Molecular Parameters for Prognostic and Predictive Assessment in Colorectal Cancer

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4.1 Introduction

Over the last several years, a large amount of information has been obtained on the molecular and genetic characteristics of colorectal cancer, especially related to the mechanisms of cancer development, invasion, metastasis and response to therapy. Part of this information can be translated into useful molecular testing, which might assist the clinician in classifying patients more effectively and developing personalized therapies. Here we review the molecular characteristics of colorectal cancer, with the specific purpose of highlighting those features currently known to possess prognostic or predictive value.

Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer worldwide and continues to be one of the most fatal [1]. The pace of genetic and molecular discovery in the field of CRC development, progression and metastasis has been impressively rapid over the last few years. Seminal discoveries in the field of hereditary CRC genetics, and later the analysis of global gene expression by microarrays or deep sequencing technologies have generated an impressive amount of information. In turn, this has inevitably raised high expectations that the knowledge gained might permit the identification of molecular markers able to assist the clinician and the surgeon in optimizing and tailoring treatment. This has not necessarily occurred in most cases, and several of the published findings still appear contradictory or redundant. The purpose of this chapter is to summarize the current knowledge on CRC, with

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the specific purpose of highlighting the molecular information that has actually turned out to be important for prognostic and predictive purposes.

4.2 Molecular Genetics of Colorectal Cancer

Colorectal carcinogenesis represents a paradigm for cancer development due to the successive accumulation of mutations in genes that control epithelial cell growth, differentiation and cell proliferation [2, 3]. Starting from the original hypothesis of multistep carcinogenesis (the so called adenoma-carcinoma sequence [4], involving the subsequent mutations of only a few genes [5]), the most recent determination of cancer genomes has revealed that at least 15 cancer-associated genes may play a role in transformation, and that no less than 80 somatic mutations in exons characterize the genetic landscape of the transformed cells [6, 7]. Some of the detected mutations are inherited and underlie a genetic predisposition to cancer development; most others arise as a constellation of genetic defects in somatic cells and are also present in sporadic CRCs.

It is currently estimated that 15-30% of CRCs have a major hereditary component; of these cases, approximately one-quarter (<5% of all CRC cases) have a Mendelian inheritance due to mutations in single genes [4]. Identification of the mutated alleles in these hereditary tumors have immensely increased our understanding of the genetic defects which also underlie sporadic cancers. The majority of the hereditary cases are attributable to hereditary nonpolyposis colorectal cancer (HNPCC) and the familial adenomatous polyposis (FAP) syndromes.

The genes mutated in HNPCC (Lynch syndrome, which accounts for ~2-5% of all CRCs), are part of a series of genes involved in DNA mismatch repair (MMR), which include MSH2 and MLH1 (70% of cases) and, less frequently, PMS1, PMS2 and GTBP/MSH6 [8]. MMR is a highly conserved - from bacteria to man - strand-specific form of DNA repair that recognizes and repairs base mismatches due to misincorporation, insertion or deletion of nucleotides occurring during DNA replication and recombination or ensuing upon DNA damage [9]. Mutations of the MMR genes account for a peculiar mutator phenotype, which is revealed by marked length variations in microsatellite DNA (microsatellite instability, MSI). HNPCC patients, having inherited a defective MMR gene allele, have a much higher probability of undergoing mutation of the other allele in somatic cells, and manifest the MSI phenotype. As a consequence, the adenoma-carcinoma transition may take 3-5 years in an HNPCC patient, compared to 20-40 years estimated for most sporadic CRCs [4].

A high frequency MSI (MSI-H) phenotype also characterizes approximately 15% of apparently sporadic CRCs [10, 11]. Rather than being due to de novo germline mutations or somatic mutations in MMR genes, this appears to be consequent to the loss of MLH1 gene expression via promoter DNA hypermethylation [8, 12].

On the other hand, FAP accounts for less than 1% of familial CRCs. It is an autosomal dominant syndrome characterized by hundreds to thousands of adenomas that develop in the colon and rectum, with a lifetime probability of malignant transformation approaching 100% [13]. The disease is caused by germline mutations in the adenomatous polyposis coli (APC) gene, a tumor suppressor gene that becomes inactivated by frame-shift or nonsense mutations. APC encodes a ~300 kDa protein involved in the regulation of the Wnt/β-catenin pathway. In particular, APC takes part, together with other cellular proteins such as GSK3β, Axin and CK1α, in the formation of a so-called "destruction complex", which induces proteasomal degradation of β-catenin. Upon Wnt stimulation, this complex is inhibited, and free β-catenin enters the nucleus and activates transcription of several genes, including those coding for factors involved in cell-cell adhesion, cell migration, chromosomal segregation and apoptosis [14]. Thus, the bi-allelic mutation of APC mimics constitutive Wnt signaling in the colon crypt cells.

Deregulation of the Wnt/β-catenin pathway is also a major determinant of sporadic CRC development. Somatic mutation of both APC alleles is an early step in the development of most adenomas; truncations of the gene are detectable in 70-80% of adenomas and carcinomas.

Over the last several years, analysis of sporadic cases of CRC, in addition to the above-described mutations that were originally identified in hereditary CRC, has also highlighted the existence of common mutations in a vast series of other cellular genes. Like many human cancers, three members of the Ras family of the small-G proteins (KRAS, HRAS and NRAS), which are involved in signal transduction from different growth factor receptors (in particular, the epidermal growth factor receptor, EGFR), are mutated in approximately 40% of CRCs [7]. Other common alterations are mutations of the PIK3CA catalytic subunit of the class I PI3Ks (15-25% of cases) and of BRAF, a protein kinase directly activated by RAS, which in turn activates the MAPKs MEK1 and MEK2 (5-10% of CRCs) (Fig. 4.1).

Inactivating mutations and loss of herozygosity (LOH) in tumor suppressor genes are also very frequent. The most common involve the PTEN phosphatase (which is also mutated in the germline of Cowden patients; 10% of CRCs), various members of the TGF-β signaling pathway, including the TGF type II receptor and the SMAD2 and SMAD4 genes, and the FBXW7 gene, which encodes an F-box protein that normally drives degradation of Cyclin E, a cofactor for the CDK2 kinase, which is essential for the transition from the G1 to the S phase of the cell cycle. Finally, approximately 70% of CRCs show LOH for the region of chromosome 17 that encodes the p53 protein, while, in most of these cases, the other allele of the gene is affected by somatic point mutations [3, 4, 8].

A characteristic common to approximately 85% of CRCs is the presence of chromosomal abnormalities, frequently associated with LOH for specific genomic regions. This characteristic chromosome instability (CIN) appears to be a distinctive trait of cancers that do not show MSI-H. The cellular and molecu-

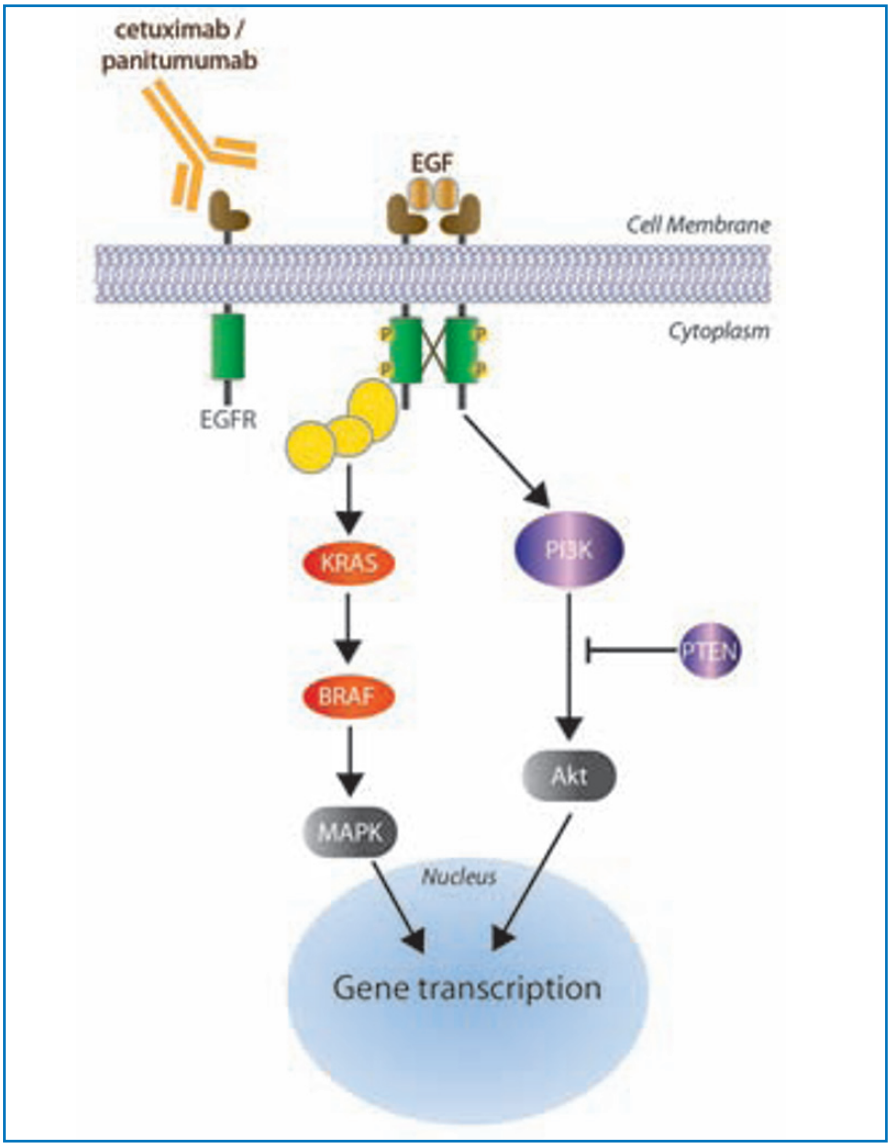

Fig. 4.1 Schematic representation of the EGFR pathway

lar events that determine CIN are still elusive, and are possibly the sum of multiple independent changes, possibly arising as a consequence of the biallelic loss of the APC tumor-suppressor gene, which eventually results in mutations of genes that control mitotic spindle formation or karyokinesis [15, 16]. A surrogate marker of CIN appears to be the partial aneuploidy of the long arm of chromosome 18 (18qLOH), observed in approximately 70% of CRCs and 50% of

large, late-stage adenomas. This chromosomal region, among several other genes, encodes for the SMAD2, SMAD4 and SMAD7 factors operating in the TGF-β pathway and for the DCC (Deleted in Colorectal Carcinoma) gene [4].

Finally, approximately 15% of CRCs show a characteristic epigenetic abnormality consisting in hypermethylation of CpG islands at gene promoters. In mammalian genomes, more than 80% of cytosines at the CpG dinucleotide are modified by methylation, with the exception of highly CpG-dense islands, mainly located in the promoters of approximately 50% of the genes. In CRC cells, there is a generalized decrease in the total level of methylation of the genome, in any case accompanied by the selective methylation of several CpG islands and the consequent epigenetic silencing of the neighboring genes [17]. Modification of the normal DNA methylation pattern defines the so-called CpG island hypermethylation phenotype (CIMP), which ultimately modifies the expression of various genes essential for cell differentiation and cell-fate determination [18]. The CIMP phenotype contributes to the global deregulation of the gene expression profile that is commonly observed by analyzing the CRC transcriptome.

4.3 Molecular Markers for Early Cancer Detection

While colonscopy is the most accurate procedure for CRC screening, it is expensive, has poor patient compliance and can be associated with procedure-related complications. In contrast, fecal occult blood testing (FOBT) is inexpensive but has low sensitivity and specificity. Instead, detection of the specific genomic changes due to DNA hypermethylation could be used for specific, sensitive and noninvasive testing for early cancer detection, especially because CIMP already shows development in early polyp lesions. Assays start from genomic DNA extracted from stool or plasma samples and detect the presence of methylated CpGs upon quantitative PCR amplification of the promoter regions of specific genes. Among the genes considered so far are those coding for Vimentin, Septin, AKAP12, TFPI2 or SPG20 [17, 19]. Of these, stool-based methylated Vimentin detection is now an early detection, clinically validated test for colorectal cancer, commercially available in the U.S (ColoSure^{TM}) [20]. This assay is reported to have a sensitivity of 83% and a specificity of 82%, with approximately equal sensitivity in patients with stage I to III colorectal cancer [21].

4.4 Molecular Markers for Prognostic Assessment

A vast number of studies have addressed the possibility of exploiting the existence of common genetic and molecular features in CRC patients (presence of MSI, CIN, CIMP, LOH at defined loci and existence of specific DNA mutations) for prognostic purposes. The overall outcome of these studies is schematically summarized in Table 4.I.

Table 4.1 Synoptic view of the most frequent molecular and genetic alterations found in CRC and their use as prognostic or predictive markers

Molecular characteristics	Prognostic value	Predictive value
Genetic and epigenetic alterations		
Mutation of MMR genes (MSI-H phenotype)	Favorable - strong evidence	Unfavorable for 5-FU; uncertain for irinotecan
CIN	Unfavorable - strong evidence	
18qLOH (positive correlation with CIN and negative with MSI)	Unfavorable - moderate evidence	Possibly unfavorable
CIMP (correlates with presence of BRAF and KRAS mutations)	Unfavorable - value as an independent marker uncertain	
Mutations of specific genes		
Mutation of APC		
Mutation of KRAS	Unfavorable in advanced disease - clinical use uncertain	Strong predictor of inefficacy of anti-EGFR therapies
Mutation of PI3CA	Unfavorable - clinical use uncertain	
Mutation of BRAF	Unfavorable - clinical use uncertain	Mutation V600E predicts resistance to anti-EGFR therapies
Mutation of PTEN		Mutation predicts resistance to anti-EGFR therapies
Amplification of EGFR, CDK8 or c-MYC		Clinical value uncertain
17qLOH and/or p53 mutation		Variant Pro72 sensitizes cells to 5-FU; clinical value uncertain
Levels of gene expression		
Oncotype DX Colon Cancer Test (7 genes) and ColoPrint (8 genes)	Predict risk of recurrence	Clinical value uncertain
miRNA expression pattern	Research stage only Prognostic significance uncertain	
TFAP2ε		Low expression predictive of unresponsiveness to 5-FU
EGFR		Overexpression predictive of unresponsiveness to cetuximab
VEGF-A, LDH5		Overexpression predictive of poor response to bevacizumab and vatalanib

(*cont.*) ↓

Genetic polymorphisms		
DPD, MTHFR		Polymorphisms predict sensitivity to 5-FU
GST-P1		Polymorphisms predict sensitivity to oxaliplatin
UGT1A1		Presence of UGT1A1*28 associated with drug-induced neutropenia
Thymidylate synthase (TS)		Polymorphisms in the non-coding regions associated with decreased response to 5-FU
ERCC-1		Polymorphisms predict sensitivity to oxaliplatin
VEGF-A		Polymorphisms in the promoter region affect levels of expression and response to bevacizumab
Infiltration by circulating cells		
CTLs (extent of CTL infiltration correlates with MSI status)	CTL infiltration favorable - negatively correlated with lymph node metastasis	
T-regs	Presence of T-regs unfavorable	
Myeloid cells		Extent of tumor infiltration negative correlates with efficacy of anti-angiogenic therapy
Other characteristics		
Intratumoral hypoxia		Predicts resistance to 5-FU and oxaliplatin
Immature and aberrant tumor vasculature		Predicts resistance to chemotherapy - Difficult to assess in vivo

4.4.1 Prognostic Value of Genetic and Epigenetic Tumor Characteristics

The most common, mutually exclusive, specific genetic features at the basis of colon carcinogenesis are MSI and CIN. MSI has a frequency of 15% and is defined by the presence of at least 30% unstable loci in a panel of 5-10 loci consisting of mono- and dinucleotide tracts [22]. CIN on the other hand is found in as many as 85% CRCs and is defined as the presence of numerical chromosome changes and structural aberrations; it is typically assessed by flow cytometry [23].

These two characteristics readily distinguish normal from transformed colonic epithelium and are discriminant in the prognosis of CRC, since several clinical studies and their meta-analyses have extensively documented that CIN-positive tumors carry a worse prognosis than MSI-positive ones [24, 25]. The hazard ratio for overall survival was estimated to be 0.65 for MSI CRCs vs. 1.45 for CIN CRC [23]. Despite the association of MSI and CIN with prognosis, however, these determinations have not yet entered routine testing for clinical decision making [26, 27].

Another prognostic marker is the deletion of the long arm of chromosome 18 (18qLOH). CRC patients with 18qLOH have a worse prognosis compared with patients with tumors without 18qLOH [26, 27]. There is a strict correlation of 18qLOH with CIN and an inverse correlation with MSI. As a consequence, it is still unclear whether 18qLOH is a truly independent maker for prognostic assessment or rather a surrogate marker for CIN/MSI assessment [23].

A third epigenetic instability marker, after CIN and MSI, is CIMP, commonly defined as the CpG methylation of at least three loci from a selected panel of five CpG islands [23]. Retrospective studies have indicated that CIMP is a negative marker for CRC progression and survival; however, its prognostic value as an independent marker is uncertain at the moment, especially because patients with CIMP also carry BRAF or KRAS mutations [26].

4.4.2 Prognostic Value of Individual Genetic Mutations

Among the specific genetic mutations detected in CRC patients, those of the genes coding for proteins involved in signal transduction from the receptor tyrosine kinases and the EGFR in particular, have been extensively investigated. These include mutations in KRAS, BRAF and PIK3CA [28-30] (Fig. 4.1). There is now limited evidence that the presence of mutations in KRAS codons 12 and 13 (which are validated predictive markers for treatment with EGFR inhibitors; cf. below), PIK3CA and BRAF are prognostically unfavorable, especially in advanced diseases; however, the clinical usefulness of these findings is uncertain at the moment [23].

4.4.3 Prognostic Value of Gene Expression Profiling

Over the last few years, a vast series of studies have assessed global expression profiles of CRCs by microarrays or, more modernly, by deep RNA sequencing, or have analyzed the levels of expression of various subsets of individual genes, with the ultimate purpose of establishing possible correlations between gene expression and prognosis.

In particular, two gene expression profiling diagnostic tests have been the object of important clinical studies. Both tests determine the risk of recurrence and relapse-free survival of colorectal cancers in stage II and III after surgical resection. This area of interest appears to be of particular importance, since better risk stratification is needed in a phase of disease when the risk of recurrence exists and the indications for chemotherapy are controversial. The Oncotype DX® Colon Cancer Test has been commercially available since January 2010, while the ColoPrint® assay was clinically and technically validated in 2012.

The Oncotype DX® Colon Cancer Test, similar to the by now clinically validated Oncotype DX® Breast Cancer Assay, uses fixed, paraffin-embedded primary tumor tissues and analyses, using RT-PCR, seven cancer-related genes selected from a panel of 761 genes recurring in CRCs. Of these seven genes, three are involved in cell proliferation (MK167, MYBL2 and MYC), three are associated with activated stroma (BGN, INHBA and FAP), and one is part of the DNA damage response (GADD45B). Expression values for these seven genes are normalized according to the levels of five reference genes, and the values are then elaborated to provide an individualized recurrence risk score [31, 32]. The ColoPrint® test, devised to follow the validated breast cancer test MammaPrint®, is a microarray assay which analyses the levels of expression of 18 unique genes associated with prognostic significance for tumor recurrence in patients who have undergone surgical resection for stage II or III colorectal cancer. Patients are divided into high and low risk of recurrence. ColoPrint® facilitates the identification of patients with stage II disease who may be safely managed without use of chemotherapy [32, 33].

As far as the expression of specific subsets of genes is concerned, different studies have aimed at identifying markers that could predict the metastatic potential, especially since deaths caused by CRC can mostly be attributed to visceral metastasis. One study identified PCSK7, which codes for the proprotein convertase subtilisin/kexin type 7, as the top upregulated gene in metastatic tumors [34]. In contrast, the expression of several genes appears to be deregulated in node involvement, including tumor suppressor genes (ST7, BAP1), OAS1 and NTRK2, PRSS8 (encoding for the prostasin serine protease) and PSMA, which was also related to node metastasis in prostate cancer [34, 35]. The expression of FOXC2, instead, was reported to be directly proportional to the aggressiveness of node metastasis in CRCs [36]. Finally,

one study also analyzed the levels of expression of approximately 30 genes involved in angiogenesis and lymphangiogenesis, and identified the levels of Plexin-A1 and stromal cell-derived factor 1 (SDF-1) as predictors to discriminate between tumor and paired normal mucosa, the former being overexpressed and the latter downregulated in tumors [37]. Collectively, these studies have provided important insights into the mechanisms of tumor development and metastatic spread. For example, it is now clear that gene expression in primitive tumors, visceral metastasis and lymph node metastasis is largely dissimilar, indicating that the two metastatic processes are biologically different and that the metastatic cells are affected by the microenvironment where they become established [38]. However, the very high inter-patient, intra-study and inter-study variability prevents the use of individual gene expression for prognostic purposes at the moment.

An essential level of gene regulation, the importance of which has been increasingly appreciated over the last few years, is the control of mRNA levels by the cellular microRNA (miRNA) network. MiRNAs as small (20-22 nt long), noncoding RNAs, produced by processing the primary transcripts of over 1,000 cellular genes. The miRNA network impacts on all aspects of mammalian biology, including cancer development and spread [39]. MiRNAs may also represent a novel class of prognostic and possible predictive biomarkers, especially because a few of them are released, and can be detected, in blood and feces [40, 41]. Although several miRNAs have been reported to be differentially expressed in specimens from CRC patients, very limited validation is currently available. As a consequence, it is too early to draw conclusions as to the extent to which some miRNAs might actually translate into specific biomarkers useful in clinical practice.

4.4.4 Prognostic Value of Immune Cell Infiltration

Human solid cancers are invariably infiltrated by various lymphoid cell populations. A direct relationship between the intratumoral presence of cytotoxic T lymphocytes (CTLs) and CRC patient survival has been detected in several analyses; interestingly, CTL infiltration appears to be more marked in MSI-H tumors [42] and is inversely proportional to lymph node metastasis [26].

Another lymphoid cell population that has been widely investigated in recent years are the CD4+ CD25+ T-regulatory (T-reg) cells. The presence of infiltrating Forkhead Box P3-Positive (FOXP3) T-regs has been associated with a worse prognosis in CRC patients, probably due to their function in suppressing antitumor immunity. Different studies have indicate that T-regs are markers of shorter patient survival and predictors of recurrence when associated with decreased levels of CD8+ CTLs [43-45].

4.5 Molecular Markers Predicting Response to Therapy

Adjuvant and neoadjuvant chemotherapy using 5-FluoroUracil (5-FU)-based regimens is often indicated for patients with stage II or stage III disease (www.asco.gov; www.cancer.gov). Clinical and biochemical parameters, such as perforation, obstruction, local and lymph node invasion, or circulating levels of carcinoembryonic antigen (CEA) have clear prognostic value, but they do not predict which patients are likely to benefit from chemotherapy [10]. In particular, approximately 25 to 30% of newly diagnosed CRC cases have node-negative (stage II) disease; with surgery alone, the overall survival at 5 years of these patients is about 80% [46]. Adjuvant chemotherapy offers most of these phase II patients a minimal incremental benefit, with improvement in survival being less than 5% [32]. Thus, defining the genetic or molecular characteristics of the subset of patients with high-risk stage II disease who benefit from adjuvant regimens appears particularly important. The most important results of the studies so far conducted are summarized in Table 4.1 and reported below.

4.5.1 Predictive Value of Genetic and Epigenetic Tumor Characteristics

Both prospective [47-49] and retrospective [50, 51] studies performed in stage II CRC patients have suggested that MSI-H is a negative predictor of 5-FU response. Furthermore, there is also evidence that 5-FU-based therapies might even be detrimental for some MSI-H stage II individuals [47]. Therefore, although neither ASCO nor the European Group on Tumor Markers currently recommends MSI testing to guide treatment selection, it might reasonably be expected that such a recommendation will be included in the guidelines in the near future. Fortunately, however, the presence of MSI-H itself has a good prognostic value for stage II patients, such as not to justify the administration of adjuvant chemotherapy. In terms of the specific response to Irinotecan, on the other hand, there is still controversy on the role of MSI-H determination [52-54].

As far as 18qLOH is concerned, this marker appears to be a powerful predictor of patients with adverse response to 5-FU-based therapy [55]. The observation that reduced levels of SMAD4, a gene located within the 18q region, are associated with a worse response to 5-FU is consistent with this conclusion [56].

4.5.2 Predictive Value of Specific Genetic Variations

As already discussed above, the EGFR pathway is often constitutively activated in advanced CRC, often correlating with more aggressive tumor pheno-

types, and is thus a well-conceived target for anti-cancer therapies. To date, two monoclonal antibodies (cetuximab – Erbitux®– and panitumumab – Vectibix®–) have been approved for use in combinatorial regimens (Fig. 4.1) [57, 58]. Their effectiveness, however, seems clear in only a small subset of stage IV CRC patients [59]. Some evidence has suggested that EGFR gene copy number might correlate with improved response to both monoclonal antibodies [60], but major technical issues hamper the clinical application of this determination.

In patients resistant to cetuximab and panitumumab, point mutations in EGFR are uncommon, unlike the situation with other types of cancers. In contrast, mutations in KRAS account for approximately 50-60% of these resistances [26]. Genetic testing for KRAS is now currently required by both the FDA and the European Medicines Agency (EMEA) to select CRC patients who would benefit from anti-EGFR therapies, and clearly stands as one the brightest examples of the potential usefulness of a biomarker to predict drug responsiveness [61]. In spite of the clear predictive value of KRAS mutations, however, no more than 50% of wild-type KRAS patients objectively respond to anti-EGFR therapies, possibly as a consequence of alterations in other members of the EGFR pathway [61].

Like KRAS, BRAF is a protein kinase frequently mutated in many cancer types. The vast majority of BRAF mutations occur at a single hotspot at position 1799, resulting in a Valine to Glutamic acid substitution (commonly referred to as V600E) [62]; as a consequence, the BRAF mutation is an ideal biomarker for routine clinical use. Both retrospective and prospective studies have in fact confirmed an association between V600E and poor response to anti-EGFR therapies [63-65]. BRAF genotyping has recently been included in the major guidelines for the selection of patients scheduled to undergo anti-EGFR therapies.

Preclinical evidence suggests that PTEN deficiency also determines resistance to anti-EGFR drugs [66, 67]. Analysis of PTEN status by tissue immunohistochemistry has indeed indicated that almost half of CRCs have impaired PTEN expression [68, 69]. Interestingly, however, only PTEN status at the level of metastasis appeared to correlate with efficacy of cetuximab treatment.

Finally, although not unequivocally, there is evidence that response to 5-FU is associated to retention of wild-type p53 status, at least for stage III patients [70]. In the p53 protein, a common polymorphism at codon 72 distinguishes two protein variants (Arg72 or Pro72), which have different biochemical properties [71]. The presence of the Pro72 variant might contribute to sensitize tumor cells to 5-FU [72]. Despite decades of work assessing the predominant role of p53 in tumor biology, however, the establishment of this protein as a biomarker is seriously hampered by major technical issues that can be overcome only with systematic gene sequencing, an approach still far from clinical routine.

4.5.3 Predictive Value of Gene Expression Profiling

Over the last few years, several small studies have profiled gene expression in CRC surgical specimens to identify possible gene combinations that might have prognostic or predictive value [37, 73-77]. Overall, these studies have led to inconclusive results, possibly due their relatively small scale, except for the fact that they indicate that there is very wide patient-to-patient variation in the levels of expression of most of the analysed genes, which essentially prevents the identification of potentially universal predictive markers. Unlike the commercially available OncoType DX® Breast Cancer, or the MammaPrint® assays, which provide both prognostic and predictive information for women with breast cancer, the above-described OncoType DX® Colon Cancer and ColoPrint® tests for gene expression profiling in CRC provide prognostic information, but their capacity to predict response to therapy appears highly uncertain at the moment [32].

As far as the analysis of individual genes is concerned, a particularly promising observation was that low expression of SMAD4 (a gene located in the long arm of chromosome 18) was associated with poor responsiveness to 5-FU-based adjuvant chemotherapy [55], especially since this observation was in line with previous data linking drug efficacy to 18qLOH [78]. However, neither low expression of SMAD4 nor 18qLOH has been consistently confirmed in subsequent studies. Enthusiasm for gene expression as a predictive biomarker has very recently been revitalized by a study showing that the low expression of Transcription Factor AP-2 epsilon (TFAP2ε), possibly consequent to promoter hypermethylation, was predictive of unresponsiveness to 5-FU [79].

Interestingly, analysis of gene expression appears rather to have an exploitable value to predict the effectiveness of a series of new generation drugs, essentially EGFR and VEGF inhibitors. As already discussed, responsiveness to anti-EGFR monoclonal antibodies (i.e., cetuximab) is well predicted by mutations in effector genes in the EGF pathway, mainly KRAS and BRAF. As a general rule, mutations that activate these genes curtail the effect of EGFR inhibitors [27]. However, there is a subset of tumors that are not sensitive to EGFR therapies despite the apparent lack of mutations of KRAS or BRAF. A few studies have indicated that, in these cases, resistance might be the consequence of overexpression of EGFR or EGFR ligands [80, 81]. Similarly, high expression of VEGF-A or LDH5 (lactate de-hydrogenase) might account for the poor response to the angiogenesis inhibitors bevacizumab and vatalanib, respectively [82, 83]. The clinical usefulness of these observations remains undefined at the moment.

4.5.4 Genetic Polymorphisms Affecting Drug Efficacy

The vast majority of chemotherapy regimens are designed as 5-FU-based therapies, hence its associated toxicity is a relevant matter in clinical management. Nearly the entire 5-FU content in the organism is catabolized by the enzyme dehydro-pyrimidine dehydrogenase (DPD). Expression of this enzyme varies significantly within the population, with a small fraction (less than 5%) being partially or totally deficient [84]. Since impairment of DPD function can lead to life-threatening 5-FU toxicity [85], it appears important to determine DPD status. Clinical application of this concept, however, is rendered difficult by the fact that about 30 different SNPs have been associated to DPD deficiency.

The role of methylene-tetrahydrofolate reductase (MTHFR) in indirectly increasing sensitivity to 5-FU is on the other hand less clear [27]. In this case, two common polymorphisms that affect MTHFR activity (C677T and A1298C) have been shown to increase responsiveness to 5-FU [86]. Despite the obvious interest in predicting 5-FU toxicity, none of these findings has so far been translated into the clinic.

Oxaliplatin, like other platinum derivatives, undergoes hepatic detoxification, through various enzymes mainly belonging to the glutathione-S-transferase (GST) family. Among these isoenzymes, GST-P1 is the most prominent in oxaliplatin catabolism. Two well-characterized polymorphisms in the coding region of the protein have been shown to significantly decrease GST-P1 activity [87]. These substitutions, which occur in approximately 15% of the entire population, severely impair drug metabolism [88], eventually resulting in oxaliplatin-induced neuropathy. Toxicity, however, appears to have importance only at high drug dosage [89].

The active metabolite of irinotecan, SN-38, is mainly detoxified by UDP-glucuronosyl-transferase-1-A1 (UGT1A1). Several studies have reported an association between a particular polymorphism (UGT1A1*28) and drug-induced neutropenia, due to reduced enzyme activity resulting in insufficient drug clearance [90]. In 2005, the American Food and Drug Administration (FDA) approved a commercial test for UGT1A1, to assist in the correct choice of irinotecan dosage [23]; the practical usefulness of this test, however, is limited by the fact that the irinotecan doses administered in combination regimens (such as standard FOLFIRI) have negligible toxicity.

Besides drug metabolism, another set of genetic polymorphisms affect the levels of expression or the function of the factors targeted by the drugs. The main target of the 5-FU active metabolite (5-FdUMP) is the enzyme thymidylate synthase (TS). A few polymorphisms located in the promoter region or in the 3' untranslated portion of the mRNA are known to modify the levels of expression of the TS gene and have been variously associated to increased or decreased response to 5-FU [91]. Multiple clinical trials are currently ongoing to further define the clinical usefulness of these findings.

Oxaliplatin mainly exerts its activity through the formation of DNA adducts, that eventually impede DNA replication but are tentatively repaired

by the cellular DNA repair proteins. Expression of one of these proteins, ERCC-1, was suggested to be predictive of drug response [92, 93], a possibility that is now being explored by an ongoing clinical trial (OPTIMOX2) [94].

Finally, bevacizumab is a monoclonal antibody that specifically targets the Vascular Endothelial Growth Factor (VEGF), approved for the combinatorial treatment of advanced, refractory CRC, in which it has so far shown a modest and rather disappointing performance [49]. A polymorphism in the promoter region of VEGF (C to T change at position -1498) appears to modulate host VEGF levels, with the C/C allelic combination significantly correlating with amelioration of the clinical outcome when bevacizumab is administered along with standard FOLFIRI regimen [95].

Collectively, these findings unveil the importance of SNP determination as an important tool to predict response to therapy. It is still early days, but it can easily be predicted that, like other malignancies, SNP genotyping will become an integral part of the clinical management of CRC patients in the near future.

4.5.5 The Tumor Microenvironment and its Predictive Potential

Formation of an abnormal vasculature and presence of white blood cells are two features that invariably accompany the development of many types of solid cancers. In particular, tumors are invariably infiltrated by a set of monocytic cells of myeloid origin, among them the tumor-associated macrophages, TAMs, which exert a pro-angiogenic function, or the myeloid-derived suppressor cells (MDSCs), which suppress the host immune response [96]. In mouse pre-clinical models, the extent of this myeloid cell infiltration correlates with poor responsiveness to anti-VEGF treatment [97]. In keeping with the poor clinical success of bevacizumab, colorectal tumors are known to abundantly mobilize these cells through the secretion of GM-CSF [98]. Thus, the extent of myeloid cell infiltration, or the circulating levels of GM-CSF, or those of other angiogenic factors that might overcome VEGF inhibition, are currently being assessed as possible markers to guide patient selection for anti-VEGF treatments.

Another common characteristics of solid tumors, particularly including CRC, is the presence of intratumoral hypoxia. Chronic low oxygen tensions activate a variegated molecular program, crucially orchestrated by the hypoxia-inducible factor 1alpha (HIF-1α), which eventually leads to chemoresistance, radioresistance, angiogenesis and invasiveness of malignant cells. The first evidence that hypoxic conditioning desensitizes tumor cells to 5-FU was produced more than two decades ago [99], and there is now ample pre-clinical evidence that hypoxia predicts both 5-FU and oxaliplatin chemoresistance. The actual translation of these findings to the clinic is however more problematic, especially because of significant inconsistencies among the different methodologies used to quantify hypoxia in tissues.

The establishment of a chronic tumor-associated hypoxic state is directly

linked to the status of the tumor vasculature, which is characterized by a poor association with perivascular mural cells (smooth muscle cells or pericytes), increased ramification and stagnant blood flow [100]. Such an inefficient and leaky vasculature represents a major obstacle to drug penetration, and its "normalization" therefore is now regarded as an important strategy to increase drug responsiveness. This is of particular relevance in the case of CRC, where bevacizumab has been demonstrated to induce vessel normalization in some settings, possibly expressing its effectiveness only in combinatorial regimens [101]. In this respect, however, the quantitative determination of vessel normalization appears difficult, as all the proposed techniques (MRI, PET, ultrasound, CT, immunostaining) still suffer from significant limitations [101].

4.6 Peculiarity of Rectal Cancer

In clinical practice, locally advanced rectal cancer is commonly considered biologically very similar to CRC, as it has a comparable molecular evolution and often carries overlapping molecular alterations [23]. However, there is no demonstration that the events leading to cancer development are superimposable in every colorectal region. In addition, pathological and molecular evidence demonstrating how colon and rectal cancers carry different characteristics is increasing.

A large randomized trial has recently started to validate the most important CRC molecular markers specifically in rectal cancer (www.clinicaltrials.gov; ID:NCT00835055). So far, the available evidence indicates that both MSI and BRAF mutations are significantly more frequent in colon cancer, but only if we compare the right-sided ones to rectal neoplasms [102-104]. While CIMP+ status can reach 40% in proximal colon tumors, approximately only 10% distal colorectal cancers are CIMP+ [102, 103, 105]. On the other hand, there is controversy concerning the presence of KRAS mutations [102-104]. The frequency of p53 mutations is higher in rectal and left colon cancer (40-60%) than in proximal CRC (25-40%), and has independent prognostic value; the types of mutations, however, appear superimposable [106, 107].

A number of studies have also analyzed the expression profiles of cancers from the distal and proximal parts of the colon, and from the rectum. These studies have reinforced the recognition that colon cancer and rectal cancer can develop through different oncogenic events, especially comparing right-sided CRCs to left-sided and rectal CRCs. Over 60 genes have been found, the expression of which is different between left- and right-sided CRCs [108, 109]. Whether some of the genes specifically expressed in rectal cancer might used as prognostic or predictive markers in the future, is a matter that must await further investigation.

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