# Colorectal Cancer Heterogeneity and Targeted Therapy: A Case for Molecular Disease Subtypes

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## Abstract

Personalized cancer medicine is becoming increasingly important in colorectal cancer treatment. Especially for targeted therapies, large variations between individual treatment responses exist. Predicting therapy response is of utmost significance, as it prevents overtreatment and adverse effects in patients. For EGFR-targeted therapy, many mechanisms of resistance have been uncovered, for example, mutations in *KRAS* and *BRAF*, and upregulation of alternative receptors. Currently, routine testing for all known modifiers of response is unpractical, and as a result, decision-making for anti-EGFR therapy is still largely based on assessing the mutation status of an individual gene (*KRAS*). Recently, comprehensive classifications of colorectal cancer have been presented that integrate many of the (epi-)genetic and microenvironmental factors that contribute to colorectal cancer heterogeneity. These classification systems are not only of prognostic value but also predict therapy efficacy, including the response to anti-EGFR agents. Therefore, molecular subtype–based stratification to guide therapeutic decisions is a promising new strategy that might overcome the shortcomings of single gene testing in colorectal cancer as well as in other malignancies. Furthermore, the development of new agents in a disease subtype–specific fashion has the potential to transform drug-discovery studies and generate novel, more effective therapies. *Cancer Res; 75(2); 245–9. ©2014 AACR.*

## Introduction

Colorectal cancer is a heterogeneous disease. Colorectal cancers differ in clinical presentation, molecular characteristics, and the prognosis conveyed to the patient. Critically, the response to therapy also greatly varies among cancers. In recent years, a wealth of insights have been obtained that link molecular features of the tumor to the efficacy of, in particular, target therapies. In colorectal cancer, these agents mainly involve antibodies that target the VEGF and the EGFR. Although for anti–VEGF-based regimens, predictors of therapy efficacy remain largely elusive, mechanisms of resistance to anti-EGFR therapy are abundant. The role of mutations in *KRAS* as a selection marker for anti-EGFR therapy has been extensively validated, but it is estimated that only 35% of *KRAS* wild-type tumors do respond to anti-EGFR therapy (1). The list of additional genetic and epigenetic characteristics that are associated with anti-EGFR therapy resistance, albeit sometimes only in preclinical models, is rapidly expanding and includes, among others, mutations in *BRAF* and *PIK3CA*, epigenetic silencing of *EGFR* expression, augmented expression of other receptor tyrosine kinases (RTK), including *MET*, and high expression of the MET ligand hepatocyte growth factor (HGF) in the tumor microenvironment (2, 3). Until it will be feasible to routinely obtain comprehensive molecular profiles of individual tumors, it will be challenging to determine the presence of all these modifiers of therapy efficacy in clinical practice. A potential approach to this problem is the introduction of a comprehensive molecular classification of colorectal cancer that integrates many aspects that contribute to cancer heterogeneity and predicts the efficacy of targeted agents. Recently, several groups have reported on such a taxonomy of colorectal cancer using gene expression data to identify distinct subtypes of this disease (4–10). Indeed, in some cases, these subtypes differ in response to anti-EGFR therapy (4, 10). Intriguingly, individual molecular features or mutations cannot identify the molecular colorectal cancer subtypes. We propose that these classifications will be very valuable in stratifying biologic subgroups with a more homogeneous biology that can help to identify patients that benefit from particular targeted agents. This will not only prevent overtreatment and adverse effects in patients but also helps to reduce healthcare costs. Furthermore, the novel molecular stratifications will facilitate development of new targeted agents. Herein, we will mainly focus on anti-EGFR therapy in colorectal cancer, but the principles can be generalized to many other cancer types and targeted agents.

## Cancer Heterogeneity and Its Origins

That no two tumors are alike is a well-known fact. There are many aspects of cancer biology that affect the detected heterogeneity. Naturally, the organ in which the cancer develops as well as the particular cell type within the organ that transforms influences the resulting phenotype of the tumor greatly. In addition, the presence (or absence) of individual (epi-) genetic aberrations affects tumor features considerably although the relationship between individual alterations and clinical behavior is not always very clear cut. For example the presence of *KRAS* mutations is associated with a slightly worse prognosis although large patient

series were required to draw this conclusion, indicating that the relevance for the individual patient is limited (11). Furthermore, microsatellite instable (MSI) colorectal cancers generally have a better prognosis and relatively rarely metastasize, yet around 5% of metastatic colorectal cancers are MSI (12). Often the combination of several genomic aberrations relates much stronger to the clinical presentation of the disease. A clear example of this notion is that *BRAF* mutations, in general, only impact modestly on disease outcome; however, when combined with MSI status, the effects are much more marked and the relatively rarer *BRAF*-mutant microsatellite stable cancers have dismal prognosis (11). Additional characteristics of tumor development and host factors that impact on colorectal cancer heterogeneity include the order in which mutations occur, the particular nondriver mutations present, genetic polymorphisms of the host, the actual cell of origin, external influences such as diet, the polyclonal composition of the tumor, the properties of the immune response, the contents of the intestinal microbiome, and many more (13–15). Furthermore, colorectal cancers that occur in a context of an inflammatory condition (colitis) have distinct molecular features, for example, frequent *TP53* mutations, and display specific clinical behavior with a poorer disease outcome (16). Interestingly, in breast cancer, the major disease subtypes are closely related to various stages of the tissue hierarchy with, for example, Claudin-low breast cancers resembling primitive mammary stem cells, basal-like cancers early (bi-potent) progenitors and luminal breast cancers differentiated luminal epithelial cells (17). It is commonly believed that these parallels might be related to distinct cells of origin although this needs to be formally demonstrated (17, 18). Compared with the impact of mutations and the organ giving rise to the tumor, these contributing aspects to the functional and phenotypical properties of the resulting tumor are much less well characterized, but there is no reason to assume that their influence is marginal.

## Colorectal Cancer Heterogeneity and Predictors of Response

Traditionally, colorectal cancer is classified on the basis of histologic characteristics (e.g., differentiation grade) and tumor stage. Increasingly, molecular features including deficiency in mismatch repair genes leading to MSI and individual mutations (e.g., *KRAS*) are assessed, as they have been demonstrated to relate to therapy response. For example, MSI tumors do not benefit from 5-fluorouracil (5-FU) containing regimes in the adjuvant setting, and *KRAS*-mutant colorectal cancers are resistant to antibodies targeting the EGFR (1, 19). Unfortunately, these classifications leave much of the heterogeneous responses to therapy unanswered. Indeed, more recent insights suggest that not all activating *KRAS* mutations provide equal resistance to anti-EGFR agents, and furthermore that other mutations can also convey resistance to this therapeutic modality. For example, mutations in the genes encoding the downstream KRAS effectors BRAF and PIK3CA are associated with a lack of cetuximab response (2). Moreover, gene expression signatures that correspond to *KRAS*, *BRAF*, and *PIK3CA*-activating mutations predict efficacy of anti-EGFR therapy, suggesting that it is a shared downstream component of these pathways that mediates the resistance (20). Recently, several other modes of

resistance against anti-EGFR therapy have been detected that involve direct mutations within *EGFR* that impair binding of cetuximab, thereby preventing its effect (21). Interestingly, tumors that harbor these mutations still respond to panitumumab as this binds to a distinct epitope of the molecule. Furthermore, silencing of EGFR by *EGFR* gene methylation has been reported to underlie the failure of anti-EGFR antibody therapy (3).

Following increased interests in the role of the microenvironment in cancer biology, it was discovered that signals produced by either the cancer cells themselves, or by stromal fibroblasts, such as HGF in some tumors, activate parallel RTK pathways that render colorectal cancer cells insensitive to anti-EGFR therapy (22). This notion was further supported by the finding that the level of serum HGF relates to the efficacy of this treatment modality in patients with *KRAS* wild-type disease (23). In addition to HGF, overexpression of insulin-like growth factor I produced by cancer cells itself has been suggested to mediate resistance to cetuximab (24). Intriguingly, besides stimulation of parallel RTK pathways by ligands, also amplifications and upregulation of alternative RTKs convey resistance to anti-EGFR therapy as the case of HER2 shows (25).

The variety of resistance mechanisms reveals that it will be extremely difficult to develop biomarkers that can be routinely used in clinical practice to identify patients that are likely to benefit from anti-EGFR therapy. In fact, even comprehensive genetic profiles of the cancer will not reveal all resistant patients, as expression of microenvironmental factors also influences response. In addition, it was recently revealed that the inefficiency of BRAF inhibitors in most $BRAF^{V600E}$-mutant colorectal cancers is due to activation of the EGFR–PI3K pathway in colorectal cancer cells (26). This feedback loop is not present in melanoma cells harboring the same mutation, thereby explaining the disparity in efficacy of these inhibitors. This example illustrates that the specific and complex context in which mutations occur, in this case cell type, greatly affects the effects of these mutations and their influence on the response of targeted agents. Therefore, we propose a less mutation-centered view of patient stratification to identify patients with cancer who are likely to benefit from particular therapeutic interventions.

## An Integrative Approach: Molecular Cancer Subtypes

Recently, following developments in other cancer types, including breast cancer, pancreatic cancer, and brain cancer, we developed an unbiased classification of colorectal cancer using whole-genome gene expression data. We identified three main colon cancer subtypes (CCS) that are each characterized by distinct (epi-)genetic and clinical properties (4). CCS1 tumors are characterized by mutations in *KRAS* and *TP53* genes, display a high activity of the Wnt signaling cascade, and show evidence of marked chromosomal instability (CIN). CCS2 cancers are strongly enriched for MSI/CpG island methylator phenotype (CIMP)-positive colorectal cancers with an immune cell rich infiltrate that are often located in the ascending colon (right sided). To conclude, CCS3 tumors consist of both MSI and CIN tumors, but are enriched for *BRAF* and *PIK3CA* mutations and display a mesenchymal phenotype. This molecular classification sustained

throughout patient-derived xenografting (4). Although CCS1 and CCS2 tumors are characterized by a fairly good disease outcome, CCS3 tumors have a dismal prognosis (Fig. 1). To explore the origins of the various subtypes, we studied the relation of the subtypes to common preneoplastic lesions in the intestine. We found that CCS1 tumors display a strong adherence to tubular adenomas and CCS3 cancers share an extensive gene expression program with sessile serrated adenomas, suggesting a role of this alternative route to colorectal cancer in the genesis of these tumors. A number of groups have reported on similar classifications of colorectal cancer using comparable strategies (5, 7–10). These additional taxonomies have been established on the basis of their customized bioinformatic analysis on alternative datasets and interpretation from different perspectives (27). In these studies, the number of subtypes ranges from 3 to 6, but evident relations between the subtypes can be detected (Fig. 1; ref. 28). These molecular subtypes do show overlap with previous used classification systems (MSI, CIMP, CIN) or mutation status (BRAF), but cannot be identified only based on single mutations or (epi-)genetic features (Fig. 1).

Currently, much needed efforts are under way to obtain a widely carried consensus on colorectal cancer subtypes to facilitate implementation in clinical practice and in prospective trials. Critically, in this effort, a balance needs to be struck between presentation of a comprehensive classification of molecularly distinct colorectal cancer subtypes, which can be very many, and clinical relevance and applicability, which favors a rather limited number of disease categories.
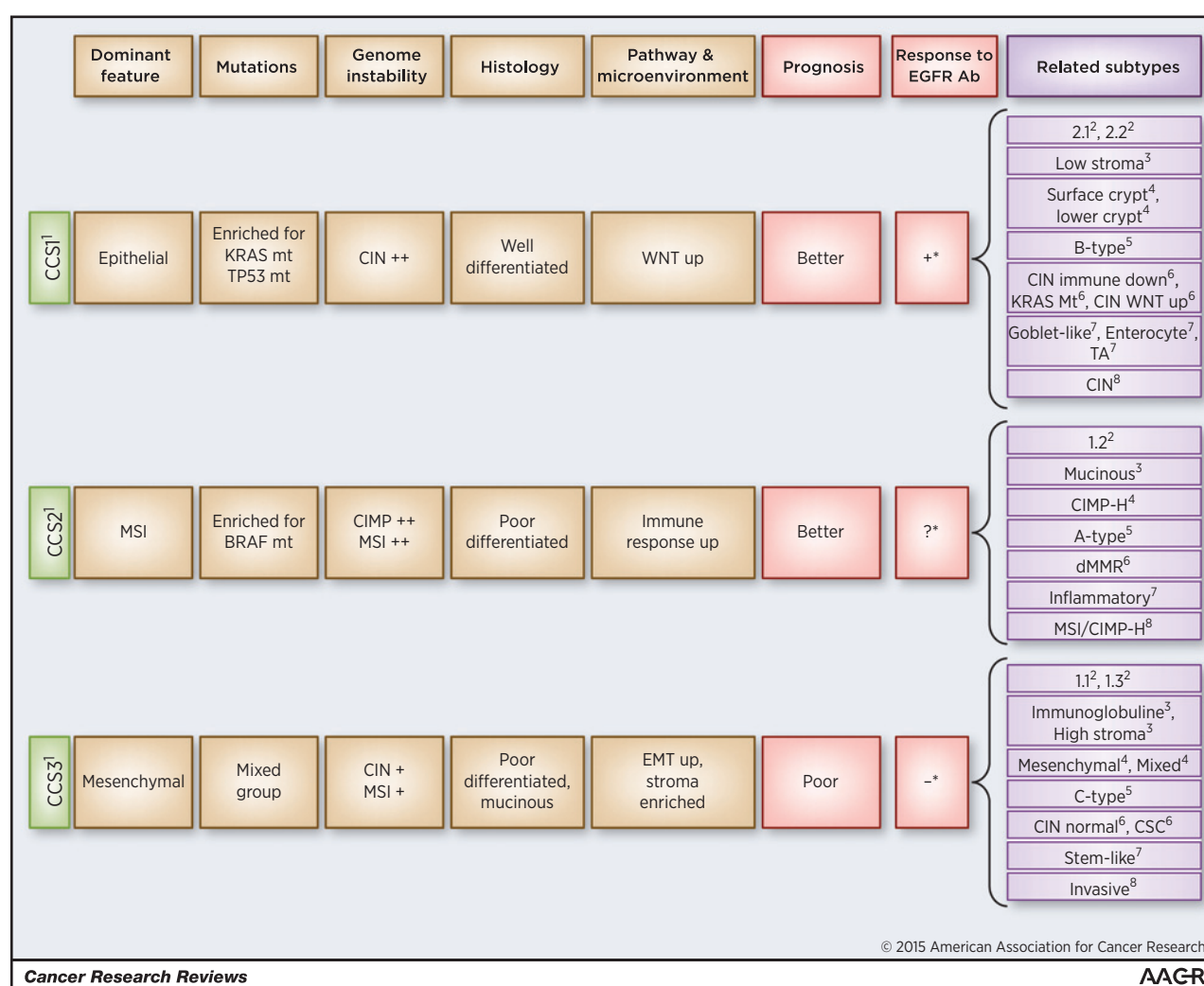


**Figure 1.**
Summary of the molecular classification of colorectal cancers as reported in De Sousa E Melo et al. (4). Although the groups cannot be identified by a single mutation or molecular feature, associations exist and are shown in orange. The red boxes represent the prognostic properties and the detected response to targeting agents of the distinct subgroups in retrospective analysis. Other groups have reported on similar taxonomies that show a great overlap with the groups we found. These relations based on similar associations with key molecular properties are illustrated in the violet column. dMMR, deficient mismatch repair; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition. [1]De Sousa e Melo et al. (4); [2]Schlicker et al. (5); [3]Perez-Villamil et al. (6); [4]Budinska et al. (7); [5]Roepman et al. (8); [6]Marisa et al. (9); [7]Sadanandam et al. (10); [8]TCGA (37). *, CCS1 are more sensitive toward monotherapy with EGFR-targeting agent cetuximab compared with CCS3 tumors independent of KRAS mutational status. CCS2/MSI tumors infrequently metastasize.

To date, most studies identifying colorectal cancer subtypes mainly focus solely on gene expression data. However, in breast cancer, for example, an increased molecular subtype resolution with clinical relevance has also been obtained by including copy number variation data in the classification algorithm (29). Furthermore, efforts emerge that use long noncoding RNA expression to identify colorectal cancer subtypes (30). It remains unresolved, however, how these subtypes relate to mRNA-based classifications.

## Disease Subtypes and Response to Targeted Agents

The colorectal cancer disease subtypes that have been identified, besides being prognostic, also predict response to targeted therapies. For example, tumors belonging to the CCS3 subtype demonstrate resistance to cetuximab monotherapy in metastatic colorectal cancer (4). Intriguingly, this effect was independent of KRAS mutational status, suggesting that the more comprehensive classification of cancers can contribute to predicting therapy sensitivity regardless of mutational status. Furthermore, also the tumor classification presented by Sadanandam and colleagues comprehends a molecular subtype that is resistant to anti-EGFR therapy (10). In addition, in this study, a further subdivision of one of the subtypes was proposed in which a previously detected gene set that predicts anti-EGFR therapy efficacy in colorectal cancer could identify patients with high anti-EGFR response with very high accuracy. This finding indicates that when specific molecular features are critically predicting therapy sensitivity, such as mutations, these are potentially subtype specific or might be more explicit predictors within molecular subtypes rather than on the population as a whole. However, it remains unknown how the other mutations that have been associated with therapy resistance, like BRAF and PIK3CA, are represented in the nonresponsive subgroups and to which degree the resistance of these subtypes can be explained by the presence of aforementioned mutations.

Besides anti-EGFR therapy, other agents have also been proposed to be specifically active within particular colorectal cancer subtypes such as detected within the studies described above using genome-wide gene expression data. These include, for example, anti-c-Met therapy to be predominantly effective in the cetuximab-resistant transit amplifying (CR-TA) subtype and FOLFIRI (a combination of irinotecan, 5-FU, and leucovorin) to be especially active within the poor-prognosis stem-like subtype (10). It will be critical, however, to validate these observations in larger retrospective studies even before prospective clinical trials can be started on the basis of these findings. Classification of cell line panels such as from the Sanger institute and Astra Zeneca, which have been studied and profiled in large pharmacogenomics studies, within the molecular subtypes such as detected in patients, identified further subtype-specific vulnerabilities. These include, for example, the sensitivity of the MSI, mesenchymal subtype 1.2 to Src inhibitors, and the good-prognosis, Wnt active subtype 2.1 to Aurora kinase A inhibitors (5). However, these interesting observations need to be further tested in preclinical models.

Molecular subtypes detected in other cancer types are also reportedly predictive of therapy response. For example, trastuzu-

mab, an antibody against HER2, is traditionally only used in patients with breast cancer with an amplification of the HER2 gene (31). More recently, however, no significant association between HER2 copy number status and benefit could be detected (32). In addition, not all HER2 amplified tumors respond equally to trastuzumab (33). This suggests that trastuzumab response might not be HER2 specific and that other tumor characteristics are involved. Again, more comprehensive molecular subtype analysis might overcome the shortcoming of single gene assessment. Indeed, breast cancer cell lines representing the luminal subtype growth can be blocked by trastuzumab, even in lines that do not display HER2 amplification (34). In other preclinical studies, response to targeted agents was also shown to be breast cancer subtype specific (35). Finally, in pancreatic ductal adenocarcinoma (PDA), an unbiased classification approach using gene expression data identified three molecularly distinct subtypes with clinical significance (36). Also, in this case, marked distinct responses to therapeutic interventions were detected. Of note, the "classical" PDA subtype with frequent KRAS mutations was demonstrated to be particularly sensitive to EGFR inhibition using erlotinib (36). This supports the notion that KRAS mutation status is an imperfect determinant of anti-EGFR therapy and that identification of subtype-specific vulnerabilities is a promising way forward. Clearly, molecular-based classification as a tool for treatment design has to be further investigated in prospective trials before it can be implemented in clinical practice.

## Outlook

In the future, it is expected that development of novel targeted therapeutic agents will take into account the extensive heterogeneity that is present in colorectal cancer. This could be achieved by performing vulnerability screens within series of cell lines or primary cultures representative of the various subtypes rather than on a cell line panel comprising the whole range of colorectal cancer subtypes and stratified on the basis of (individual) genetic aberrations. In this respect, it is interesting to note that the colorectal cancer subtypes are so diverse that the number of differentially expressed genes is >30% of all the genes expressed. In comparison, the number of differentially expressed genes between colorectal cancer and breast cancer approaches a similar number (L. Vermeulen and X. Wang; unpublished data). This fact stresses the notion that the molecular subtypes such as detected in colorectal cancer, most likely benefit from different therapeutic regimes, in a similar fashion as patients with colorectal cancer benefit from other therapies than patients with breast cancer. Indeed, molecular colorectal cancer subtypes should be considered distinct disease entities and novel therapeutic modalities should be developed and tested in clinical trials as such.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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