain the striking and apparently irreversible dependency on a single genetic change. Microbiome and cancer remain very much in the line of fire toward to get a cancer solution.

Table 10.1 Objectives of an Oncobiome project

A grand challenge for oncobiomics[a] would be to develop new cancer diagnostics based on the application of microbiomics technologies to the tumoral tissue. Such a challenge would comprise:
Comprehensive quantitative analysis of microbiome constituents among subjects that were later diagnosed with cancer that were part of large population cohorts to identify proteins that might predict the onset or risk of major common cancers.
Comprehensive quantitative analysis of microbiome constituents among newly diagnosed subjects with cancer to define subsets that provide information about lineages and signaling pathways that drive tumor development, progression and response to targeted therapies.
Elucidation of microbiome subsets that vary with biological parameters including age, sex and ethnicity.
Elucidation of microbiome subsets that vary with diet and with common exposures, notably tobacco smoke.
Development of a knowledge base of microbiome alterations in cancer.
Development of affinity capture agents for microbiome with cancer relevance.
Development of protective microbiomes for colonize and modified microbiomes associated with cancer.
Development of standardized specimen reference sets to be made available to investigators.

[a]This new concept it must be integrated at the broad spectrum of "omics" definitions

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Nutrition, Cancer and Personalized Medicine

11

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Abstract

Cancer is a complex group of diseases where different signaling pathways have been found to be deregulated, mainly related to cell proliferation, angiogenesis, metastasis, evasion of apoptosis and insensitivity to anti-growth sings among others. Diet plays a fundamental role in the treatment of the oncological patients, we must be aware that food can interact with certain types of cancer therapy. On the other hand, cancer therapies sometimes affect the patient's sense of smell, taste, appetite, gastric capacity or nutrient absorption, which often results in malnutrition due to the lack of essential nutriments. In this chapter we will review the effect of different metabolic disorders in cancer and mechanisms of action of some phytochemicals found in different foods like resveratrol, EGCG, curcumin and lycopene.

Keywords

Cancer · Diet · Nutrition · Precision therapy · Personalized therapy · Biomarker · Targeted therapy · Resveraterol · Lycopene · Curcumin · EGCG

11.1 Introduction

Cancer is among the leading causes of morbidity and mortality worldwide. The number of new cases is expected to rise significantly in the next few decades [1]. A third of cancer deaths are due to a poor nutritional status; a mere reflection of poor dietary habits, tumor activity and treatment related adverse events [2].

Worldwide, having a higher body mass index (BMI) is among the leading risk factors associated with cancer after smoking and infections [3]. Obesity appears to be a principal risk factor for many types of cancer, including breast, endometrial, liver, ovarian, kidney, colon and rectum. Obesity-related metabolic alterations, such as insulin resistance, manifest several pathophysiological changes that may cause an inflammation of the microenvironment, which is associated with a worse prognosis and greater tumor growth [4]. Likewise obesity promotes leptin dysregulation, which has a role in the development of a large variety of malignancies

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like breast cancer, thyroid cancer, endometrial cancer and gastrointestinal cancer [5].

It has been estimated that 30–40% of all tumors can be forestalled with a correct lifestyle and diet. A high intake of carbohydrates with a high glycemic index (GI) and a lack of fiber are considered to cause hyperglycemia and hyperinsulinemia, along with a higher risk of insulin resistance and type 1 insulin-like growth factor (IGF-1) production. Both insulin resistance and high levels of IGF-1 are related to alterations in cell proliferation and apoptosis. Also, a high intake of saturated fat and trans fatty acids may cause proinflammatory effects and alter the cell-mediated immune response by macrophages.

On the other hand, malnutrition is often identified when diagnosing cancer, or during treatment. It varies in frequency between 31 and 87%, depending on the stage, type of cancer and treatment. Weight loss can occur as a result of low energy intake or a deficiency in nutrient absorption. In cancer patients, weight loss may be caused by a variety of factors, such as inflammation, tumor-induced catabolism, and treatment side effects (anorexia, early satiety, nausea, vomit, mucositis, dysphagia, diarrhea, hemorrhoids, anal fissures and alterations in taste and scent). Toxic effects from chemotherapy may also affect food intake and nutrient absorption. Therefore, weight loss and/or malnutrition are linked to progression of the disease, poor prognosis, worse quality of life, lower levels of physical activity and reduction of pathological response to treatment [2].

Finally, while nutritional problems like obesity can promote carcinogenesis; cancer treatment can promote both weight loss and malnutrition, leading to treatment failure and poor quality of life. Compounds like retinoids, vitamins C, D and E, polyphenols and polyunsaturated fat acids such as Omega-3 may inhibit carcinogenesis. But what is the link between nutrition and cancer? Could food modify the effect of anti-tumor therapy making it more effective? Can a good diet act as a protective factor against cancer?

It's imperative to implement a periodic evaluation of both cancer patients and cancer survivors' nutritional status, which should include promoting healthy nutritional habits and adequate physical activity.

11.2 Metabolic Disorders and Cancer

The main metabolic alterations found in cancer patients are obesity, insulin resistance, type 2 diabetes mellitus (DM), systemic arterial hypertension (SAH), dyslipidemia, metabolic syndrome (SM), malnutrition, sarcopenia and leptin dysregulation.

Adiposity is associated with adipose tissue disfunction, adipocyte death and low-grade chronic inflammation. Under these conditions, adipose tissue develops inflammation and present characteristics of a chronically damaged tissue, such as infiltration and immune-cell remodeling [5]. Metabolic disfunction of adipose tissue increases the production of proinflammatory mediators, among them leptin, resistin, vascular endothelial growth factor (VEGF), tumor necrosis factor alfa (TNF-α), plasminogen activator inhibitor-1 (PAI-1), and interleukins 6 (IL-6) and 1β (IL-1β), along with an increase in the local production of estrogens and chemotactic factors, such as monocyte chemotactic protein-1 (MCP-1) [6, 7]. MCP-1 promotes the attraction of monocytes and macrophages to the affected tissue. This infiltration increases the release of proinflammatory mediators, which can induce carcinogenesis progression [7].

A high BMI is associated with adipose tissue hypertrophy, as well as an increase in its secreting function. Currently, adipose tissue is considered an endocrine organ with important metabolic activity by producing cytokines and adipokines, including leptin and adiponectin. These adipokines have an important participation in regulating inflammation and insulin resistance. Dysfunction in these adipokines induces an increase in local production of estrogen, as well as some cytokines such as IL-1 and TNFα, which have endocrine, paracrine and autocrine activity. Locally, hypertrophy and metabolic dysfunction of the adipose tissue modulates and

maintains microenvironment inflammation that promotes tumor growth [4, 6, 8].

An increase in leptin production is related to growth, proliferation and survival of the tumor cell and angiogenesis, through the activation of the MAPK and PI3K signaling pathways [7, 9, 10]. Similarly, leptin increase is associated with the activation of macrophages able to produce TNF-α and IL-6. TNF-α activation begins when it is coupled with its primary receptor (TNF-R1), capable of activating transcription factor NF-κB, which promotes gene expression of proinflammatory cytokines. IL-6 activates the JAK-STAT3 pathway, which is involved in regulating proliferation and inhibiting apoptosis of neoplastic cells [4, 9, 10].

Meanwhile adiponectin production is affected by TNF-α and IL-6 production by hypertrophic adipose tissue and M1 macrophages. The decrease in adiponectin production inhibits its anti-inflammatory effect and protection against carcinogenesis [7, 10, 11]. Therefore, normal adiponectin production is thought to act as an antagonist to leptin's proinflammatory effects through the activation of AMP-activated protein kinase (AMPK). Some of adiponectin's protective actions include inhibiting proliferation and migration of endothelial cells to avoid angiogenesis, plus inducing apoptosis of neoplastic cells [10].

Another factor involved in carcinogenesis is PAI-1. This factor presents certain negative effects related to tumor migration, progression, growth and invasion, by inhibiting apoptosis and favoring angiogenesis of the tumor cells [4, 9].

An increase in proinflammatory mediators produced by hypertrophic adipose tissue not only affects carcinogenesis, it also causes certain metabolic alterations related to obesity; among them, insulin resistance, hyperglycemia and dyslipidemia, which promote a decrease in muscle mass. Insulin resistance is the main cause of deficient glucose metabolism by tissues, especially muscle tissue, which causes an increase in hepatic gluconeogenesis from amino acids released during muscle atrophy [12, 13].

Therefore, disfunction of the previously mentioned physiological mechanisms, as well as hyperinsulinism secondary to insulin resistance, favor an increase in IGF-1, which is associated with cancer progression, increase in the number of mitosis and apoptosis inhibition. This metabolic disfunction is also linked to PI3K, AKT, RAS, RAF and MAPK signaling pathways, which are implicated in neoplastic cells proliferation [4, 9, 10].

Additionally, production of TNF-α, IL-1, IL-6, catabolic hormones (glucocorticoids, glucagon and catecholamines) and proteolysis inductor factor (PIF), is related to skeletal muscle wasting [11]. Production of proinflammatory regulators and inadequate nutrient intake contribute to fatigue and lower levels of physical activity [13]. These alterations induce the presence of sarcopenia and, in some patients, sarcopenic obesity. Sarcopenic obesity is characterized by an increase in fat mass and a decrease in muscle mass, almost always without any weight change [14]. The presence of sarcopenia or sarcopenic obesity in cancer patients is directly related with a higher toxicity and a worse prognosis [14, 15].

11.3 Malnutrition or Cachexia

Another health-threatening manifestation is malnutrition or cachexia. Cancer cachexia is defined as a multifactorial syndrome, characterized by the presence of anorexia and a decrease in body weight, mainly due to skeletal muscle wasting and fat loss. Cachexia affects around 50–80% of cancer patients and is indirectly responsible for at least 20% of all cancer patient deaths [16, 17]. Incidence of anorexia-cachexia syndrome is very high, although it varies depending on the type of tumor. It is present in 80% of gastric and pancreatic cancer, 50% in lung, prostate and colon cancer, and approximately 40% in breast cancer or leukemias. Anorexia-cachexia syndrome presents clinical symptoms such as asthenia, anemia and fatigue, altogether resulting in a lower quality of life [16].

Cancer-related anorexia-cachexia syndrome is characterized by an increase in growth hormone, insulin-like growth hormone-1 (IGH-1), resistin, leptin, adiponectin, ghrelin and insulin consid-

ered as responsible for energy balance [18]. Despite high levels of ghrelin, there is no increased appetite in cachexia patients [19]. Leptin, besides having neuroendocrine and immune action, performs a central role in controlling body weight and energy homeostasis. In gastric and pancreatic cancer patients, reduced plasma levels of leptin have been reported due to a decrease in fat mass; the opposite occurs in breast and colon cancer, where leptin plasma levels are elevated. According to Garofalo and Surmacz, it's possible that local leptin concentration is related to tumor progression and presence of tumor metastasis [19]. Therefore, accumulation of HIF-1α, produced by hypoxic tumoral environment, promotes leptin production, insulin increase, IGF-1 and VEGF, which are implicated the regulation of glycolysis, angiogenesis, proliferation, apoptosis and metastasis. Similarly, a hypoxic state in tumor cells attracts M2 macrophages, which favor the production of proinflammatory cytokines (IL-6, leptin, TNF-α) and angiopoietin, which are involved in developing cachexia and favor tumor metastasis [19, 20].

As mentioned, diet and nutritional alterations are considered to have a direct relationship with cancer etiology through immune and inflammatory responses. Risk factors related to cancer development are divided in modifiable and non-modifiable (Fig. 11.1) [21].

Cancer patients present multiple difficulties in managing their diet, and nutritional problems are a common complication. Some of these issues are induced by neoplasia, surgery, chemotherapy and radiotherapy side-effects which, in many occasions, make nourishing the patient difficult or impossible.

Due to these alterations, a systemic evaluation of cancer patients' nutritional state is recommended, including a periodic weight control and evaluation of biochemical parameters that facilitate detection of any deficiency or metabolic alteration in a timely manner.

During chronic inflammation, pro-inflammatory compounds, like cytokines, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and NF-kB are up-regulated. These molecules provide the microenvironment that promotes DNA instability and growth of malignant cells.

Recently it was shown that there is a mechanism linking carbohydrate-derived metabolites with carcinogenesis and cancer progression which may share light on the biological consequence of consuming a pro-inflammatory diet which can affect tumor biology. Advanced glycation end-products (AGE) are reactive metabolites produced as a by-product of sugar metabolism. Failure to remove these highly reactive metabolites can lead to protein damage, aberrant cell signaling, increased stress responses, and decreased genetic fidelity [22]. AGE accumulation was demonstrated in larynx, breast and colon tumors by immune-histochemical staining. On the other hand exogenous treatment of breast and prostate cell lines promotes migration, invasion and cell growth. Recent studies found that the dietary derived AGE carboxymethyl-lysine was associated with an increased risk of pancreatic cancer suggesting the positive association between red meat and pancreatic cancer [23]. The common american diet comprised of red meat, refined grains and high fat/high sugar foods are associated with systemic disease and are particularly AGE-laden, contributing as much as 30% of the AGEs accumulated within our bodies (Fig. 11.2) [24].

11.4 Anti-Inflammatory Food

Vitamins C and E, selenium, and carotenoids provide antioxidants that act in different body compartments to reduce development of reactive species that can initiate cancer development through direct DNA damage or inflammation (Fig. 11.2).

Foods high in omega-3 fatty acids, principally EPA and DHA, provide the starting point for production of anti-inflammatory eicosanoid compounds, the n-3 Polyunsaturated fatty acids (n-3PUFAs), which are considered to have anti-inflammatory effects if the intake of omega 6 PUFAs is higher than that of omega 3

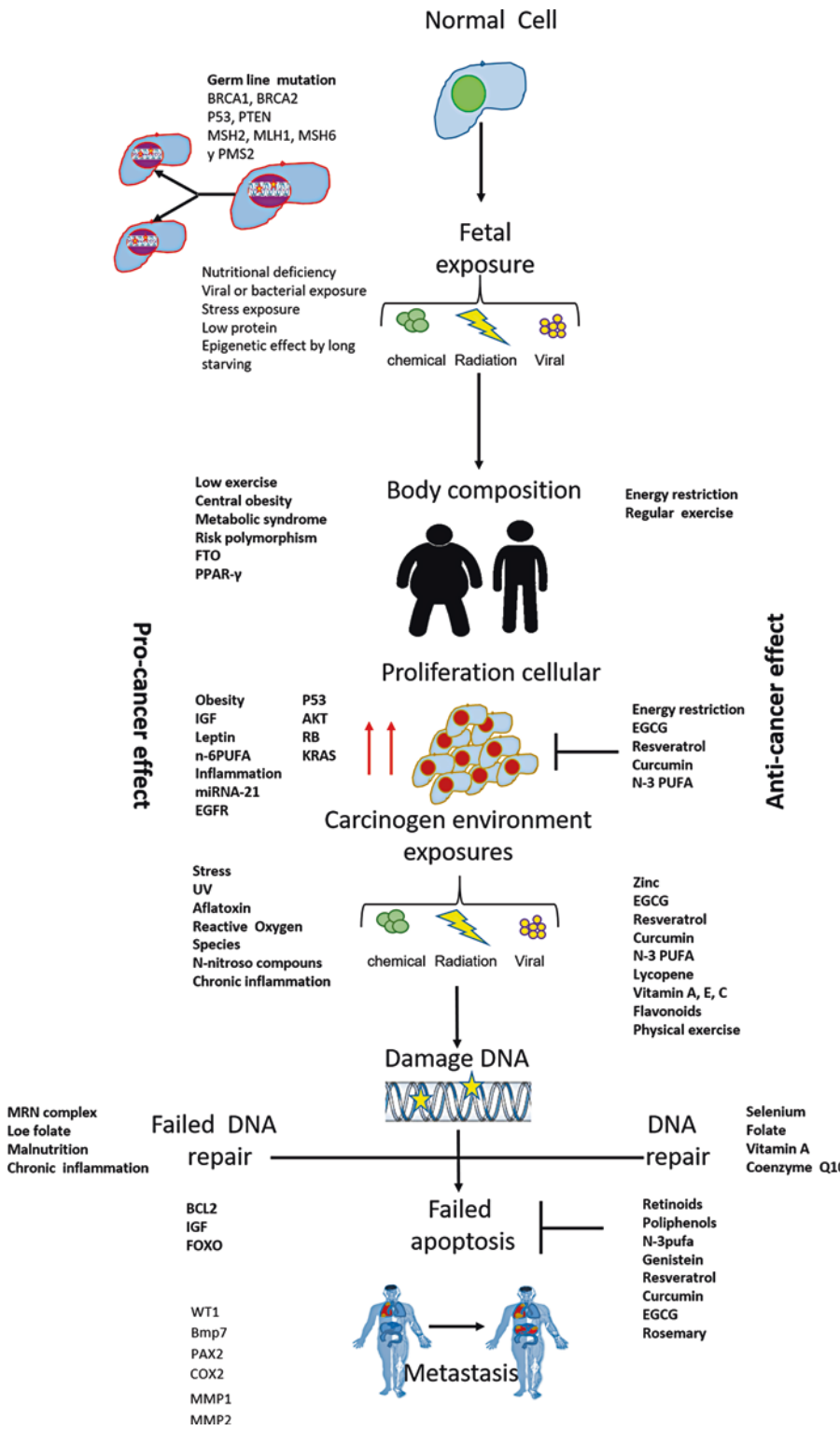

Fig. 11.1 Carcinogenesis processes. The protective effect of nutrient, exercise and phytochemicals are enlisted at the right side, the left side show genes, molecules and processes related with an increased risk of cancer

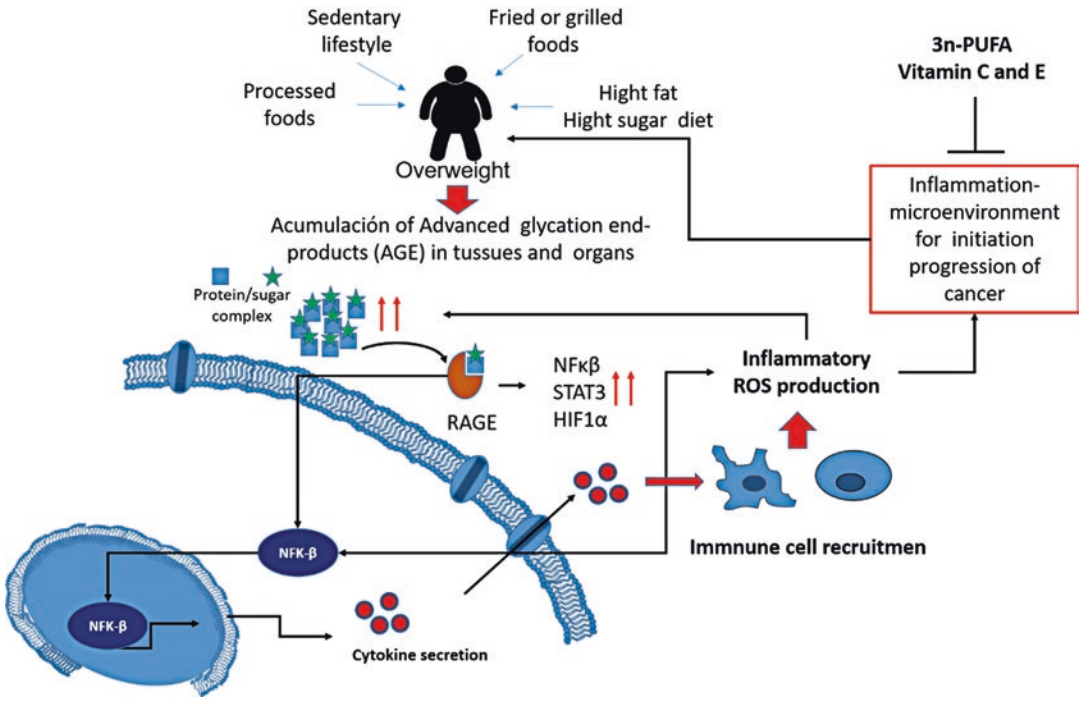

Fig. 11.2 Pro-inflammatory Effect between food and cancer. AGEs promote immune mediated chronic inflammation through binding to the receptor for AGE (RAGE) which is over-expressed in cancer. It leads to an increase in the activation of pro-inflammatory transcriptional regulator such NFkB,STAT3 and HIF1. Over-expression of these critical transcription factors increases the secretion of cytokines/chemokines like L1β, IL6 and TNFα. These increased recruitment of lymphoid and myeloid immune cells into the tumor microenvironment lead to an elevated ROS production and an inflammatory response

PUFAs, due to the former being ant-inflammatory and the latter, pro-inflammatory [25].

Clinical and experimental evidence in several studies show that supplementing patients' diets with omega 3 PUFAs improves response to cancer therapies, diminishes treatment side-effects and reduces cell proliferation. In breast tumors, omega 3 PUFAs induce apoptosis and lower risks of relapsing; these effects are associated with regulation of inflammation [26].

Omega 3 fatty acids' anti-inflammatory effects are thought to work through multiple mechanisms, such as disruption of oncogenic proteins' signaling pathways, plus inhibiting omega 6-derived proinflammatory eicosanoid production, which is associated with tumor growth [26, 27].

Results in experimental studies performed by Bougnox refer that omega 3 PUFAs supplementation improves sensibility to chemotherapy or radiotherapy in neoplastic cells [28]. It is thought that omega 3 fatty acids' biological effects work by regulating inflammatory mediators' production, positively inducing expression in genes implied in cell control and DNA repair [26, 27]. It has also been suggested that incorporating omega 3 fatty acids to cell membranes contributes to lipid peroxidation inducing cytostatic or cytotoxic effects inhibiting tumor cell growth [27].

On the other hand, lowering E2 prostaglandin production diminishes aromatase activity, which directly influences estrogen synthesis and signaling. PUFAs also lower A2 thromboxane and B4 leukotriene production, as well as monocytes and macrophage intercellular adhesion molecules 1 (ICAM-1) expression, which in turn diminishes IL-1B, IL-6, TNF-α and platelet-derived growth factor (PDGF) produced by MI macrophages, all related to tumoral carcinogenesis [29].

Likewise curcumin has an anti-inflammatory effect, this natural compound works in reducing inflammation *in vitro* and *in vivo,* it inhibits expression of NFkB and AP-1 transcription factors and decrease the production of tumor necrosis factor-α and pro-inflammatory cytokines, interleukin-1β, interleukin-8 and INOS.

11.5 Phytochemicals in Cancer Treatment

The number of patients with a poor prognosis, with tumor resistance and antitumor treated associated toxicity is increasing. Therefore it is necessary to find new strategies to minimize the toxicity of the chemotherapeutic agents and increase their antitumor effects. It is known that there are a large number of natural compounds obtained from plants that have anticancer activity and inhibiting processes such as angiogenesis, proliferation, and metastasis and promoting apoptosis *in vitro* and *in vivo* in animal models; however, the number of these natural or phytochemical compounds tested in cancer patients is small.

11.6 Resveratrol

Resveratrol (trans-3,4,5trihydroxystilbene) is a natural polyphenol extracted principally from red fruits and skin of grapes, peanuts and other food products. Over the past few years, it was revealed to have potent antioxidant and anti-inflammatory effects, which help inhibit tumor angiogenesis, reduce antiestrogen activity, lipid synthesis in liver, eicosanoid synthesis and platelets aggregation. Resveratrol can also activate several systems, including expression of p53, protein kinase C (PKC), DNA polymerase and cyclooxygenase (COX), cell cycle progression, and cell apoptosis [28, 30]. The resveratrol anti-proliferative activity has been related with the inhibition of several transcription factors and up-regulation of P53, BAX and caspases that lead to apoptosis and down-regulation of BCL2and cyclins [31]. On the other hand, many studies have related an aberrant DNA methylation with epigenetics alterations in cancer. Epigenetic regulation is positioned as a new strategy for the treatment of cancer, this strategy represents a new field of research focused on the silencing of oncogenes and the activation of tumor suppressor genes through the modulation of the epigenetic state in tumor cells. It has been observed that resveratrol modulates DNA methylation through of decrease DNMT activity and down-regulation of DNMT1, DNMT3A and DNMT3B using 15 μM of resveratrol in HCC1806 breast cancer cell. Interestingly, modification in DNMT activity were not detected in non-tumorigenic MCF10A cells even after 72 h of the treatment [32], moreover resveratrol treatment was able to reduce DNMT enzymatic activity and modify DNA methylation patterns by decreasing 5-methylcytosine levels, which in turn, led to a significant reduction in DNA methylation in MDA-MB-157 breast cancer cell, an estrogen receptor- α negative (ERα-Negative) cell which is clinically more aggressive and does not respond to hormone-direct therapies. Other studies demonstrate that this polyphenol can inhibit oncogenes or cancer related pathways such as NF-κB, BRCA1 [33] PI3K/Akt signaling, mTOR signaling, MAPK signaling, cyclooxygenases, phosphodiesterases, estrogen receptors, microRNAs and several protein kinases. In colorectal cancer, resveratrol can lead to the overexpression of Bax, P53 and glutathione reductase while down regulating TNF-α and PKC-β2.

Finally some semi-synthetic resveratrol analog molecules have been found to have pharmacological effec including a chemopreventive function, anti-oxidant effects, and anti-aging properties, as well as a reverse in drug resistance in different cancer cells by chemo sensitizing. For example trans-resveratrol and glucoside have a cardioprotective effect, an anti-inflammatory and an estrogenic/anti-estrogenic effect.

The resveratrol effect has been used in some clinical trials. For example, 80 g per day of frozen -dried grape powder was used for pancreatic and colon cancer for 2 weeks and resulted in a decreased Wnt target gene expression at the regu-

lar mucosa, but not in cancerous mucosa. Likewise 0.5–1 g of resveratrol per day has an effect on proliferation in colorectal cancer. On the other hand SRT501 (micronized resveratrol formulation from GSK company) increased cleaved caspase-3 in the hepatic tissue, suggesting an increase in the apoptosis of the tumoral tissue [34].

11.7 Lycopene

Lycopene is a natural carotenoid pigment compound that gives fruits and vegetables its red color. Approximately 85% of dietary lycopene comes from tomato products such as ketchup, tomato juice, sauce, or paste. It is a highly unsaturated acyclic isomer of β-carotene; its hydrocarbon chain contains 11 conjugated and 2 nonconjugate bonds, various carotenoids lycopene have been shown to be protective, particularly for prostate cancer [35]. Lycopene is associated with a 35% lower risk of prostate cancer. In a study, 32 patients were administered 30 mg of lycopene per day before undergoing a radical prostatectomy. After 3 weeks, serum and prostate lycopene levels increased two-fold, while PSA levels decreased by 17%. In vivo studies have revealed that lycopene treatment also inhibits tumor growth in the liver, lung, breast, and colon.

One of mechanism of lycopene activity is through its antioxidant activity. This could be because lycopene contain many double-conjugated bonds. Lycopene has been shown to be two times more effective than β-carotene and 10 times more efficient than α-tocopherol in its ability to trap O_2+ [36]. The administration of lycopene (2.5 mg/kg) can suppress gastric cancer *in vivo* by reducing lipid peroxidation, increasing the levels of the antioxidants vitamin C, vitamin E, and reduced glutathione (GSH), and increasing the activity of circulating GSH-dependent enzymes, like glutathione reductase, and glutathione-S transferase (GST) [37].

In breast and colorectal cancer, lycopene reduces IGF-1 levels, increases IGFBPs and inhibits cell proliferation mediated by PI3K/AKT/PKB and Ras/Raf/MAP kinase signaling pathways [38]. Lycopene has a synergistic effect with anticancer compounds. The combination of low concentrations of lycopene with 1,2,5-dihydroxyvitamin D3 in HL-60 cells produced a higher inhibitory effect on cell proliferation and cell differentiation compared to the inhibitory effect caused by lycopene r 1,2,5-dihydroxyvitamin D3 alone. Some studies suggest that lycopene enhanced the effect of docetaxel on cellular growth in prostate cancer, as well as enhancing the antitumor activity of platinum-salts decreasing the severity of radiation-induced acute toxicity of the gastrointestinal tract, weight loss, and diarrhea in rats [39].

11.8 Curcumin

Like resveratrol, curcumin is a polyphenol (diferuloylmethane) derived from the rhizome of a turmeric (curcuma longa Linn), a principal component of the Indian spice. Curcuma has anti-cancer effects in colon cancer, breast cancer lung metastases, and brain tumor. One of the mechanism includes its capacity to induce apoptosis in cancer cells without affect normal cells. Curcumin can inhibits inflammation by interfering with NF-κB, it is able to dissociate raptor from mTOR, and silence the mTOR complex. Curcumin also modulate growth and cellular proliferation by interacting with Cyclin D1, and c-myc. Curcumin can inhibit cell invasion by down-regulation of COX-2 and MMP2 expression and suppression of EGFR [40].

Curcumin has been reported to be nontoxic in doses of 12 g per day. In vitro curcumin, increased the expression of AP-1 in a dose-dependent manner in HT-29 colon cancer cell line using 1–25 μM. It inhibited NF-κB and the phosphorylation of AKT and mTOR in PC-3 prostate cancer cells [41].

Finally during past years a large number of nano-formulations have been developed with the objective of enhancing curcumin use in vitro, in vivo, and in pre-clinical settings that involve the use of adjuvants, stabilizers, conjugates,

polymer conjugates nano-gels and nanoparticles. Nanoparticles in general can increase the circulation time of the loaded therapeutic molecule and improve its residence at the pathological site by enhance permeation and retention. Maitras group has designed a curcumin nano-formulation (NanoCurc™) [42]. This formulation consist in micellar aggregates of 50 nm amphiphilic polymers which release 40% of curcumin at physiological pH, this formulation showed tumor growth inhibition through a reduction in the activation of NF-κB, inhibition in the expression of MMP-9 and cyclin D1. The combination of NanoCurc™ and gemcitabine indicated an additive therapeutic effect in pancreatic cancer [43].

11.9 Epigallocatechin-3-Gallate (EGCG)

Green tea is the second most commonly consumed beverage in the world. The infusion of leaves from the *Camellia sinensis* plant contains proteins, amino acids, carbohydrates, minerals, lipids, vitamins and volatile compounds but is rich in polyphenolic compounds known as catechins, with the most abundant being (−)-epigallocatechin-3-gallate (EGCG). It has recently been studied as a health-promoting beverage that may prevent several human diseases through its biological proprieties such anti-inflammatory anti-arthritic, antimicrobial, anti-oxidative, neuroprotective, antidiabetic, anti-angiogenesis and anticancer effects [44]. A number of epidemiological, preclinical and in vitro studies have demonstrated that green tea exhibits potential preventive and chemotherapeutic effects against a wide range of human malignancies. These studies indicate that EGCG may alter the hallmarks of cancer by suppressing apoptosis, cell proliferation, angiogenesis and invasion [45, 46]. All these EGCG-induced cellular effects are mainly due to the modulation of biological pathways including growth factor-mediated pathway, the mitogen-activated protein (MAP) kinase-dependent pathway, and ubiquitin/proteasome degradation pathway. EGCG has gained increased attention in lung cancer research. In consequence, potential therapeutic applications of EGCG intervention have been recently reported for this neoplasia. For instance, EGCG enhances the anti-proliferative activity of c-MET and EGFR inhibitors in non-small cells lung cancer (NSCLC) [47]. In addition, EGCG induces the reversion of cisplatin resistance mediated by down-regulation of AXK and TYRO 3 receptor tyrosine kinases in chemo-resistant lung cancer cells. On the other hand, a schedule-dependent effect of EGCG and paclitaxel on growth inhibition of NCI-H460 lung cancer cells was also reported [48]. Finally, it has been reported that EGCG can inhibit HDGF in lung cancer cells and promotes by increasing cisplatin induced apoptosis trough the disruption of the mitochondrial membrane potential, and activation of caspase 3 and caspase 9 to sensitize A549 cells to cisplatin treatment.

11.10 Conclusions

Diet plays a fundamental role in the treatment of the oncological patient, this is because some foods can function as chemo-protector or can increase the response to a treatment and make synergy to suppress some mechanism linked to cancer as cell proliferation; but it can also generate toxicity or interact to a certain type of therapy for example oral chemotherapy.

Food can interact with oral chemotherapy through reduction of the bioavailability and/or by induction or inhibition of the metabolism of the administered drug often due to the metabolism by the cytochrome P450 system affected by grapefruit interaction; for example a study showed that concurrent intake of 240 mL of grapefruit juice increased by 60% nilotinib systemic exposure through Inhibition of CYP 3A4 and P-glycoprotein thus administration of nilotinib with grapefruit juice is not recommended [49] on the other hand in vitro results shown that calcium supplementation combined with inhibitor of EGFR results in an additive effect on tumor growth inhibition in CCR. However, the combined use of dietary calcium supplementation and EGFR inhibitors also resulted in elevated

toxicity suggesting that careful consideration be given when combining dietary supplements with prescribed cancer therapies [50] similarly has been reported that alpha linolenic Acid (ALA) treatment dramatically suppressed the at the transcriptional level, the expression of HER2 in breast cancer cellular lines and have a synergic effect with trastuzumab increasing the effect reducing cell viability in *vitro* [51] other study shown that green tea enhanced the inhibitory effect of tamoxifen on the proliferation of the ER positive breast cancer cells in *vitro, In vivo* on animal models green tea and tamoxifen reduce tumor size, and the increase apoptosis in tumor tissue, as compared with either agent administered alone [52] by the counterpart grapefruit juice may potentially reduce the effectiveness of tamoxifen through Inhibition of CYP 3A only one 8-oz. glass of grapefruit juice will inhibit CYP3A for 24–48 h [53]. Finally one report show that an alkaline diet may increase the effect of EGFR TKI treatment in NSCLC patients with EGFR mutations through of change in the tumor microenviroment [54].

So, it is vital to know these interactions and design personalized and comprehensive treatments that take into account not only the histopathological and molecular characteristics of the tumor but also the environment and the nutritional requirements of the patient.

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