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Chapter 17

The Complexity of Colorectal Cancer Biology — Putting Bricks on the Path to Personalized Medicine

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http://dx.doi.org/10.5772/57172

1. Introduction

Boveri's hypothesis about the genetic basis of cancer, a century ago, subsequently confirmed by Loeb et al. in 1974, opened the gate for genetic studies that became essential for cancer management. [1, 2]

Cancer is a disease that arises from altered cell due to multiple genetic and epigenetic alterations that confer them the properties of apoptosis evasion and growth advantage. These properties represent a competitive advantage over normal cells, leading to the expansion and colonization of other tissues by these cells, which cause patient´s death by interfering with the normal function of its organs. [3]

Introduction of chemotherapy in cancer treatments has supposed an important improvement in progression-free survival but, it is also associated to life threatening secondary effects, in the worst scenario, and an important quality life reduction in the best one. Furthermore, due to the difficulty to stratify patients in low and high risk, there are a substantial number of them that will receive the therapy but will not experience any benefit from it. These consequences, underline the importance of personalized treatment in cancer management. [4, 5]

The first chemotherapy treatments were based on a frequently observed characteristic of cancer cells, this is, a high proliferation index. Their effectiveness rates vary among cancers, but all of them are characterized by important secondary effects as consequence of their low specificity, since these chemotherapeutics also affects normal cells.

The use of this traditional chemotherapy effectively shrinks tumor mass but, observation of tumor metastases and recurrences led to the idea of the existence of cell populations unaffected by the treatment, either because they are a type of cells with different characteristics from the

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cells that the drug was designed to, because they have undergone genetic changes that confer them resistance or because the microenvironment protects them. Identification of the resistance cause is one of the cornerstones of cancer treatment and in this attempt, identification of cells that have tumorigenic potential to sustain cancer is fundamental. [6]

Based on this, studies of tumor organization were performed. As indicated by Shackleton et al, research on tumor organization intends to determine which cells have tumorigenic potential to sustain cancer, this is, all cells in a cancer or only a specific population of them. There are two principal models of tumor organization, not mutually exclusive, the clonal evolution model [stochastic] and the stem cell model [hierarchical], that have been subjected to deep examination owing to their implications in cancer management. [6]

The clonal evolution model was described by Nowell in 1976, and it is quite verified that it seems to be ubiquitous in all cancers. It describes cancer development by the successive acquisition of differential features in the derived cells giving rise to the formation of cell clones, this is, group of cells with common features because they originate from the same progenitor cell that can be a stem cell or not. Some of these features can be positive and provide a selective advantage to these cells over the others, with the consequent establishment of these clones. This model supports the idea that all cells in a tumor are important since the cancer can be sustained due to the possible acquisition of resistance or advantage features in the derived cells. On the other hand, this process is not random, tumors share similarities, but these similarities will be modulated by the tumor environment turning each tumor into unique entities. [3, 7]

To establish a model of tumor organization is fundamental since it should drastically change treatment cancer approach. Identification of cells that drive tumor progression will allow us to design target drugs against this cells instead of nonspecific drugs that treat all cancer cells, even normal cells. Tumors that have a hierarchical organization from stem cells to more differentiated cells are said to follow the stem cell model that have been recently proved in a few cancers, among them, the colorectal cancer. [3, 6-13] Stem cells are a very specific type of cells that possess differential capabilities such as being pluripotent, remain in a quiescent state, have a long life as well as self-renewal capacity which allows them to perpetuate themselves and repopulate different cells linages.

Nonetheless, both models are not incompatible, mutation causing clonal expansion may happen in the stem cell compartment and manifests its effects on the progenitors cells or may happen in the progenitors cells that can re-activate the auto-renewal machinery to generate stem cells. [14] As long as the disease progresses, these changes can induce alterations in the normal patterns of development of these cells, reducing their ability to differentiate and increasing their auto-renewal capacity, causing the uncontrolled increase of undifferentiated cells, as it happens in leukemia. [15] As Nowell denoted the observation of non-differentiation of tumor cells is explained by focus the cell resources in increasing cell proliferation and invasiveness. [3]

Both models describe a scenario of intratumor genetic heterogeneity that reproduces the tumor tissue heterogeneity observed in patients, and also describes the heterogeneity observed in the different stages of cancer as consequence of selection in different environments. [3, 7]

http://dx.doi.org/10.5772/57172

Nonetheless, cells in this tissue are not independent units, there is a communication between them and even collaboration has been proved in leukemogenic cells. [16] This communication is performed through the extracellular matrix by different signals to which cells respond and its behavior is modulated by these signals as them change over time. Hence, during cancer development, cells in a tumor, experiment different genetic alterations selected by their fitness which is determined by the tumor microenvironment and tissue characteristics where the tumor is being developed. All these characteristics explain the observed intra-tumor heterogeneity in cancer and the different subtypes identify in a specific type of cancer, which added to the modification performed by the individual genetic background makes each cancer a unique entity. [7]

To add more complexity to the system, not all the mutations within a tumor are important for its progression or survival. Due to alterations in DNA repair systems, numerous mutations are produced that do not provide any advantage nor disadvantage to cells at a given moment but, its presence and proportion in the tumor mass will depend on if they are produced in clones which have driver mutations, this is mutations that will lead tumor development and response to the environment. Differentiate which mutations are drivers from passengers it is another important and confounding factor for both, identification of tumor and pharmacogenetic markers. [7, 17, 18]

Equally importantly is to determine the global effects that mutations cause, since understanding the aims of the tumor through the modulation of the pathways that performs will help us to predict the compensatory mechanism that executes. [3] Knudson´s two hits hypothesis postulates that more than one mutation is needed in cells to become malignant. [19, 20] The fine regulation that tumor cells exert, and the importance of the molecular pathways implied is exemplified by the °Just-right hypothesis° that postulates that the second mutation in the adenomatous polyposis coli [APC] gene [which is a gene where germline mutations are found to be associated to an hereditary form of colorectal cancer] produces in a tumor is dependent on the type and localization of the germline mutation in a patient in order to maintain some basal APC activity, which it is needed for cell functioning. [21]

Apart from all these convolutions, each tumor has an inherent progression; there is a pattern of genetic alterations typical of each tumor, whose establishment is a priority when designing cancer treatments. The goal is to increase drug efficiency along with its specificity in order to diminish the secondary effects. In this road, there have been two important events that have helped us to achieve this ultimate goal.

Firstly, advances in knowledge of genetics have allowed us to discover the hereditary component of cancer as well as the steps that follow in cancer development, the more important pathways trigger in them and have pointed out the key deregulated molecules against to specific drugs can be designed. The studies realized to determine the cancer characteristics have also contributed to the discovery of specific and differential patterns of each cancer. [22-25]

Secondly, drug development research has focused their efforts in the development of target drugs that act specifically on those molecules in order to avoid the important secondary effects of classical chemotherapy.

Molecular specific drugs, like tyrosine kinase inhibitors or antibody-based therapies are the next-generation cancer treatment. [4] As result of this molecular specificity, effectiveness of these selective treatments is more dependent on the biology of the target cell. Consequently, the pace of the improvements in cancer treatments is highly dependent on the knowledge of cancer cells biology.

But, as stated above, cancer cells are not islands, its behavior is modulated by the signals that its surroundings emit, as well as its surroundings control the quantity of nutrients, oxygen and chemicals that reach the tumor. Therefore, treatment efficiency is influenced by drug pharmacokinetics, that is dependent of the biology of the normal cells, as well as the secondary effects are determined by drug pharmacodynamics, that is subject to drug specificity and the inherited sensibility of normal cells to chemotherapeutic agents determined by different mechanisms, for example, detoxifying mechanisms. [3] Subsequently, when trying to personalize treatments it is important to identify both, the genetic of the tumor and the genetic of normal cells. This is one of the causes that explain the variability in response to chemotherapy observed in patients with similar tumor characteristics. [4]

Despite the efforts realized, the only pharmacogenetics markers used today in clinic are KRAS and BRAF mutations. Some of the causes of this delay in markers discovery are then. No intention of this chapter is to provide a deep review of the different subtypes but offer an outline of the principal characteristics of the diverse subtypes according to the literature, in order to expose the cause of the delay in markers discovery that was previously mentioned, and reflect that problems in pharmacogenetic studies obtained are due, in part, to the high heterogeneity in colorectal cancer which makes difficult to establish clearly differentiated groups of study and at the same time, to reflect that intense research has a positive point of view, since important advances in tumor characterization and target molecules discovery have also been done.

2. Colorectal cancer biology — Heterogeneity of colorectal cancer

Colon tissue is organized in a repetition of structural subunits called crypts. Cells in each crypt have an ordered configuration, being the stem cells at the base of the crypt and the subsequent differentiated cells upwards along the crypt. The main conductor in the colonic cell differentiation is the Wnt/β-catenin signaling pathway. [26] β-Catenin is a transcriptional co-activator of genes implicated in cell growth and differentiation. Activation of the Wnt pathway disrupts the cytoplasmatic complex that marks β-catenin for degradation allowing it to enter the nucleus were exerts its activity. [27-29] APC protein forms part of the degradation complex. Concordant to their function in the differentiation and growing pattern, there is an inverse gradient of APC/β-catenin expression along the crypt axle, being APC mostly expressed in the upper part of the crypt and β-catenin in the lower part on the crypt. [30]

Colorectal cancer is a highly heterogeneous malignancy caused by genetic and epigenetic alterations in the stem cells of the crypt of the bowel which give rise to precancerous lesion, aberrant crypt foci, that overgrow usually forming polyps that after a successful accumulation

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of genomic alterations become tumor cells. [11-13, 31, 32] It can arise in a sporadic form or it can have a hereditary component.

Identification of the cause that predisposes to cancer in the hereditary syndromes have given us clues, due to the similarities of the mechanisms identified, to understand what happen in the sporadic tumors, that involves the majority of the colorectal cancer cases. Germline mutations in known oncogenes or DNA repair genes have been identify as causative of a predisposition to endure colorectal cancer but the origin of the sporadic form is still unclear and it is attributed to genetic alterations caused by the environmental as chemical carcinogens, age related factors which increase DNA errors rate and nutritious factors. All these conditions will probably determine the activated cancer mechanism depending on which factor has triggered the cancer. But, environment is also an important factor that modulates colorectal cancer appearance in patients with a hereditary component since germline mutations cause a predisposition to cancer, but another mutation is needed in the cells for them to become malignant, as it is postulated in Knudson´s two hits hypothesis. [19, 20]

In recent times it has become evident the need of establish subtypes in colorectal cancer due to the differential characteristics of the proximal and distal segments of the colon as well as diverse features, at both histological and molecular level, observed in colorectal cancer.

Proximal and distal colon, proceed from a different embryological origin, midgut and hindgut respectively. Besides the embryological origin, its distinctions span from innervation and blood supply to functional differences and differential gene expression. [33, 34]

At histological level, most the polyps are adenomatous [95%], but only a small percentage of them progress to cancer and not all the colorectal cancer cases are presented with polyps. [35-37] Actually, prevalence of each type of polyp is only rough since it is population dependent, due to its genetic background and environmental dependence apart from the expertise of the pathologist to identify the polyp, which sometimes is difficult. [37, 38] Histological features of the tumors are associated to the underlying molecular pathway. So far, two main types of carcinoma have been identified, traditional and serrated carcinomas, that have several subtypes and whose development is driven by three main molecular mechanisms, chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). Combinations of these mechanism and some additional genetic and epigenetics changes according to the tumor environment give raise to the large varieties of histological forms observed. [38]

On molecular basis, colorectal cancer development is mainly, but not exclusively, driven by the deregulation of one of these mechanisms (CIN, MSI or CIMP), CIN being the most prevalent. [23, 39-44] But it not clear if these mechanisms are the cause or the major alterations trigger in cancer. [2]

The predominance, of one of these mechanisms over the others, since they are not mutually exclusive, provides the tumor their differential features, which includes morphological characteristics, cancer prognosis and treatment efficiency. This heterogeneity observed in colorectal cancer is due to the diverse scenarios in which colorectal cancer develops so,

characterization of the colorectal cancer in different subtypes, according to its distinctive signatures, will help us to stratify cancer progression risk and personalize treatments. [45-47]

But these subtypes are not mutually exclusive, the intra-heterogeneity observed in a patient´s polyps reflects the heterogeneity of the molecular pathways and mechanisms that can be implicated. [37]

Despite this overlap, predominant characteristics can be distinguished into the subtypes and even alterations that are, in principal, mutually exclusive have been identified, that reflect the unique signature of each tumor. [48, 49]

2.1. Molecular mechanism

2.1.1. Chromosomal instability mechanism – CIN

Chromosomal instability mechanism (CIN) is detected in the 65-70% of the colorectal cancers. It is defined by the identification of changes at chromosomal level in tumor cells that cause gene dose variations. [2] This mechanism is associated to the major hereditary syndrome, the polyposis adenomatous familiar (FAP) as well as its attenuated variant (AFAP), that accounts for 1% of the colorectal cases and it is the most frequently detected in the sporadic form [38, 50-52]. Less commonly, CIN is categorized in subtypes as CIN-high and low. [47, 52, 62]

The molecular steps that describe this mechanism were proposed by Fear and Vogelstein in the °adenoma-carcinoma sequence°, where mutations in the APC gene (chr.5q) is the first step identified in a sequence of genetic alterations on oncogenes and tumor suppressor genes that leads to its keys characteristics, the aneuploidy and loss of heterozigosity. [22, 52-54]

Germline mutations in the APC gene are associated with these hereditary syndromes, as well as mutations in this gene are detected in the 72%-85 of sporadic colorectal cancer cases and hypermethylation of its promoter in the 18%. According to the importance of the Wnt pathway in the colorectal cancer, in half of the patients where genetic alterations in APC are not detected, gain of function mutations in the β-catenin gene have been found that account for 10% of colorectal cases. [52, 55-57]

Besides its function in the repression complex of the β-catenin, APC has numerous functions, among which highlights its implication in chromosomal segregation and, according to its parallel increasing expression with cell differentiation, plays a role in different features of cell differentiation. [52, 55, 56]

Mutations in APC are followed by genetic alterations with a different frequency, in KRAS, DCC, SMAD4 and p53 that are detected in the progression of a tumor from adenoma to carcinoma. [22, 58] All these genes are key points of regulation of important pathways that control cell behavior thus, KRAS [chr.12p] is member of the Ras and PI3K pathway, which are usually dysregulated in cancer and is implicated in cell proliferation, differentiation, survival, metabolism and apoptosis. Its activation triggers both pathways. [58, 59]

SMAD4 belongs to the transforming growth factor β pathway signaling which is a tumor suppressor pathway. Its inactivation is related to tumor progression and invasion, being

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associated to the transition from adenoma to carcinoma. This pathway has also a function on the microenvironment regulation of cell by autocrine and paracrine factors. [58, 60]

Because of its important and numerous functions in cells, like cell cycle regulation or maintenance of genomic integrity, p53 (chr.17p) is called the guardian of the genome. Being one of the most frequently mutated genes in cancer, its inactivation is associated to metastasis. [58, 61]

Nonetheless, the establishment of the adenoma-carcinoma sequence does not implied that all mutations described are needed for the progression of cancer nor the uniques but the more frequently ones detected, with different prevalence across the stages. [22]

2.1.2. Microsatellite instability (MSI)

Microsatellite instability (MSI) is defined by the detection of alterations in the length of short repeated sequences known as microsatellite, which is indicative of defects in the DNA mismatch repair system (MMR). These alterations influence expression of the affected genes. [63] The genes that have been found associated to this altered mechanism are MLH1, PMS2, PMS1 and MSH6. This mechanism is detected in 15-20% of colorectal cancers in both sporadic and hereditary tumors. It is the main feature of another hereditary syndrome, Lynch syndrome that accounts for 2-3% of colorectal cases. [38, 64] The number of altered microsatellites, is also important, defining two subtypes as this number is high, MSI-H, or low, MSI-L. [65]

Mutations in MUTYH are associated to another hereditary syndrome, rarely found in sporadic cases, called adenomatous polyposis associated to Mutyh (MAP). This protein belongs to the DNA base-excision repair system, which is important in DNA oxidative damage repair that causes guanosine (G) to thymidine (T) transversions. [66] Molecular mechanisms are not yet totally clarified and polyps from MAP have some of the features but not all of both, CIN and MSI mechanism. [67, 68]

2.1.3. Methylator Phenotype (CIMP)

CpG island methylator phenotype (CIMP) is defined by the detection of high degree of methylation. [69] Hypermethylation of the promoter region causes silencing expression of affected genes. It is generally age-related and it is related to cell response to inflammation. [65]

This mechanism is frequent in sporadic tumor and has also been detected associated to hereditary mechanisms with a non-Darwinian patter of inheritance. [58, 70-75] There are two panels of genes studied to identify the CIMP state, which define two subtypes, CIMP-high and CIMP-low. CIMP-H is associated to neoplastic methylation meanwhile CIMP-L are age related.

2.2. Molecular pathways

2.2.1. Traditional pathway (Chromosomal instability mechanism – CIN)

The traditional pathway of colorectal cancer is the most prevalent, 60% of CCR arise by this pathway. [76] Histologically, tumors that follow the traditional pathway are tubular polyps that can be subdivided into tubular, tubulovillous and villous, being the former the most

prevalent. Colorectal cancer from FAP and AFAP patients, are the canonical tumors develop by these pathway. This type of adenomas affects the epithelial layer and are originated from dysplastic aberrant crypt foci. [77]

CIN is the most prevalent molecular mechanism in the traditional pathway of colorectal cancer, which is also characterized for being microsatellite stable (MSS) and CIMP-. Tumors driven by this mechanism are more frequently detected in distal localization and have a worse prognosis than MSI tumors. They recently have showed the use of CIN as useful marker for predicting survival, being patients that harbors CIN high tumors associated with a poor survival. [62]

Adenoma – carcinoma sequence also lies beneath Lynch syndrome polyps but develops at a faster tempo. [78, 79] Lynch syndrome polyps are more frequently associated to the proximal colon, and even though it can be tubular, are more frequently associated with a mucinous or signet ring histology and villous structures. These polyps show a high grade dysplasia, poorly differentiated cells and Crohn's-like infiltration of lymphocytes which has been found associated to an increased survival. These polyps have higher risk of cancer but are less invasive, have a better prognosis and a different response to chemotherapeutics. Less frequently these polyps have KRAS or p53 mutations. [64, 65, 78-80]

Although, presence of this mechanism in tumors has been reported to be inversely related to CIMP, either CIMP-L or CIMP-H, [81, 82] there is a subgroup of CIN/MSS tumors characterized by the presence of BRAF mutation that seems to be correlated with CIMP and poor survival. [83] BRAF is implicated in MERK-ERK activation pathway by its recruitment by KRAS. Confirming previous reports of KRAS and BRAF mutations as mutually exclusive, these tumors are wild type KRAS. [84]

2.2.2. Alternative pathway (KRAS)

The alternative pathway is still not very well characterized. Some studies indicate the presence of KRAS as its hallmark. [85] Histologically, the presence of KRAS, p53 mutation and recently GNAS, has been associated to villous histology and a high grade dysplasia [86-88] and the presence of CIMP+ to tubule villous size, right side localization and amount of villous, [89, 90] as well as to a differential pattern of the Wnt pathway genes. [91] Villous polyps are also characterized for being microsatellite stable [MSS] and CIMP-L.

2.2.3. Serrated pathway CpG Island [methylator phenotype (CIMP)

The serrated pathway underlines the 20-35% of colorectal cancer cases. [37, 76] Histologically, it is characterized by hyperplastic polyps, sessile serrated polyps or traditional serrated adenomas, originated from non-dysplastic aberrant crypt foci that can either be mucinous or not mucinous. [77, 85, 92] This type of tumor is mostly localized in the proximal colon and has bad prognosis. [37]

Serrated polyps can be hyperplastic (HP) (20-30%), sessile serrated adenomas (SSA) (2-9%) and traditional serrated adenomas (TSA) (0.3%) that are subsequently divided into subtypes.

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HP can distinguish polyps of microvesicular type (MVHP), Goblet cell–rich type (GCHP) or Mucin poor type (MPHP), according to their content in mucin. SSA can be with/without cytological dysplasia as well as TSA can be with/without conventional dysplasia. [37, 76]

The molecular characteristic of the majority of serrated polyps is the BRAF mutation and CIMP + mechanism. [93]

The CpG island methylator phenotype (CIMP) is detected in 15%-40% of the colorectal tumors. [94, 95] This kind of tumor is mostly related to ageing and environmental factors. One of these genes identified as susceptible of hypermethylation is MLH1, which belongs to the DNA mismatch repair system (MMR), being the causal mechanism of MSI in the serrated pathway. [37, 65, 69]

The sequential steps described in this pathway from normal mucosa to carcinoma goes from the presence of BRAF mutation in microvesicular hyperplastic polyps and posterior acquisition of CIMP phenotype in sessile serrated polyps to a co-occurrence of both genetics alteration leading to sessile serrated polyps. The acquisition of MLH1 promoter methylation is related to the carcinoma stage. [76]

Goblet cell-rich hyperplastic polyps (GCHP) and traditional serrated adenomas (TSA) are associated to KRAS mutations and while it has not been demonstrated a progression of GCHP to carcinoma, it was proved in TSA. It has also been suggested MSI-L after MGMT methylation or partial MLH1 methylation. [76]

Tumors associated to MUTYH can be since hyperplastic and sessile serrated polyps to no polyps at CRC presentation. A higher association of KRAS mutations (70%) has been found in MAP tumors that follow the serrated pathway compared to sporadic (17%), as well as an increased on G:C to T:A transversions (94% vs. 29%). The findings of mutations on APC in adenomas indicate two pathways of development of MAP tumors. [67, 96]

These data seem to confirm that are, at least, as many cancers as patients.

3. State of the art of pharmacogenetics

Personalized medicine is based on the clinical use of molecular biomarkers. A biomarker is any specific physical trait or measurable biological change in the organism related to disease or health conditions, being a very broad concept that includes many different measurements of a biologic status.

Besides being diagnostic [used for the establishment of a particular disease present in the patient sample [97]] or prognostic [used for the establishment of an association with clinical outcomes, such as overall survival or recurrence-free survival independently of the treatment [98] biomarkers can be predictive, by the assessment of the likely benefit of a specific treatment to a specific patient, [99] or pharmacodynamic, by the measurement of the drug effect in a disease [100].

The term, Pharmacogenetics, was first used by Vogel in 1959 as the science about the effects of heritability on drug response [101]. According to the definitions approved by the US Food and Drug Administration (FDA), pharmacogenetics is 'the study of variations in DNA sequence as related to drug response' [102] while the more comprehensive term Pharmacogenomics is defined as 'the study of variations of DNA and RNA characteristics as related to drug response' [103]. Sometimes used indistinctly, Pharmacogenomics is related to the study of whole genome, the gene transcripts and population variability, with the aim of predicting the right treatment in individual patients and designing new drugs.

In last years work in the field has grown almost exponentially [104], at the same pace as the expectations about the increasing of clinical benefit and reduction of the risk of adverse drug reactions (ADR), at least in outliers, i.e. people whose drug responses are not "average" [105].

Nonetheless, adoption of validated pharmacogenetic markers into routine clinical practice has been slow, mainly in the oncological field. Pharmacogenetics has mainly focused on the association between monogenic polymorphisms and in variations in drug metabolism. [102] But, limitations exist on the role of pharmacogenetics in cancer therapy, mainly because of the non-concordance at genetic level between germinal and somatic line of patients [106]. Heritability can be used to assess toxicity, but there are major concerns in their use to assess effectivity.

Today, more than a 100 drugs have pharmacogenomic biomarkers in drug labels approved by the U.S. Food and Drug Administration (FDA), being 35 oncology drugs [107]. In the opposite sense, the European Medicines Agency (EMA) has been more conservative in the implementation of pharmacogenetic markers in drug labels.

In the oncology field, hematology has been the more rewarding, due in part to the lack of some of the architectural barriers found in solid tumors. As result of the pharmacogenetic management, survival rates of some leukemias have improved drastically. Albeit the efforts realized to infer drug efficiency from germline markers, the only ones that have been consistently replicated across the studies for efficiency, are tumor markers, leaving germline markers for the identification of patients with toxicity risk and posterior evaluation of risk/benefit of the drug.

3.1. Colorectal cancer treatment efficiency

In colorectal cancer, 75% of patients with stage I to III can be treated with surgery alone or in combination with chemotherapy, with a 5-year survival rate of 93.2%, 82.5%, and 59.5%, respectively, in contrast with only 8.1% survival rate of patients harbouring stage IV disease. [108]

For patients management, the probability of distant metastasis and response to chemotherapy, are the most important clinical variables. With or without surgery, adjuvant chemotherapy is routinely employed to treat those colorectal cancer patients at high risk of developing recurrence or, those who already have metastatic disease at the time of diagnosis (up to 20 %).

The initial standard treatment with 5-fluorouracil (5-FU), with a median overall survival of 12 months or less and an overall response rate of 10%, has evolved to combinations with oxali-

http://dx.doi.org/10.5772/57172

platin or irinotecan that have dramatically improved survival. [109-111]. The preoperative application of radiotherapy with infusional 5-FU have significantly decreased, the rate of local recurrence [112-114]. In advanced colorectal cancer fluoropyrimidine chemotherapies are basics for treatment in combination with oxaliplatin or irinotecan [115]. The integration of biological agents with conventional cytotoxic drugs has expanded the treatment of metastatic disease, resulting in an increased response rate and survival and achieving downstaging for surgical resection and potential cure. The currently approved and widely used targeted treatments are the monoclonal antibodies bevacizumab that recognizes the vasculature endothelial growth factor (VEGF) and cetuximab and panitumumab, targeting the epidermal growth factor receptor [EGFR]. These combinations reach response rates of up to 50% with a median time of progression free survival of 10–12 months for patients with advanced CRC.

Biomarker development is now essential to aid selection of patients likely to respond to therapy, rationalizing treatments and improving outcomes. But the different approaches used in order to establish biomarkers of the response to treatments in patients with CRC are not lacking in controversial. Even though numerous biomarkers have been postulated to be used as pharmacogenetic markers, only a few of them are actually being used to manage cancer treatment [116].

3.2. Biomarkers of 5-FU response

Thymidylate synthase (TS) is the primary intracellular target of 5-FU. 5-FU acts by preventing the methylation of the deoxyuridine monophosphate to deoxythymidine monophosphate by forming a stable complex, 5-FU–TYMS, causing a thymine deficiency. [117] In CRC, the overexpression of TS has been associated with 5-FU resistance. [118] Several studies analysed genetic polymorphisms as potential predictive factors to 5-FU response [119] but the association between TS expression or TS polymorphisms and response after 5-FU adjuvant treatment is still largely unclear. Thus, although most studies report a TS expression decrease with a better response, [120] there are studies that contradict this association, regarding both the genotype and level of expression, and that link genotypes of high expression of TS with a better response [121] and even a lack of association. [116, 122] Among the several factors that can explain the variability of results, a relevant role might be played by the use of germline genetic data despite the target of 5-FU is the tumor cell. In fact, the TS genotypes from germline and tumor cells from a single patient can differ widely in rectal cancer, distorting the influence of TS on the response to 5-FU. [116]

Although MSI CRCs have a prognostic advantage, [123] mainly due to the minor metastatic potential of MSI CRCs, [124] the predictive value of MSI is still controversial. [125-127] MSI is a strong and well validated prognostic marker to be used in the decision making process in an appropriate clinical setting, for example in stage II, the favourable outcome of patients with MSI CRC suggests that adjuvant chemotherapy could be avoided. [128]

Loss of heterozygosity (LOH) or allelic imbalance (AI) is common in chromosomal instability (CIN) CRC. LOH of chromosome 18q leads to the loss of the tumor suppressor gene Deleted in Colon Cancer (DCC) and had been associated with a poor prognosis of stage II and III CRC patients in several studies [129], but these data were not confirmed. [130] In addition, their role

as predictor of outcome in CCR patients following 5-FU based adjuvant therapy, is controversial too.

Near 30–50% of colorectal tumors harbour mutations in the KRAS oncogene, 90% of the mutations occurring either in codon 12 or 13. [131] However, there is no consensus about the role of KRAS as a prognostic marker [132] and KRAS mutation can hardly be expected to be a predictive marker of response to standard chemotherapy. [132, 133]

Global hypomethylation and hypermethylation of tumor, characterize epigenomic instability. Due to this context of genomic instability is difficult to know how the hypermethylated phenotype "CpG island methylator phenotype" (CIMP) affect survival rate. Despite initial results, [134] CIMP positivity in CRC seems not to be a significant independent predictor of survival benefit from 5-FU chemotherapy. [135]

3.3. Biomarkers of platinum response

Glutathione, a ubiquitous tripeptide thiol, is a vital antioxidant and has a protective role against a range of toxins including metal compounds such as cisplatin. Glutathione S-transferase P1 (GSTP1) acts directly in the detoxification of platinum compounds so it is an important factor related to resistance to platinum [136]. However, and despite initial studies [137] reporting the association between GSTP1 Ile105Val and oxaliplatin efficacy and toxicity, results of subsequent studies were inconclusive. [138, 139]

High levels of excision repair cross-complementing 1 (ERCC1), an endonuclease of the nucleotide excision repair (NER) system, are associated with an increased platinum resistance. [140, 141] The x-ray repair cross complementing group 1 (XRCC1), a member of the base excision repair (BER) pathway, links to other proteins related to the BER pathways and repair specific base damage, caused by oxaliplatin. [142] XRCC1 polymorphisms increase the risk of oxaliplatin resistance, via inadequate repair or increased damage tolerance. [143] XPD (ERCC2) has an important role in DNA repair by removing bulky DNA adducts produced by environmental toxins and xenobiotics. The XPD Lys751Gln polymorphism has been associated to clinical outcome following platinum-based chemotherapy. [136, 139, 144]

3.4. Biomarkers of monoclonal antibodies response

There is a wide consensus on the predictive value of KRAS mutations in response to treatment with anti-EGFR drugs. Interestingly, a single first study, in barely 30 patients with metastatic CRC treated with cetuximab, demonstrated the relation between KRAS mutation and non-response: KRAS mutations were found in 68% of non-responding patients but in none of the responders. [145] [145]. The fact is that KRAS is downstream in the EGFR signalling pathway and that pathway is activated by KRAS mutations irrespective of the receptor status, overriding the efficacy of anti-EGFR therapy. The Food and Drug Administration (FDA) approved, in a record time, label changes to cetuximab and panitumumab to advise against their use in patients with KRAS positive metastatic CCR [146, 147]

BRAF mutations also affects the EGFR signalling pathway and are found in CRC at lower frequency than KRAS (≤10%), in fact BRAF and KRAS mutations are mutually exclusive events

http://dx.doi.org/10.5772/57172

in tumors. [148] The most frequent BRAF mutation (V600E) represents 50% of BRAF mutations in CCR, being more common in MSI CRC, than in microsatellite stable tumors. [128] BRAF mutation is involved in MEK-ERK pathway activation and CRC carcinogenesis. BRAF V600E is also associated with the CIMP phenotype, 70% of CIMP CRC can harbour BRAF mutations. [93] BRAF mutations have been associated with poor prognosis in patients with stage IV CRC. In agreement with the role of BRAF mutations in enhancing stimulation of downstream MEK-ERK signalling, in patients with metastatic CRC, BRAF mutations are predictive of non-response to EGFR-targeted agents. [149]

3.5. Biomarkers of toxicity

As stated previously, more than a 100 drugs have pharmacogenomic biomarkers in drug labels approved by the FDA. It is paradoxical that, while germline genetical markers should be better for the identification of patients with toxicity risk, the efforts in pharmacogenetic studies were realized to infer drug efficiency. Only six of the FDA approved oncology biomarkers are associated with toxicity. DPYD*2A for capecitabine and UGT1A1*28 irinotecan are the biomarker associated to CRC chemotherapy. [107]

With this data, the problem related to discovering biomarkers of ADRs is clear. Most studied biomarkers related to ADRs are reflected in Table 1, but they are not extensively used in clinical practice.

Gene	Treatment	Toxicity	Review
DPYD	fluoropyrimidine	myelotoxicity	Amstutz U et al. Pharmacogenomics. 2011, 12 [9]:1321-36 FDA: XELODA® [capecitabine] Label [http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020896s026lbl.pdf]
UGT1A1	irinotecan	Myelotoxicity severe diarrhea	Marques SC, Hum Genomics. 2010, 4 [4]:238-49 FDA Camptosar® Label [http://www.accessdata.fda.gov/drugsatfda_docs/label/2006/020571s030lbl.pdf]
EGFR	EGFR inhibitors	Skin rash	Galvani E, Future Oncol. 2012, 8 [8]: 1015-29
VEGF	VEGF inhibitors	hypertension	Schneider BP, J. Clin. Oncol. 2008, 26 [28]: 4672–4678
ABCB1	capecitabine	neutropenia	Gonzalez-Haba E, Pharmacogenomics. 2010, 11 [12], 1715–1723.
GSTP1 GSTM1 ERCC1 XPD [ERCC2] XRCC1	platin-based platin-based oxaliplatin oxaliplatin platin-based	Neurotoxicity neutropenia hematotoxicity hematotoxicity gastrointestinal	Cortejoso L, López-Fernández LA. Pharmacogenomics. 2012, 12 [10]:1173-1191

Table 1. Association of genes to toxicity in colorectal treatments.

3.6. Biomarkers used in clinical practice

In conclusion, currently the UICC/AJCC Tumor Node Metastasis (TNM) stage system remains the only valid prognostic marker for predicting the outcome of CRC patients. [150-155] Besides different histomorphological, immunohistochemical and molecular biomarkers have been proposed [156, 157] to improve stratification of CRC patients into prognostic subgroups. But, if no additional prognostic and predictive factors were included in the pre- and postoperative management of non-metastatic CRC until now, for metastatic CRC patients gene mutations are arising as predictive biomarker, mainly the KRAS mutational status, with the implementation of anti-EGFR therapy.

4. Establishment issue of genotype-phenotype correlations in cancer

In the last years, numerous candidate prognostic and predictive markers have been reported in hundreds of studies and failed to demonstrate clinical utility. It is difficult to review, even monthly, all described molecular markers of prognosis. Clearly a validation is necessary to establish an association between any of these markers with prognosis or with the response to therapy but the validation itself is not an explanation of why so many potential markers fail to be validated. Inconsistencies can arise between initial reports and subsequent studies because differences in assays, study design, genetic substructure of human populations, statistical power or methodologies. But the establishment of clear genotype-phenotypes correlations, mostly in solid tumors, is still a wide and difficult field of study due to several reasons:

4.1. Architecture of solid tumor

Hematologic cancers treatments have turned them, in some cases, in chronic diseases with the appropriate treatment. Even though they share a common problem with solid tumor, this is, localization and properties of the cancer stem cells, majority of leukemia cells are located in the bloodstream so once drugs reach the bloodstream do not have additional barriers to trespass but the cellular membrane. This more easily access by the drug allows reduce cancer cells load.

But in solid tumors, drugs have to overcome several barriers to access the cells. To get access to tumor drugs first have to extravasate and diffuse across the extracellular matrix to reach all the cells in a tumor, included not well irrigated zones where transportation into the cells is even more difficult due to the extracellular acidic pH. [158, 159]

Tumor mass is a not equally organized mass of cells with an equally distributed blood capillary network that supplies all cells in a tumor but a disorganized mass of different cells with unequally blood supply subject to a different interstitial fluid pressure that produce differential gradient of molecules distribution, among them, drugs. Thus, obstruction of an adequate intratumoral drug delivery in one the cause is one of the causes for cancer recurrence. [158, 159]

Indeed, modification of tumor microenvironment is one of the novel mechanisms to overcome drug resistance. [160, 161]

4.2. Tumor microenvironment

It is already established that tumor microenvironment has an important role in tumor behavior as well as physical impediment of drug delivery but its function in promoting cancer development and drug resistance by segregating molecules by stromal cells have been recently proved. [161-164]

In colorectal cancer, presence of tumor-infiltrating lymphocytes, as part of the immune system response, has been associated positively to both survival and chemotherapy response. [165, 166]

Straussman et al. showed that resistance to RAF inhibitors are not only due to gene activated mutations but mediated by the segregation of hepatocyte growth factor (HGF) by the stromal cells. Their work confirmed the microenvironment as a frequent cause of chemotherapy resistance, principally to targeted drugs [164].

4.3. Intertumor heterogeneity

Similar to other natural ecosystems, tumor growth and development is dependent on the niche conditions. In the case of a tumor, these conditions are primarily determined by the genetic background and physiological state of the patient and lately, determined by the genetic of the tumor itself. And as it happens in all ecosystems, tumor is not in a static state, as the environment changes, the tumor will be adapted to the changed conditions. The different conditions in which a tumor will be developed [a unique mixture of genomic and epigenomic features] determine its specific features, which are improbable to be parallel in another organism. [167]

Albeit colorectal cancer is governed by the general mechanisms described above, the existence of a different mutational spectrum between patients with the same type of cancer has been broadly reported. [168] The experimental confirmation of the 'just right hypothesis' [169] provided new insights into the importance of the genetic background of the patient in determining tumourigenesis and tumor progression.

Even the triggered mechanisms into the tumor cells contribute to such divergence: Bielas et al. determined the mutational rate in tumors and found that was, on average, 200 times greater than in normal cells. This finding uncovered a novel cancer mechanism called point mutation instability (PIN) [170, 171], and can have consequences over pharmacogenomic assays.

4.4. Intratumor heterogeneity

As stated before [116], intratumor heterogeneity may explain the difficulties encountered in the validation of oncology biomarkers owing to sampling bias. As consequence of the different conditions that tumor cells undergone, tumor cells have to adjust their behavior. The adaptation process to these variable circumstances is accompanied by a differential mutational process that results in a genetic heterogeneous blend of cells.

Gerlinger et al [172] demonstrated performing exome sequencing in primary renal carcinomas that 63 to 69% of all somatic mutations were not detectable in all the samples from different tumor sections. They also found biomarkers of good and poor prognosis in the analysis of different regions of the same tumor and high intratumor heterogeneity when ploidy was measured. [172] These findings indicate that intratumor heterogeneity is one of the most important obstacles in the establishment of biomarkers of both prognosis and response to treatment, what implies that the approaches that, so far, have been realized have to change.

4.5. Stem cells

Other possible pitfall can be the contribution of cancer stem cells to drug resistance. As explained before, identification of the appropriate cell to direct therapy is essential to eradicate cancer. Even though treatments can decrease tumor burden, if drugs do not target the appropriate cell, chemotherapy can, as much, become a disease in chronic but not eliminate it from the organism, as it has proved in leukemias. Stem cells are the only tumor-initiating cells within a malignancy and therefore have been shown to maintain colorectal cells population, [173] in fact, they account for about 2.5%% of cancer cells in CRC. [13] But the current chemotherapy is not specific for these cells, and possibly cancer stem cells are naturally resistant to chemotherapy through quiescence, capacity for DNA repair or expression of genes affecting transport and effective drug release into the cells as ABC-transporter. [174]

Even more, stem cells itself are related to genetic background of the tumor. Mutations in APC gene can increase the number of stem cells via Wnt signaling, promoting tumorigenesis [169].

4.6. Response to chemotherapy

Introduction of chemotherapy is a determinant selection factor of cell survival. The appearance of resistance cells to treatments due to mutations that prevent treatment efficiency either by selection of pre-existent resistant clones, either by the emergence of mutants clones induced by the drug, either by inducing the segregation of protective molecules by autocrine or paracrine mechanism or molecules that bypass the activity of the drug, adds new difficulties to both, discovery of clear biomarkers and development of drugs that cure cancer.

4.7. Difficulty of identification or characterization of specific histologic subtypes

Histological identification of different colorectal cancer subtypes can be tough due to its heterogeneity which makes it hardly dependent on the pathologist to identify the polyp. [37] This fact introduces an error in the genotype-phenotype correlations that obscures biomarkers identification.

4.8. Techniques limitations

The existence of high intratumor heterogeneity reveals the scarcity in the information that can be obtained from a tumor and the impossibility, so far, of study bigger regions of the tumor either because of the limited laboratory resources and high cost disclose high intratumor heterogeneity is a difficult obstacle to overcome.

Detection of genetic abnormalities is subject to the proper target identification and design of the methodologies used. For example, one of the difficulties to categorized CIMP is the choice of the appropriate panel of loci to study methylation. [175, 176]

4.9. Studies design

The classification of the groups is often different among studies. To analyse the statistical association of a biomarker, some studies group patients in I-II stage and III-IV of cancer, having each group a different association [positive, negative or not association with respect to the analysed trait] meanwhile others does that with II-III stages. The different criteria used to group patients, is obviously a source of contradictions, since studies compare results obtained from patients with different characteristics. The different methodologies used between studies is another source of confuse. [62, 83]

Some limitations in studies design are an obviously consequence of the resources restraints, either economic or by shortage of the sample but extrapolation of results from these studies are more hazardous.

The reality of high tumor heterogeneity and dynamic change of tumor behaviour makes easy to understand the very little information we can acquire when study only one, two or three markers in one slice of the tumor and this is even worse when study not targeted drugs since cell have more options to overcome their effect like compensatory mechanisms.

As Greaves and Maley expose °genome profiles under-estimate complexity° and continue ° It may be that only a modest number of phenotypic traits are required to negotiate all constraints and evolve to full malignant or metastatic status but the inference is that this can be achieved by an almost infinite variety of evolutionary trajectories and with multiple, different combinations of driver mutations° [7]

The limited capacity to detect low- prevalence clones are another source of a possible future selection of resistance. [7]

Another reason for inconsistencies is small study sample sizes. Typically biomarker studies are done in a subset of patients enrolled in a main study and, therefore, often not statistically adequate to answer clinical questions. Analysis is hampered further by multiple comparisons in correlative studies. Although this approach is crucial to sound statistical methodology, correction for multiple comparisons [or the failure to do so] has probably led to heterogeneity. A major statistical flaw is the potential for false-positive associations because of assessment of multiple SNPs. The opposite is a concern too; biologically important associations frequently cannot be detected after stringent correction because the selection of SNPs is too broad. Study power might also be inadequate if SNPs with excessively rare minor allele frequency are selected. Finally, racial heterogeneity within the trial is important to take into account, and proper correction or analysis of patients in subgroups by ethnic origin must be done.

Due to the need of major research projects, both in number of samples as in appropriate resources for their study, consolidate research consortia has become imperative.

The establishment of clear genotype-phenotypes correlations is still a wide and difficult field of study due to the previously exposed heterogeneity and overlapping characteristics observed

in colorectal cancer in both histological and molecular level, exacerbated by the confusion in identifying some histologic subtypes of polyps and the designing problem of the studies, which often compare only a few markers and patients in different stages, and with different treatments, that as previously show have an impact in the molecular mechanism trigger. As consequence, some of the published associations are not lately replicated

5. Conclusion

The clinical application of pharmacogenetic tests is still limited to a few drugs. But, the fact that a significant number of patients obtain no advantage receiving chemotherapy encourage us to increase the efforts to get more and better biomarkers. Knowledge of the problems outlined above gives us a better understanding of the challenges of pharmacogenetics and allow us to reach a better understanding of the biological basis of cancer treatments. While that work continues, new genomic technologies now in development are enabling to bring useful biomarkers from the bench to bedside in a more rapid and effective way.

Acknowledgements

This work was supported in part by a grant from Acción Estratégica de Salud (ISCIII-Mineo) (PS09/02368)

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