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Introduction

Recent advancements in medical research brought to a better understanding of the molecular bases of diseases and the interindividual variability in drug response, opening a new era in the management of patient care, known as the *precision medicine*. In this view, new approaches to patient diagnosis, monitoring or treatment can benefit from the integration of information deriving from different technologic approaches such as high-throughput *omics* (next-generation sequencing, metabolomics, proteomics, epigenomics, bioinformatics, system biology, and medicine biobanks) in order to allow the implementation of a truly tailored therapy [1]. In fact, for a specific disease, a multidisciplinary approach will allow a more accurate prediction of treatment and strategy, differently from the traditional “one-size-fits-all” approaches [2]. Systems pharmacology and pharmacogenomics (PGx) helped the understanding of the clinical impact of genetic-determined interindividual differences in pharmacokinetics (PK) of many drugs especially

for antineoplastic agents, in which the patient risk is due to the narrow therapeutic index. On the other hand, in the era of precision medicine, the understanding of the tumor molecular profile has the potential to drive clinical decisions for tailored treatment options with improved efficacy. Consequently, the interindividual variability in drug response, in terms of efficacy and toxicity, due to the interaction of genetic, pathophysiological and environmental factors, has a relevant effect on cancer treatment. Cancer is not a single disease but is a series of genome-based diseases and its treatment activity is conditioned by disease diffusion and individual patient-related factors. In fact, genomic deregulation at different levels is involved in tumorigenesis and includes different events such as gene inactivation (promoter silencing, deletion, mutations), alterations in gene expression (copy number variation, methylation), and mutations or rearrangements responsible of protein activation [3]. The transition from conventional cytotoxic drugs to molecular biomarkers-driven decision for the selection of cancer therapeutic options improved the management of many advanced-stage tumors. In fact, the identification of somatic and germline genetic biomarkers provides information about the likelihood of response to treatment and offers therefore predictive and prognostic information for the selection of patients. The frequent exposure to endogenous and exogenous reactive chemicals can alter the DNA sequence as well as

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chromatin structure and bring to somatic genomic and epigenomic abnormalities. In most cases, no cellular abnormalities occurs, while in some cases in a prone tissue, the clonal transformation of a cell takes place and consequently begins the development process, which will finally drive to a malignant lesion. In many cancers, including chronic myeloid leukemia, colon, breast, lung and melanoma, predictive biomarkers are currently in use to select patients, which might benefit of targeted therapy and avoid toxic side effects of chemotherapy. Biomarkers, providing information on cancer molecular signatures, may allow treatment tailoring and are distinguished into: diagnostic, prognostic, treatment and prevention subgroups. Key mutations and molecular pathways involved in tumor development and proliferation can be identified by predictive biomarkers, which are measurable and linked to relevant clinical outcomes. They have undergone a validation process for use as predictive tool within clinical trials. Instead, prognostic biomarkers identify somatic and germline mutations, alterations in DNA methylation, microRNA (miRNA) and circulating tumor cells (CTC) in blood and provide information on tumor outcome independent from treatment. Today, diagnostic companion assays undergo validation for biomarker value for treatment decision-making. High-throughput technologies provided the opportunity to identify genomic changes conditioning development and progression of a tumor (“driver” lesions) with a selective growth advantage and *addiction* of the cancer cell to a particular molecular pathway, despite other quantitatively preponderant and concomitant armless *passenger* alterations [4]. Consequently, genes identified to have a driver role in at least one cancer type are considered oncogenes [5]. A subset of the driver aberrations could have significantly diagnostic, prognostic or therapeutic potential and are often indicated as *actionable*; a subset of mutations may also be *druggable* as target for drug development [6]. Today, tumors molecular characterization and predictive/prognostic biomarker discoveries have allowed better understanding of the complex mechanisms of carcinogenesis and have fueled the development

of novel drug targets and new treatment strategies to enhance patient care. The hallmarks of precision medicine rely on genomics and clinical data integration based on cancer molecular characteristics in order to personalize oncology and to design new clinical trials. In order to study targeted therapies in different tumor types expressing low-frequency mutations (<5%) it is possible to design *basket trials* where are enrolled a small number of patients with different kind of cancer expressing the same genetic alteration, while in an alternative approach, *umbrella trials* recruit patients with a single cancer type but different actionable mutations. Drug structure analysis allows the design of new studies to test new drugs and biomarkers. In *basket trials*, a hypothesis-driven strategy is implemented and can be the proof-of-principle validation of a putative target and offer the opportunity to integrate a classical clinical trial design with the knowledge of molecular expression at tumor level. The limit of this trial design is that a mutation can act differently as driver druggable target in a given tumor, while it can be a passenger lesion in other tumor contexts. Another aspect emerging and in contrast with the performance of basket and umbrella trials is the role of tumor stroma in conditioning therapeutic choices and future drug development [7, 8].

In our chapter, as a prototypical condition, we will discuss the current scenario of personalized treatment of colon–rectal cancer, including molecular cancer-related and patient-related biomarkers, the emerging molecular landscapes and finally we will discuss the new approach of integrative genomics, as emerging vision based on large biological annotated datasets and bioinformatics tools.

Current Status: The Case of Colorectal Cancer (CRC)

Metastatic colorectal cancer (mCRC) is characterized by several molecular lesions involving activation or loss-of-function mutations, which occur in receptor tyrosine kinases (RTKs) and more frequently in downstream components of

RTK-activated intracellular pathways. Therefore, treatment effects of the target therapy can be considered as strictly related to specific molecular alterations.

The epidermal growth factor receptor (EGFR), expressed on the cell surface, belongs to the ERBB-family, a subfamily of RTKs. The anti-EGFR cetuximab and panitumumab mAbs prevent activation of EGFR [9, 10]. They block ligand-stimulated EGFR signaling and they probably stop activation of phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/MAP2K (also called MEK)/MAPK1/3 (also called ERK2/1) signaling pathways, leading to inhibition of cellular proliferation and induction of apoptosis [11].

One of the most important molecular mechanisms of primary resistance to EGFR mAbs (cetuximab and panitumumab) is KRAS mutation. In fact, the mutation in KRAS appears to hold a negative predictive value for the response of anti-EGFR therapy [12, 13]. At the beginning, only the mutation in exon 2 of KRAS was considered [14, 15] and then the research for mutations was expanded to the exons 3, 4 of KRAS and 2,3,4 of NRAS, also involved in the resistance to anti-EGFR drugs [16].

In patients with mCRC the efficacy of chemotherapy can be, in fact, implemented by biological drugs based on the molecular status of RAS, in particular cetuximab and panitumumab for wild-type RAS status and bevacizumab for both RAS wild type and mutated [17–22].

The correlation between the molecular status of KRAS and the survival endpoints in first-line mCRC treated with cetuximab and standard chemotherapy regimens was initially demonstrated by a retrospective analysis of the Crystal study [23].

In patients with PAN-RAS mutations the best standard first-line treatment is represented by the association of chemotherapy with bevacizumab [17–20], whereas in mutated patients has not been established the best sequence for the use of anti-EGFR drugs in first line rather than in the second one [24–26].

During the carcinogenesis trajectory, genetic aberrations accumulate and this process leads to the so-called genetic heterogeneity resulting in the selection of clones with different functions

including the ability to respond to a specific treatment and to generate metastases [27]. For this reason, patients with RAS wild-type mCRC could present mutated subclones that induce resistance to treatment with anti-EGFR under the selective therapy pressure [28, 29].

It is known that in patients RAS wild-type molecular alterations of BRAF [30, 31] and PIK3CA [32, 33] genes might be present, which may cause primary resistance to anti-EGFR.

BRAF is a human gene that encodes a protein called BRAF and it is a member of the RAF gene family. BRAF protein is a serine–threonine protein kinase involved in RAS-activated pathway. BRAF mutation is found in 15% of colorectal cancers, and it is known that this alteration is linked to a poor prognosis [31, 34].

The most frequent BRAF mutation is V600E, located in the kinase activation domain and it leads to an increased activity of MAPK1/3 pathway. BRAF-mutant tumors have dissimilar clinical and histological characteristics from RAS-mutant tumors [35]. It was found that the CpG island methylator phenotype (CIMP) and microsatellite instability are observed in BRAF-mutated tumors [31, 35].

In a retrospective consortium analysis it was revealed that only two patients out of a total of 24 patients with BRAF-mutated cancer responded to the treatment with cetuximab [32].

Only a small sample of patients with BRAF-mutated cancer benefit from treatment with panitumumab or cetuximab [35].

PIK3CA is part of lipid kinase family involved in various cellular processes regarding growth, proliferation, differentiation, motility, survival and intracellular trafficking [36].

PIK3CA mutations can occur more frequently (80%) in exon 9 (60–65%) and 20 (20–25%) [32]. In a study it was shown that only mutation in exon 20 of PIK3CA is associated to a resistance to cetuximab activity in population KRAS wild-type [32]. Moreover, PIK3CA has a negative prognostic value because it is associated with a shorter survival in tumors RAS wild-type stage I–III [37].

Another important molecular lesion involves PTEN gene that encodes the phosphatase and

tensin homolog protein. PTEN mutations are present in nearly 5% with high microsatellite instability. PTEN role in colorectal cancer is not clear, but it was shown that PTEN loss is associated with a reduced response to cetuximab [30, 38–40].

Other important factors are prognostic for survival in colorectal cancer in addition to defined molecular defects [41, 42].

The importance of the clinical and biological difference between proximal and distal cancer is becoming now clear. Right- and left-sided CRCs are characterized by different carcinogenesis trajectories, mucosal immunologic microenvironment and gut microbiota [43]. Right-sided cancer is most frequently diploid and has a mucinous histology, high microsatellite instability, CpG island methylation and BRAF mutations [44, 45], while the left-sided one is characterized by chromosomal instability. These peculiarities reflect a different embryonic origin [46, 47].

The analysis of the correlation among tumor sidedness and survival after chemotherapy+/- bevacizumab was performed in three independent cohorts in a study. According to this, patients with right colon cancer have a lower recurrence, but they show a more aggressive behavior in relapsed disease [48]. In this group of patients, the role of BRAF is clear as a negative prognostic factor [49] in a more advanced phase of the carcinogenesis process and, with other factors, might play a role in chemoresistance, while the left colon cancers have an increased benefit from treatment on activity and efficacy endpoints [48].

About the benefit of the biological treatment according to the tumor site, it was found an increased activity of anti-EGFR drugs in the left-sided primary tumor location, demonstrated in terms of PFS [50].

It is important to consider that the tumor microenvironment is different between the left and right colon. Indeed the right colon cancers have a higher share of eosinophils and intraepithelial T cells [51, 52].

It has been speculated that this could be the result of a homeostatic balance in T cells between tolerance for the commensal microbiota and the immune response against pathogens [53].

Currently major attention is focused on the mismatch repair (MMR) gene deficiency, which can be sporadic or occurs within the Lynch syndrome. It is found in 1 out of 35 patients with colon-rectal cancer [54] and it leads to microsatellite instability (MSI) represented by alterations in the length of tandem nucleotide repeats [55, 56].

MSI overall predicts for a better prognosis. The correlation between the microenvironment rich in lymphocyte cells, the immune-score and the favorable outcome in tumors with MSI needs additional investigation [57].

The immune-score is characterized by the determination of the number of cytotoxic and memory T cells represented in intra-tumor and peri-tumor infiltration and it is considered a biomarker with prognostic relevance [58, 59].

The presence of high levels of CD8 + lymphocytes in the microenvironment that express the chemokine-receptor-7 (CCR7) is found to influence the prognosis increasing the overall survival and progression-free survival after a first-line chemotherapy [60].

Moreover, high levels of FOXP3+ T lymphocyte correlate with the outcome of patients who undergo chemotherapy or chemo-immunotherapy [61].

All together, these findings open a new biological scenario where the immune system plays a substantial role. In fact, there is now a renewed interest for the immunotherapy which has opened the way for immune checkpoint inhibitors development that modulate immune response against tumor cells. While in some tumors, such as malignant melanoma, immunotherapy has produced highly successful results, in others unfortunately did not reach the same activity, such as in mCRC. In fact, only a small subgroup of mCRC patients with deficiency of the MMR mechanism benefit from treatment with programmed death-1 (PD-1) checkpoint inhibitors (5–10% of all mCRC patients) [62].

A phase 2 trial showed the efficacy of treatment with pembrolizumab in tumors with MMR deficiency [63]. Tumors with defective MMR are more responsive to the PD1 block confirming the successful advantage of high density of immune

system cells in the microenvironment and the mismatch repair deficiency [64–66].

Another potential predictive biomarker is represented by mutation in exonuclease domain of DNA polymerase epsilon (Pol-ε). This mutation correlates with a higher immune infiltrate (like MMR deficiency) and a better disease-free survival in MSI-proficient tumors. Both MMR deficiency and Pol-ε mutation lead to increased tumor mutation burden and to the onset of tumor specific neo-antigens, which could activate the immune system in a tumor specific response [67].

Recently, it has been focused on HER2 gene alterations (HER2 over-expression or amplification) that make the cancer sensitive to a specific combination of direct molecular targeted drugs against this target [68].

To conclude, the selection of the most appropriate treatment should be based on the patient, on the biological characteristics of the tumor, on the objectives to be achieved, on the toxicity of the treatment, and finally on the continuum of care, which indeed needs to be also considered.

At present only negative predictors of response to various treatments are available and validated for the clinical scenario. The biomarker that has demonstrated a deep impact in the history of colorectal cancer is the RAS mutational status, which is indeed a negative predictor.

To guide the oncologist in the decision-making process of treatment of colorectal cancer, positive predictive biomarkers are eagerly awaited for treatment individualization and need validation in prospective trials (Fig. 2.1).

Future Perspective: Molecular Landscape of Colorectal Cancer

Genomic Classification of Colorectal Cancer

Surgery is the mainstay treatment for CRC patients although, at the time of diagnosis, CRC is often a systemic disease and therefore adjuvant chemotherapy is the best choice for preventing disease relapse. The standard classification of CRC considers pathological staging a clinical

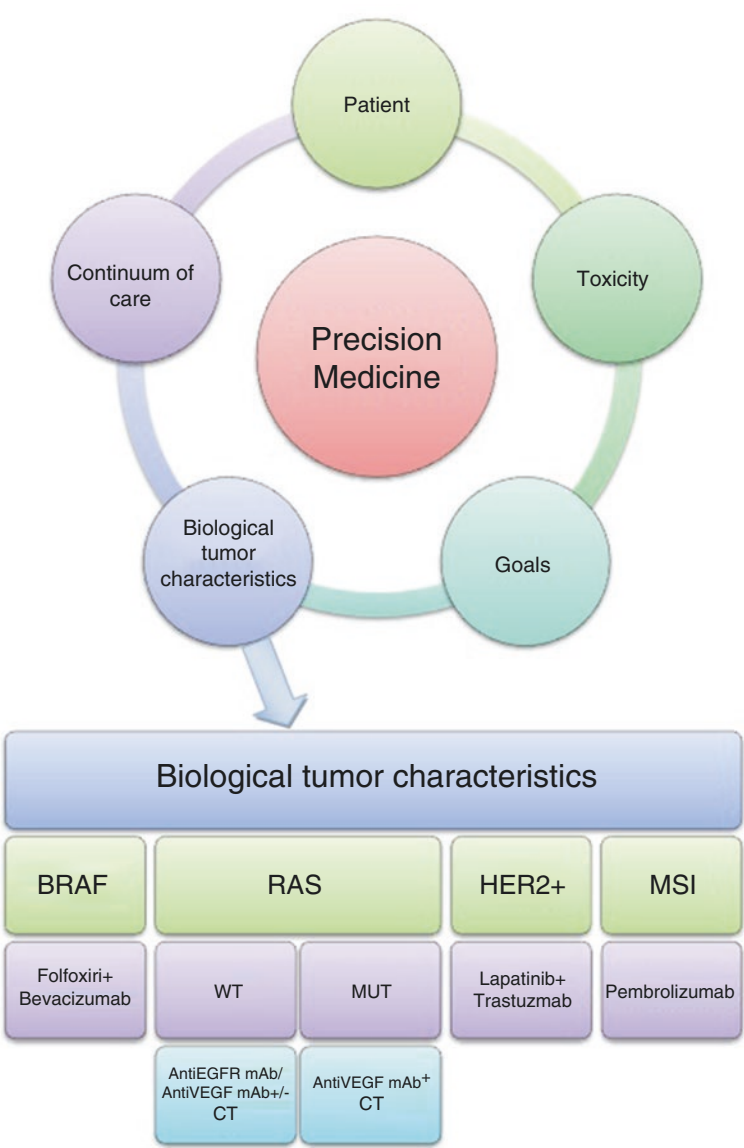
prognostic factors to select patients for adjuvant chemotherapy.

For this lethal disease, with an estimated heritability of approximately 5%, exists a classification based on molecular profiling and linkage studies. In fact, germline mutations on APC gene and DNA MMR genes characterized the hereditary colorectal cancer syndromes, while other low penetrance genetic variants have been correlated to approximately 20% of the familial association in CRC [69]. Inherited CRC syndromes are classified based on the presence of large numbers of adenomatous polyps like familial adenomatous polyposis (FAP), attenuated FAP and MUT-Y-homolog-associated polyposis (MAP) and the presence of hamartoma polyps like primary lesions in Peutz–Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS) as well as non-adenoma syndromes Lynch 1 and 2. Hyperplastic polyposis (HPP) is a condition that produces substantially increased cancer risk. Somatic mutations and polymorphic features in TP53 gene impact susceptibility to sporadic CRC, prognosis and response to therapy [70].

According to gene expression profile, supervised approaches contributed to identify signatures related to relevant outcomes such as recurrence, metastasis and overall survival, while semi-supervised approaches refined outcome prediction according to patients selection based on stage disease [71, 72].

Recently, an unsupervised analysis considers inherent molecular subtypes for CRC classification and correlates them to prognosis [73, 74], while recent studies proposed a consensus classification system identifying three groups: the Goblet/Inflammatory group, the TA/Enterocyte group, and the stem/serrated/mesenchymal (SSM) group [75, 76]. However, it has been proposed also a sub-classification of CRC that distinguishes those with MSI (which arises on a hereditary and sporadic basis, located primarily in the right colon and associated with the CpG island methylator phenotype (CIMP) and hypermutation) and those that are microsatellite stable (MSS) but chromosomally unstable (CIN) [77]. Barat et al. utilized microarray-based gene expression and methylation dataset to identify

Fig. 2.1 This chart describes the possible molecular alterations that lead to the therapy’s customization based on the molecular profile of each patient. The center of our attention is precision medicine that has to guide the oncologist’s decision in order to provide the best choice based on the characteristics of patient, tumor, and treatment



methylation-based subgroups and distinguished three main clusters: highly methylated (HM), intermediately methylated (IM) and large clusters with both lower and rarer locus-specific methylation (LM) [78]. The study provides evidence that integration and combination of gene expression and methylation datasets analyses could better described the CRC subtypes. Gene expression profiles and genomic characterization influence CRC outcome (Fig. 2.2).

Critical genes and pathways, including the WNT, RAS–MAPK, PI3K, TGF- β , P53 and DNA MMR pathways, are involved in the initiation and progression of CRC [77, 79]. They are associated with different mutation frequencies of the main oncogenes RAS, BRAF, APC and other genetic events, whose expression redefines treatment selection. With the exception of hyper-mutated cancers, CRC have similar patterns of genomic alteration, and there is evidence of sig-

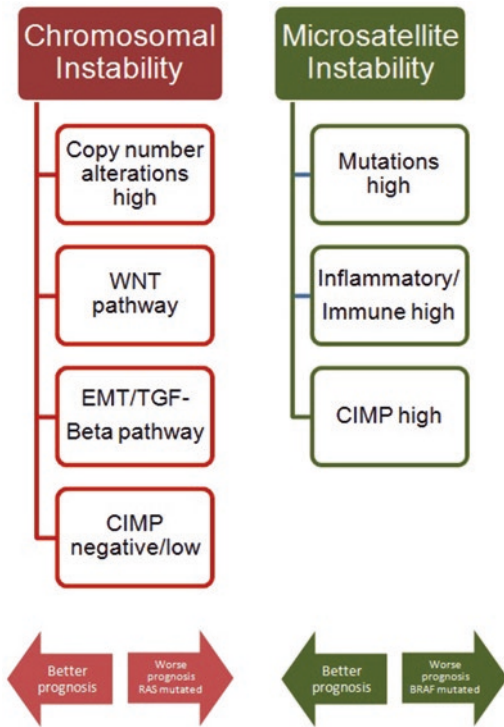


Fig. 2.2 Diagram of gene alteration pathways based on genomic characterization: the related outcome according to CRC subtypes

nificant intra-tumor genetic heterogeneity due to variations in localized somatic mutations and copy number abnormalities [80].

Through bioinformatics tools in 750 patients with stage I to IV CRC, undergone to surgical treatment, it has been possible to stratify CRC by transcriptomic-based classification on the bases of clinical-pathological features and common DNA markers [76]. In fact, six prognostic molecular subgroups of CRC sample have been identified and validated on the bases of gene expression data, associated with clinical and pathological characteristics, molecular alterations, specific gene expression signatures and deregulated signaling pathways. Today, although official guidelines indicate a risk stratification, no clear recommendations for adjuvant chemotherapy in stage II disease are available, and molecular technologies are strictly required to improve the selection of individualized therapeutics [81]. Promise derives now from validation clinical trials evaluat-

ing two prognostic tests, based on the expression of different gene panels like ColoPrint (Agendia, Amsterdam, the Netherlands), which are based on 18 genes, and Oncotype DX (Genomic Health, Redwood City, CA) which includes 12 genes (seven recurrence risk genes and five reference genes) and represents the individual prognostic score most widely retrospectively evaluated with a little overlapping [82, 83]. Until now ColoPrint and Oncotype DX were available to improve risk prediction in early-stage CRC [83, 84] and have been investigated in three independent datasets of stage II–IIIA CC and as a prognostic score in the QUASAR and CALGB9581 trials, respectively [76]. Presently current pathological staging is not able to predict recurrence in a phase of curable disease, so it is necessary to take benefit from additional tools. Nomograms such as “Adjuvant Online” or Memorial Sloan Kettering Cancer Center (MSKCC) and Bayesian Belief Network (BBN) can be used in clinical practice to show outcome of patients in the same disease condition and predict the probability of CRC patient’s to 5 years OS after surgical removal of all cancerous tissue [85]. Another prognostic nomograms was developed by Peng et al. for predicting outcome in patients with locally advanced rectal cancers without preoperative treatment, while no nomogram can predict long-term outcome after CRC surgery for all disease stages [86, 87].

It is clear that all the above-described tools represent sound decision supporting instruments but cannot be defined bona fide precision medicine approaches, taking into account the intrinsic heuristic nature. Despite this complex scenario, presently there isn’t an integrated view of the CRC genetic and genomic changes in initiation and subsequent different stages of disease progression. Further insight may help the understanding of CRC pathophysiology and the identification of potential therapeutic targets.

Recently, Dalerba et al. identified a subgroup of stage II CRC patients who might benefit from adjuvant chemotherapy for the lack of caudal-type homeobox transcription factor 2 (CDX2) expression in their cancer stem cells [88]. By a bioinformatics approach, the authors, in order to identify a single prognostic biomarkers for stratification of

CRC undifferentiated tumors, have analyzed a large database of gene expression arrays obtained from populations of stem and progenitor cells and searched for genes associated with differentiation processes. Among the 16 selected candidate genes for identification of predictive biomarkers, negatively linked to the activated leukocyte–cell adhesion molecule (ALCAM/CD166) in CRC patients with stage II or stage III, they selected the homeobox transcription factor CDX2 strictly correlated to ALCAM expression and tested for the association with disease-free survival and a benefit from adjuvant chemotherapy. In particular, it was identified that subgroup of high-risk stage II CRC patients benefit from adjuvant chemotherapy and was characterized by lack of CDX2 expression and high levels of ALCAM.

The translation of this knowledge in CRC has had an important impact into drug development and biomarker discovery for the different subtypes and examples of molecular targeted therapies are tyrosine kinase inhibitors, regorafenib and bevacizumab.

Pharmacogenomics and Irinogenomics

In CRC, despite the standard chemotherapy and novel targeted drugs provided an improvement in terms of response rate and patient's survival, toxicity remains an unsolved problem and PGx has helped the routinely administration of drugs in CRC patients [89]. In CRC as well as in other cancers, the treatment paradigm is to give the dose which achieves the best drug exposure and effectiveness, with an acceptable risk of toxicity [90]. Unluckily, the inter-patient PK variability is a limiting factor due not only to differences in body size but also to variability in absorption, metabolism, distribution and/or excretion (ADME) of the drug and metabolites. In fact, several enzymes and transporters that are part of the ADME processes can condition drug efficacy and toxicity because their expression and activity are highly variable between patients, partially due to germline genetic variability. Germline variants in the coding region can change protein

activity, while variations outside of the coding region could influence protein expression [91]. Another important aspect to consider is patient's germline variation underlying sensitizing condition that mimics the toxicity and can be worsened by the drug. Thus, a patient who carries a sensitizing germline variant would not be able to tolerate the dose required for treatment efficacy and might require to receive a dose adjustment or the selection of an alternative treatment agent. The most frequent type of genetic variants among people (10 million in the human genome) associated with the interindividual variability in drug response are the single nucleotide polymorphisms (SNPs) which represent a difference in a single nucleotide in certain stretch of DNA sequence between two genes. Frequently they are devoid of a functional role but, if a SNP occurs within a gene or in a regulatory region near a gene, they could play a more direct role in disease or in drug metabolism by affecting gene's function. Most of identified SNPs are in linkage disequilibrium with gene variants with higher or lower activity and serve therefore as markers predictive of activity or toxicity due to different enzyme function. SNPs linked to genes coding for enzymes involved in drug metabolism and transport affect therefore the body response and PK profile influencing the efficacy and toxicity of treatment. The possibility to identify SNPs as predictive biomarkers of response to antineoplastic agents by classical approaches like candidate-gene-based research and the genome-wide association study (GWAS) or by technologic advances like the Affymetrix (Santa Clara, California, USA) Drug Metabolizing Enzymes and Transporters (DMET™) microarray platform will allow an improvement of patient care in the optic of personalized therapy. In particular by DMET™ platform is possible to investigate 1931 SNPs and five copy number variations (CNV) in 231 genes related to drugs metabolism contributing to discover polymorphic variants associated to the individual risk of adverse drug reactions (ADRs) and to drug efficacy. By this technology, in case-control studies we identified several polymorphic variants associated with toxicity in different diseases and added novel information on irinoge-

nomics (see below) [92–97]. DMET™ platform offers wide opportunity to identify and validate biomarkers of drug sensitivity for tailored treatment of CRC patients.

Pharmacological treatment of CRC is based on cytotoxic agents like fluoropyrimidines (FdUMP (fluorodeoxyuridine monophosphate, fluorouracil (5-FU), and its oral precursor, capecitabine), irinotecan (IRI, CPT-11), and oxaliplatin (OX), used either alone or in combinations in FOLFIRI (folinic acid, fluorouracil (5-FU), and irinotecan) and FOLFOX (folinic Acid, 5-FU, and oxaliplatin) regimens, and novel targeted agents. Recently, CRC treatment has benefited of novel biological agents as monoclonal antibodies (mAbs) targeting VEGF (i.e. bevacizumab, aflibercept) and EGFR (i.e. cetuximab, panitumumab) pathways or agents leading to a multiple-kinase inhibition (regorafenib) [98]. Cytotoxic drugs have a narrow therapeutic index and strictly dose-related effect also conditioned by interindividual variability in their metabolism. Therefore PGx knowledges, validated biomarkers, integrative genomic approaches and the availability of genetic testing could allowed the identification of subgroups of CRC patients with benefits in terms of prognosis and drug efficacy in the aim of precision medicine. In cytotoxic CRC therapy, important PGx studies have been done on highly polymorphic specific targets, whose genetic or molecular deregulation might correlate to treatment efficacy. Unfortunately, the translation of PGx researches into clinical practice is presently limited with small exceptions regarding the metabolism of 5-FU/capecitabine and irinotecan. For 5-FU SNPs in two important metabolic enzymes have a relevance in clinical practice: the thymidylate synthase (TYMS) and dihydropyrimidine dehydrogenase (DPD), while for irinotecan polymorphic variants in uridine diphosphate glucuronosyltransferases (UGTs) influence variability in biliary excretion and the degradation of irinotecan is conditioned by inherited variations in metabolic pathway. 5-Fluorouracil (5-FU) or its prodrug capecitabine is a cytotoxic drug, classified as “antimetabolite,” and represents the main chemotherapeutic regimen adopted in CRC treatment, having an

improving impact on survival and other solid cancer [99].

The activity of this pyrimidine analog is due to the incorporation of fluoronucleotides into RNA and DNA and to the irreversible inhibition of its target enzyme the thymidylate synthase (TS). Three major active metabolites derive from 5-FU intracellular metabolism: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). Genetic variants in the three drug-metabolizing enzymes thymidine phosphorylate (TP), TYMS and DPD are responsible for variability in response, toxicity and overall survival (OS) in 5-FU-based treatment schedules [100].

5-FU cytotoxic activity is mediated by its methylation to dUMP with 5,10-methylenetetrahydrofolate (CH₂THF) as cofactor, which forms in the cell a stable ternary complex with TYMS enzyme and supplies the only de novo source of thymidylate. Consequently, its cytotoxicity is due to the blocking access of dUMP to the nucleotide-binding site and to the inhibition and depletion of deoxythymidine monophosphate (dTMP) production, important for DNA replication and repair [101, 102]. In 5-FU metabolism in normal and cancer cells its conversion in dihydrofluorouracil (DHFU) is mediated by dihydropyrimidine dehydrogenase (DPD) and represents the rate-limiting step. DPD is abundant in the liver where is normally catabolized more than 80% of administered 5-FU [100]. The administration of the oral prodrug of 5-FU, capecitabine, has revealed a 5-FU comparable efficacy but a lower toxicity [103, 104]. In the liver, capecitabine is converted to 5'-deoxy-5-fluoruridine (DFUR) by carboxylesterase and cytidine deaminase and then converted to 5-FU by thymidine phosphorylase (TP) and/or uridine phosphorylase (UP) [105, 106]. The tumor-selective activation of capecitabine might be explained by the higher expression of both TP and UP in tumor tissue compared to normal tissue [107]. Patients with a decrease activity of catabolic enzymes in 5-FU pathway revealed an interindividual variability to cytotoxic chemotherapy with an increase in drug concentration and consequent high toxicity risk. DPD catalyzes

5-FU and eliminates >80% of administered drug. Its activity is influenced by dihydropyrimidine dehydrogenase (DPYD) gene which is variable at tumoral tissue level and can influence drug efficacy in consideration that intra-tumor drug concentration is fundamental for drug efficacy and antitumor activity. Mucositis, granulocytopenia and neuropathy are the most frequent toxic effects for which might be necessary a dose reduction [108].

In 5–10% of the general population, a partial DPD activity deficiency is demonstrated and only in 0.2% a total loss of enzyme activity [109]. However, DPD polymorphisms influenced the 23–38% of 5-FU toxicity [110]. The most common polymorphic variant recognized to be associated with partial DPD deficiency and consequent 5-FU toxicity is IVS14+1G>A mutation in intron 14 coupled with exon 14 deletion (DPYD*2A), together with the SNPs at 496A>G in exon 6, at 2846A>T in exon 22, and at T1679G (DPYD*13) in exon 13, also recognized to be associated with 5-FU toxicity [111–113].

The US Food and Drug Administration (FDA) has underlined, in the drug labels for 5-FU and capecitabine, that their use should not be allowed in carriers of high-risk alleles. The Dutch Pharmacogenetics Working Group has recommended an alternative treatment in patients homozygous for the high-risk allele and almost a dose reduction of 50% or an alternative drug in patients heterozygous for a decreased-activity allele [114, 115] (in agreement with the more recent Clinical Pharmacogenetics Implementation Consortium Guidelines for Dihydropyrimidine Dehydrogenase Genotype and Fluoropyrimidine Dosing).

Polymorphic variants in TYMS gene are responsible for an increased expression of the enzyme with a consequent high risk of 5-FU toxicity and reduced drug efficacy. TS overexpression is frequently associated with a reduced response to 5-FU treatment based both in adjuvant and in advanced CRC patients with more severe side effects [116, 117].

In CRC patients carrying low levels of TYMS gene product, a significantly higher rate of treat-

ment response and a prolonged overall survival compared to CRC patients with higher TS expression in tumor tissue have been described [109].

Two meta-analyses supported the role of TS expression on overall response rate and overall survival [118, 119]. However, further analyses are necessary to allow a better identification of TYMS transcription regulatory mechanisms and the understanding of the role played by genetic different SNPs combinations in several metabolic enzymes and their frequency in general populations to better clarify the interindividual variability to drugs response. Until now, no recommendations are suggested according to TS phenotype in CRC patient underwent to fluoropyrimidines treatment and although an assay for DPD and TYMS polymorphisms testing is commercially available, pre-emptive testing is not recommended. No recommendations have been issued on dosing of fluoropyrimidines by TS phenotype.

Other gene polymorphisms possibly important for fluoropyrimidine efficacy and toxicity for various enzymes have currently been explored (e.g., dihydropyrimidinase, beta-ureidopropionase, methylenetetrahydrofolate reductase), but available research data are insufficient for conclusions on their potential clinical usefulness. Several other polymorphic variants in enzymes involved at different levels in 5-FU metabolic pathways probably influenced intrinsic and acquired 5-FU pharmacoresistance in CRC patients, but no translation in clinical practice is validated, until now [120].

In CRC treatment another widely used anticancer drug is irinotecan, a camptothecin analog and inactive prodrug, activated at liver level via human carboxylesterases CES1 and CES2 into the active form SN-38, subsequently inactivated through glucuronidation via members of the UDP-glucuronosyltransferase (UGT) enzyme family catalyzing also bilirubin glucuronidation. Somatic tumor-specific mutations seem to influence irinotecan toxicity and efficacy as well as interindividual variability limited its PK and PD [121–123]. Severe diarrhea and neutropenia represent dose-limiting toxicities. Despite the

unequivocal confirmation of the role of somatic mutations on patient's outcome who underwent to irinotecan treatment, scientific evidences confirmed a role of polymorphic variants in UGTs family members, especially for UGT1A1 isoenzyme and other isoforms [103]. Polymorphic variants in UGT1A1 enzyme are responsible for impaired glucuronidating activity and consequent toxicity due to elevated serum levels of SN-38 and bilirubin [124, 125]. Ando et al. published the first evidence on the role of *UGT1A1**28 (*UGT1A1* 7/7 genotype) in the development of irinotecan toxicity [126]. The homozygous *UGT1A1**28 allele phenotype, responsible for increased risk for severe neutropenia and diarrhea, is represented in the 8–10% of the population and according to the FDA treatments in combination with other agents or as a single agent requires a reduction in the starting dose [127]. Dias et al. put in evidence an association between *UGT1A1* genotype and overall response rate in patients treated with irinotecan, but no direct evidences confirm that a dose reduction in *UGT1A1**28 homozygous phenotypes will not lead to an important reduction in overall response rate [128]. Despite FDA recommendations, in clinical practice the preemptive *UGT1A1**28 allele testing is not yet applied although commercial assays for *UGT1A1* testing are available. There are other important polymorphic genes involved in irinotecan metabolic pathways under investigation for their role as putative biomarkers of hematological and gastric toxicities, but further validations are necessary for their potential clinical utility in irinogenomics [93, 129, 130].

Future Perspectives: Precision Medicine Based on Integrative Genomics

In the recent years, the development of a variety of high technology platforms has led researchers to produce large amount of data at different molecular levels and network, in different disciplines of the *omic* world. Traditionally, approaches of bioinformatics analysis were

focused on the use of single classes of data (i.e. genomic data or proteomic data). The rising number of data has made clear that the integration of data at different levels could produce more relevant results. Consequently, many different approaches have pointed to such kind of integration, leading to the rise of a novel discipline, often defined as *integromics*, or integrated analysis of *omic* data, in which computer science, bioinformatics, and mathematical modeling have the main role. This discipline focuses on the elucidation of basic principles of the interplay among different biological molecules (such as proteins or genes), where the network theory plays a synergistic role [131–133]. The focus of computational integrative genomics is to identify basic principles of interplay of different molecules in order to better elucidate the molecular mechanism. This is under the assumption that the information gathered from integrated analysis is higher than in the single and separate study of any data source [131]. It usually utilized a common approach for findings that share a common flow of information. The flow starts from gathering data of different data sources. Then all data are integrated into a single network model, and the model is analyzed with different algorithms tailored to the specific application. Data sources of integrative omics mainly reside on messenger RNA (mRNA), miRNA and protein expression, DNA copy number, SNPs and may be produced in dedicated experiments or extracted from different available databases. Specifically, miRNA therapeutics is emerging as a valuable tool in translational precision oncology [134–139]. The scientific community has recently produced a large number of different databases useful for integrated analysis. In addition to academic data, pharmaceutical and biotech companies retain large amounts of “proprietary data” – inherited from their own and other sources. Most of the data is stored in older types of databases designed to manage a single type of data; therefore, the integration of these data source into a single comprehensive one is a relevant challenge [140].

From a biological point of view, it is clear that the main actors of this process are mRNAs, miR-

NAs, and transcription factors (TFs), those play an interacting role in the regulation of gene expression that results in variable levels of gene transcripts and proteins. Usually, the integration of such datasets relies on the formalism provided from graph theory. As a result, bioinformatics approaches for the integrated analysis usually build comprehensive graphs in which nodes are mRNAs, miRNAs, and TFs (or other molecules) and edges represent the interactions among them. Edges include two main categories: (i) activation edges modeling the interplay between molecules, among whose one may increase the level of another one, and (ii) inhibition edges that model the action of inhibition. The analysis of such graphs uses different algorithms tailored to the specific application. For instance, the individuation of small and connected subgraphs with three different classes of nodes is often used for the identification of loops (feedback and feed-forward) in which the regulation of the expression of a gene could be related to a synergistic action of both miRNA and TF.

All the methods of analysis available share some specific characteristics. First, the use of an internal knowledge base containing information collected from literature and from different databases. The knowledge base usually stores association among mRNAs, miRNAs, and TFs modeled as graphs. This internal knowledge base guides the analysis of experimental data. Second, the approaches enable the user to take external experimental datasets from a pool of samples extracted from patients in case-control or time-series experiments. Then, data of knowledge bases allow to build the association graph including experimental data. Finally, this association graph is mined to extract knowledge.

We here list some main approaches of integrative analysis focusing only on freely available tools.

MAGIA2 is the evolution of the precedent *MAGIA* web tool for the integrated analysis of both mRNA and miRNA. *MAGIA* receives as input, expression level data obtained by case-control or time-series experiments. In this way, it

is able to integrate literature evidence, prediction algorithms, and mRNA and miRNA experimental data based on anticorrelation of miRNA-target expression, using four different relatedness measures. It is able to highlight different regulatory circuits involving either miRNA or TF as regulators: (i) a TF that regulates both a given miRNA and its target gene and (ii) a miRNA that regulates both a given TF and its regulated gene. Furthermore, this tool provides functional enrichment of the gene network using DAVID platform [141].

The *dchip GEMINI* is a freely available web server that receives as input expression levels of miRNA and mRNA obtained from time-series experiments analyzing two conditions, e.g., normal and cancer conditions. It is able to individuate Feed-Forward Loops (FFLs) consisting of TFs, miRNAs and their common target genes. The association among miRNA and their target (TF and mRNA) information is extracted from the literature and stored into the web server. TFs derived from literature used as null model to statistical ranks predicted FFLs from the experimental data [142].

mirConnX is a software tool based on a web interface to build gene regulatory networks starting from mRNA and miRNA expression data on a whole-genome scale. It based on a network built using as a priori model consisting of TF-gene associations and miRNA target predictions for human and mouse derived by computational methods and literature. Experimental data allow inference of experimental associations among TF, miRNA and genes. These associations allow to weight the predefined network and the resulting weighted network can be visualized by the user [143].

miRIN is a web application designed for the identification of the modules of protein-protein interaction networks regulated by miRNAs. The approach of analysis consists of the integration of miRNA target data from literature, protein-protein interactions between target genes from literature, as well as mRNA and miRNA expression profiles provided as data input. The output of

Table 2.1 Available software tools that integrate in a single model miRNA and mRNA data

Tool	Input	Output	Model	Website
MAGIA2	miRNA/mRNA Expression Data Time Series	Feed-forward loops (FFL) Ontological Analysis	Statistical model and literature evidence	http://gencomp.bio.unipd.it/magia2/start/
dCHIPGemini	miRNA/mRNA Expression Data Time Series	Feed-Forward Loops (FFL)	Statistical model and literature evidence	http://www.canevolve.org/dChip-GemiNi
mirConnX	miRNA, mRNA time series	Regulatory Networks	Pre-built network	http://www.benoslab.pitt.edu/mirconnx
miRIN	miRNA, mRNA	Regulatory networks of miRNA, mRNA, TFs, and proteins	Associations derived from literature	http://mirin.ym.edu.tw/

miRIN is a set of regulatory networks involving miRNAs, mRNAs, TFs, and proteins (Table 2.1).

We should note that the literature also reports an approach of integration available for Ingenuity Pathway Analysis (IPA®, Qiagen, Hilden, Germany). The IPA® platform enables the reconstruction of causal networks constructed from individual relationships providing a set of tools for inferring and scoring upstream regulators of gene expression data [144]. This approach has been presented in a previous work by Di Martino et al. and has been applied to the analysis of multiple myeloma data [145]. With respect to the prior work, the authors first applied the integrated analysis into a clinical relevant scenario by applying results to the profiling of MM patients. The workflow of analysis was based on the use of publicly available published by Wu et al. [146]. Data were, initially, preprocessed by Affymetrix proprietary software and filtered using the freely available DChip tool. Through the use of DChip, the authors identified significant differentially expressed (SDE) miRNA and mRNA in two subgroups of multiple myeloma patients: hyperdiploids (HD) MM versus non-hyperdiploids (nHD) MM. These data (SDE genes and SDE miRNAs) were integrated into a single model by using the approach of Kramer et al. implemented into the IPA® software [144]. This approach also enabled to consider the role of TFs and to extract causal relationships among them. The authors also ana-

lyzed data into a functional space looking at canonical pathways and bio-functions, carried out by SDE genes and miRNAs. The main result of this analysis was the identification of different biological events related to the two MM subtypes, while the upstream regulator analysis enabled to identify URs related to the identified transcription events, drawing a new molecular scenario of the two main disease subgroups (Fig. 2.3).

Conclusions

Precision medicine is a reality, but the shift from single gene analysis to multilayered approaches as integrative genomics is likely to produce a novel way to identify targets and individualize treatment. The growing interest for immunotherapy makes this point even more compelling taking into account that each therapeutic approach needs to be personalized based on the immunobiology of the individual patients, which will drive to another shift to tumor analysis to tumor/micro-environment axis evaluation. These perspectives need not only robust technologies but also a novel way to validate findings and novel research approaches which are mostly based on Bayesian design.

Precision medicine does not substitute for good clinics but even allow better and wiser clinics.

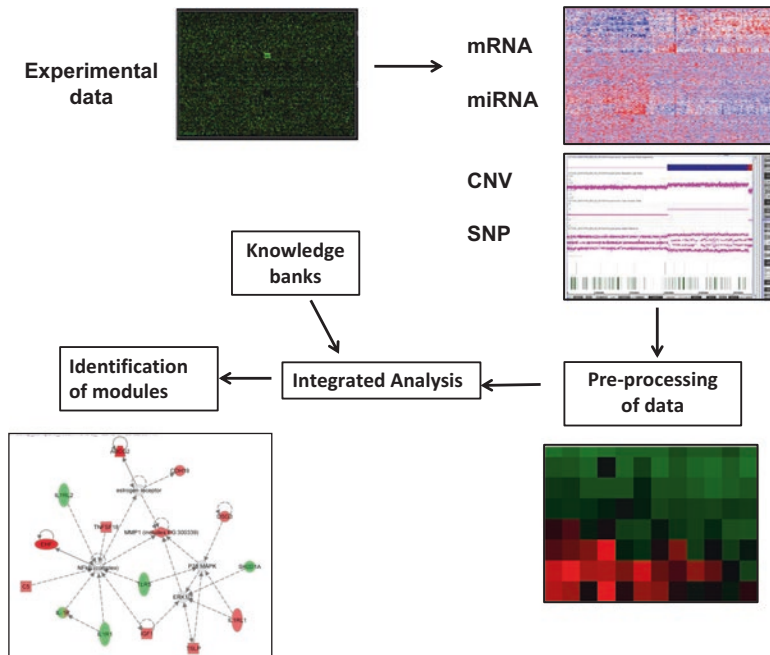


Fig. 2.3 This picture depicts the flow of data in integrative genomics. Different experimental data are collected from the investigator. The data span from classical microarray technologies (e.g. mRNA or miRNA expression) to next-generation sequencing techniques as well as genomic technologies such as CNV or SNP data. The whole set of data is then pre-processed in order to select only signifi-

cant subsets of data or to evidence difference among classes. Then data are integrated into single theoretical models and analyzed with respect to data and information annotated in existing knowledge repositories. Finally, results are presented to the users by supporting models usually coming from graph theories

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