Tumor Heterogeneity in Colorectal Cancer: What Do We Know So Far?

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Keywords

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Abstract

Colorectal cancer is not one disease but rather a collection of neoplastic diseases. Due to heterogeneity in the disease biology, therapy response, and prognosis, extensive disease stratification is required. Therefore, TNM stage, microsatellite status, tumor grade, lymphovascular invasion, and other parameters are assessed in the pathology report to indicate the extent and prognosis of the disease. The mutation status of *KRAS*, *BRAF*, and *NRAS* is also investigated in a metastatic context to predict the response to anti-EGFR therapy. Recently, 4 distinct molecular subtypes of colorectal cancer have been described that have both prognostic and therapeutic relevance. In addition, characterization of the inflammatory infiltrate revealed major differences in the amount and location of inflammatory cells in distinct colorectal tumor types. Together, all of these parameters help to stratify patients into different therapeutic and prognostic subgroups. However, this stratification is not unambiguous since tumors often display intratumoral heterogeneity, whereby several subpopulations within one tumor show differences in morphology, inflammatory infiltrate, mutational

status, or gene expression profile. This article gives an overview of all of the current known data with regard to tumor heterogeneity at both inter- and intratumoral levels.

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Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men [1]. The incidence rate worldwide amounts to nearly 1.4 million cases, with more than 90% of new cases diagnosed in adults aged 50 years and older. The incidence rates vary among different regions of the world, with a 10-fold higher incidence rate in Western countries than in developing countries. This wide geographical variation indicates that environmental factors (e.g., meat consumption, smoking habits, and alcohol consumption) might play a key role in the etiology of CRC. Inverse associations include vegetable consumption and physical activity. Other well-established risk factors for developing CRC are: age, a family history of CRC, a personal history of cancer (e.g., colon, rectum, ovary, and endometrium), chronic inflammatory bowel diseases, and a history of polyps in the colon.

The TNM classification system, designed by the American Joint Committee on Cancer and the Union for International Cancer Control, remains the most robust prognostic parameter to stratify patients. The TNM system describes the depth of tumor invasion throughout the wall of the colon (T), the number of affected lymph nodes (N), and the presence of metastasis (M) [2]. Based on the T, N, and M classifiers, tumors are divided into 4 different stages with different prognostic and therapeutic consequences. Stage 1 tumors have a 5-year survival rate of 94%. Stage 2 tumors have a 5-year survival rate of 82%, which drops to 67% for stage 3 patients. Metastatic or stage 4 colon cancers have a 5-year survival rate of only 11%. The therapeutic strategy is highly dependent on the cancer stage at time of diagnosis. Surgery is considered a first-line treatment for stage 1, 2, and 3 tumors, and it is followed by adjuvant chemotherapy for stage 3 tumors and stage 2 tumors with adverse risk factors. In stage 4 tumors, treatment options for resectable metastases include neoadjuvant chemotherapy, metastectomy, and colectomy. If extensive, unresectable metastases are present, palliative chemo(radio)therapy can be considered for symptom relief. Despite advancements in the treatment of CRC, survival rates remain highly variable for different patients, even within the same TNM stage. During the last decade it became clear that these differences in oncological outcome can be explained at least partly by differences in tumor biology, and that CRC should be considered a highly heterogeneous and dynamic disease characterized by multiple molecular pathways throughout its evolutionary process [3]. Recent insights into the genomic differences in CRC and the emerging concept of tumor heterogeneity led to a shift in the treatment paradigm towards the use of personalized medicine.

Tumor heterogeneity has been subject of many recent studies and remains a hot topic in cancer-related research. Tumor heterogeneity can be divided into intertumor and intratumor heterogeneity. Intertumor heterogeneity comprises differences in tumors of the same histologic type between patients, and, more rarely, differences in synchronic colorectal tumors occurring within a single patient. Intratumor heterogeneity can be subdivided into spatial and temporal heterogeneity [4]. Spatial heterogeneity refers to differences that can be observed within a single tumor, i.e., either different genetic subpopulations within the primary tumor site or differences between the primary tumor and its metastatic lesions [5, 6]. Temporal heterogeneity refers to the dynamic nature of CRC, with genetic alterations developing within individual tumors over time.

Tumor heterogeneity has been investigated on different levels including genomics, transcriptomics, histopathologic features, and characterization of the inflammatory infiltrate. In this review, we provide an overview of the current knowledge regarding intertumor and intratumor heterogeneity in CRC and discuss the prognostic value of different biomarkers and the influence of tumor heterogeneity on treatment decisions.

Intertumor Heterogeneity

CRC differ on various levels, resulting in differences in prognosis and therapeutic response, even for cancers with the same TNM stage. These differences highlight the need for robust classification systems in order to stratify colorectal tumors into groups with direct therapeutic and prognostic consequences. Diagnostic work-up protocols in CRC include TNM staging, analysis of histopathologic markers, and microsatellite instability (MSI) analysis. In a metastatic context, *KRAS*, *BRAF*, and *NRAS* mutation analysis is added to the pathology report, since this has direct therapeutic implications.

Histopathologic Heterogeneity

World Health Organization Subtypes

The World Health Organization (WHO) defines different subtypes of CRC based on their histopathologic appearance. Tumors are assessed as glandular if gland formation is observed without excessive mucus production (Fig. 1a). When mucus production occurs in more than 50% of the tumor mass, the tumor is designated a mucinous carcinoma (Fig. 1c). These distinct histopathologic features led to the hypothesis that mucinous carcinomas develop along a distinct oncogenic pathway. Mucinous carcinomas, which account for 10% of all CRC, are predominantly located in the right colon, and high rates of MSI, *BRAF*, *KRAS*, and *PIK3CA* mutations have been reported. The prognostic outcome of mucinous tumors is somewhat controversial. Mucinous morphology was initially considered a negative prognostic marker; however, when corrected for tumor stage, no difference in survival rates between glandular and mucinous tumors was observed. The prognosis of mucinous carcinomas in the rectum remains highly controversial, with low response rates to neoadjuvant chemo(radio)therapy and high rates of incomplete surgical resection [7, 8].

Medullary carcinomas are poorly differentiated and characterized by the presence of solid sheets or nests of malignant cells with pushing borders, prominent intraep-

Fig. 1. Histologic subtypes observed in colorectal cancer. **a** Adenocarcinoma with formation of glandular structures. **b** Medullary carcinoma, characterized by a solid growth pattern, without gland formation. **c** Mucinous carcinoma with the presence of large extracellular mucin pools that comprise more than half of the tumor

mass. Stripes of tumor cells float in these mucin pools and partially line the mucin pools. **d** Signed ring cell carcinoma. This epithelial malignancy is characterized by the proliferation of signet ring cells with intracellular mucin pools that displace the nucleus to the side. There is no evidence of gland formation in this lesion.

ithelial lymphocytic infiltration, and no to rare gland formation (Fig. 1b). The incidence rate of this relatively new histologic type has not been well established, but it has been observed that medullary carcinomas are rare tumors, constituting approximately 5–8 cases for every 100,000 colon cancers. Similar to mucinous carcinomas, medullary carcinomas are more prevalent in the right colon and strongly associated with MSI (in up to 60% of cases) and *BRAF* mutations (in approximately 85% of cases) [9]. Medullar differentiation is considered a favorable prognostic marker in CRC and it is often associated with a lack of nodal positivity or extramural invasion. The lymphocytic infiltration in medullary carcinomas promotes an antitumoral inflammatory response that prevents tumor spreading and the occurrence of micrometastases. The loss of *CDX2* and cytokeratin-20 expression indicates a loss of the intestinal phenotype, which has a prominent role in the development of medullary carcinomas [10].

Signet ring cell differentiation is another rare histologic subtype which accounts for less than 1% of colorectal carcinomas [11]. It is characterized by proliferation of signet ring cells with intracellular mucin pools that push the nucleus to the periphery (Fig. 1d). Signet ring cell tumors are generally diagnosed in younger patients, and an association with the right colon and MSI status has been observed in up to 40% of cases. Signet ring cell carcinomas display heterogeneous morphological patterns, distinct from signed ring cell carcinomas in other parts of the gastrointestinal tract, and can be subdivided into tumors surrounded by large amounts of mucin (mucin-rich signet cell carcinomas) and the mucin-poor type. These mucin phenotypes gained attention in recent literature because of the possible clinicopathologic and prognostic implications. Mucin-rich signet cell carcinomas are strongly associated with MSI in up to 40% of cases and display a better overall survival than mucin-poor types [12]. Mucin-poor types follow an aggressive clinical

Table 1. Prognostic value of histologic markers in colorectal cancer [17]

Category	Definition	Histologic markers
1	Markers with proven prognostic value and already integrated into routine pathology reporting	Local tumor extent (T) Nodal involvement (N) Distant metastasis (M) Tumor deposits Lymphovascular invasion Residual tumor
2A	Extensively studied markers with a prognostic value sufficient for pathology reporting; however, clinical relevance is not established	Tumor grade Circumferential margin Tumor regression grade after neoadjuvant therapy
2B	Prognostic value is promising in multiple studies but not sufficient for category 1 or 2A	Histologic type Tumor infiltrating lymphocytes Tumor border configuration Perineural invasion
3	Markers not sufficiently studied to determine their prognostic value	Peritumoral fibrosis and inflammatory response Microvessel density Proliferative activity
4	Markers proven to have no prognostic value	Tumor size Gross tumor configuration

course and a distinct pattern of metastatic progression compared to glandular tumors. Signet ring cell tumors have the tendency to metastasize at an early stage and to multiple sites such as the liver, ovaries, and peritoneum, while metastases of glandular tumors mostly metastasize to the liver only [7, 8].

The different histologic WHO subtypes illustrate the heterogeneity of the histopathologic features observed in CRC. Histologic subtypes gained interest in recent literature because they are believed to result from different developmental pathways and may predict the treatment response. While several studies have addressed the prognostic value of different histologic subtypes, few studies exist about their role in therapeutic decision making. With the exception of mucinous carcinomas, only limited data is available regarding the response rates to chemotherapy for different histologic subtypes [13]. Recent studies concerning the use of tumor histology in treatment decisions have shown that medullary histology is an independent predictor of programmed cell death ligand (PDL)-1 expression in tumor cells [14]. Programmed cell death 1 (PD-1) and its ligand (PD-L1) suppress the CD8 cytotoxic immune response and weaken the antitumoral immune response. Anti-PD-1 and PD-L1 agents are considered potential targets for cancer therapy and they are the subject of several ongoing clinical trials. However, the

prognostic value of PD-L1 expression in colorectal carcinomas remains controversial, with conflicting results being published [15, 16].

Furthermore, integration of the use of different histologic subtypes in the optimization of treatment protocols would provide a readily accessible marker, since it can be observed directly on hematoxylin-and-eosin-stained slides. Pathologists should be encouraged to mention the histologic subtype in each pathology report, and further research, in a multidisciplinary setting, is required to establish the prognostic and therapeutic consequences of different histologic subtypes.

Histopathologic Biomarkers

Several histologic biomarkers that contribute to the biologic behavior of colorectal tumors, and thus contribute to tumor heterogeneity, have been identified. In 1999, the College of American Pathologists summarized and grouped all of these histologic markers into 4 categories depending on their proven prognostic value (Table 1). Category 1 comprises all markers that have been proven to be prognostic based on large cohort studies and are already integrated into routine practice. Category 2A includes all markers that have been extensively studied and have shown to be of prognostic or predictive value in multiple statistically robust studies. These markers are of

Table 2. Different prognostic groups in colorectal cancer staging based on the TNM classification system [2]

Stage	Definition
0	Carcinoma in situ: invasion confined to the mucosal lamina propria (Tis)
1	Invasion of the submucosa (T1) or muscularis propria (T2)
2A	Invasion through the muscularis propria (T3)
2B	Invasion of the visceral peritoneum (T4a)
2C	Invasion of other organs or structures (T4b)
3A	– T1 or T2 tumor with the presence of metastasis in 1–3 regional lymph nodes (N1) – T1 tumor with the presence of metastasis in 4–6 regional lymph nodes (N2a)
3B	– T1 or T2 tumor with the presence of metastasis in 7 or more regional lymph nodes (N2b) – T2 or T3 tumor with the presence of N2a metastasis – T3 or T4a tumor with the presence of N1 metastasis
3C	– T3 or T4a tumor with the presence of N2b metastasis – T4a tumor with the presence of N2a metastasis – T4b tumor with the presence of N1 or N2 metastasis
4A	Presence of metastasis confined to one organ without peritoneal metastases
4B	Presence of metastasis in more than one organ
4C	Presence of metastasis to the peritoneum with or without other organ involvement

sufficient prognostic value to be integrated into pathology reporting, but their importance for clinical care has not yet been validated in large patient cohorts. Category 2B includes all markers that have been shown to be promising in multiple studies but without sufficient evidence for inclusion in category 1 or 2A. Markers in category 3 have not yet been sufficiently investigated to demonstrate a prognostic or predictive value. Category 4 comprises all extensively studied markers that lack prognostic significance [17, 18].

TNM staging, a category 1 prognostic marker, is widely used and accepted to stratify patients in different prognostic groups (Table 2). The presence of lymphovascular invasion, another category 1 marker, is a negative prognostic marker in CRC and it is defined as the presence of tumor cells in the lymphatic system and vascular structures. These tumor cells are considered to initiate lymph node involvement and distant metastasis [19, 20].

Tumor grading, a category 2A marker, is routinely assessed in pathology reporting. Colorectal tumors can be graded as well, moderately, or poorly differentiated based on the percentage of gland formation. Well-differentiated tumors show glandular structures in more than 95% of the tumor mass (Fig. 2a). Moderately differentiated tumors show glandular differentiation in 50–95% of the tumor mass, and poorly differentiated tumors display a loss of glandular structures in at least 50% of the tumor mass (Fig. 2b). Poorly differentiated tumors have a worse prognosis than moderately and well-differentiated tumors (Fig. 2c) [21]. Histologic grading can only be accurately interpreted in surgical resection specimens due to its heterogeneous nature. Therefore, preoperative tumor grading is not accurate and its role in preoperative treatment decision making is restricted [22].

Tumor budding, first described in 1954, has been reported as a negative prognostic marker in CRC. Depending on the study cited, tumor budding is observed in 20–40% of the diagnosed CRC cases. The definition of tumor budding and the method to score tumor budding were subject of change [23]. Today, tumor budding is defined by the presence of single cells or small groups of up to 5 tumor cells at the invasive border of the tumor (Fig. 3). The presence of 5 or more tumor cells lacking a gland-like structure is defined as a poorly defined cluster [24]. The “10-high-power-field (HPF) method” is the preferred scoring mechanism and it is used in pre-keratin-stained sections [25, 26]. In this method, the invasive margin of the tumor is scanned at a low magnification to find the densest budding region, and subsequently all tumor budding foci in 10 HPF within this region are counted. Tumors are classified as high budding if there are more than 10 budding foci per HPF. By counting multiple fields, this method has the advantage of assessing the heterogeneity observed in tumor budding and there is evidence of a high interobserver correlation. However, in 2016, the International Tumour Budding Consensus Conference recommended the use of the “hotspot method” for tumor budding assessment [27]. In this method, tumor bud counts are performed in a single HPF with the highest density of tumor buds. The hotspot method is considered to reflect the maximal extent of tumor budding compared to counting multiple fields, which might “dilute” the counts in cases with local high-budding foci. On the other hand, the 10 HPF method is more representative of the entire invasive border.

Tumor budding is considered to be the first step in cancer metastasis, as budding cells are thought to migrate through the extracellular matrix, invade lympho-

Fig. 2. Assessment of tumor grading in colorectal cancer. **a** Well-differentiated adenocarcinoma with formation of glands in more than 95% of the tumor mass. **b** Moderately differentiated adenocarcinoma with formation of glands in 50–95% of the tumor mass. **c** Poorly differentiated adenocarcinoma that consists of sheets of cells with formation of glands in less than 50% of the tumor mass.

Fig. 3. Tumor budding in colorectal cancer. This hematoxylin-and-eosin-stained section displays budding tumor cells, which detach from the main tumor at the invasive border and invade the extracellular stroma.

vascular structures, and form metastatic tumor colonies in lymph nodes and at distant sites and therefore reflect a poor prognosis. Tumor budding is strongly associated with the presence of poorly differentiated clusters and it is suggested to represent a histologic spectrum of the same biological process. A recent study demonstrated that tumor budding and poorly differentiated clusters are stage-independent predictors of the clinical outcome in mismatch repair (MMR)-deficient CRC [28]. However, the role of tumor budding in the prediction of response rates to therapeutic treatment has not been established.

Molecular Heterogeneity

Heterogeneity in Pathogenesis

There are 3 major pathways in the development of CRC: chromosomal instability (CIN), CpG island methylation phenotype (CIMP), and MSI [29, 30]. CIN tumors are characterized by numerical and structural chromosomal aberrations and mutations in different proto-oncogenes and tumor suppressor genes such as the adenomatous polyposis coli (*APC*) tumor-suppressor gene, the Kirsten-ras (*K-ras*) proto-oncogene, the p53 tumor suppressor gene, and the deleted in colon cancer (*DCC*) tumor suppressor gene [31]. CIMP CRC are characterized by widespread methylation of CpG islands in promotor regions, resulting in inactivation of tumor suppressor genes such as *MLH1*.

MSI Status

The MSI pathway, which accounts for 15% of sporadic CRC cases, is characterized by a deficient MMR system, which causes genomic instability at the microsatellite level. Microsatellites are short repetitive DNA sequences scattered throughout the genome. Because of their repetitive nature, DNA polymerases slip easily in these regions, and they are prone to mutations and mismatch. The MMR system consists of 6 enzymes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, and *PMS2*) that detect and excise the wrongly inserted base pair with the help of exonuclease enzymes. DNA polymerase and DNA ligase will help to resynthesize and relegate the DNA strand to correct for the mismatch. Defects in the MMR system cause inadequate DNA repair, which results in high levels of DNA replication errors, shortening or lengthening of these microsatellites, and the production of frame shift peptides. In a sporadic context, MSI tumors are charac-

terized by hypermethylation of one of the MMR genes, whereas in a familial context (e.g., Lynch syndrome) inactivating mutations are present in one of the MMR genes [32, 33]. Mutations in *BRAF* and promoter hypermethylation of the *MLH1* gene are often associated with CIMP [34].

Differences in genetic background are associated with a distinct clinical and histologic presentation. Compared to CIN tumors, MSI tumors are often located in the proximal colon and typically poorly differentiated, with high numbers of tumor-infiltrating lymphocytes and a peritumoral Crohn-like reaction. They are less prone to lymph node and distant metastases, resulting in a better prognosis [35]. Furthermore, the effect of 5-fluorouracil, irinotecan, and anti-VEGF treatment is expected to vary between MSI- and CIN-associated tumors. Due to the interplay between MSI and CIMP status, CIMP tumors are also associated with proximal tumor location and poor differentiation [36–39].

KRAS, NRAS, and BRAF Mutation Status

The family of RAS proto-oncogenes, including *KRAS*, *NRAS*, and *HRAS*, plays an important role as a downstream regulator of the epidermal growth factor receptor (EGFR), thereby regulating cell growth, proliferation, migration, and differentiation [40]. Forty to forty-five percent of CRC are characterized by mutations in the *KRAS* proto-oncogene, most frequently affecting codons 12 (G12D, 13%; G12V, 9%) and 13 (G13D, 8%) of exon 2 [15]. *NRAS* mutations, mutually exclusive with *KRAS* mutations, are only detected in 1–3% of all CRC. Activating *HRAS* mutations have not been reported in CRC [41]. Mutation of *KRAS* or *NRAS* results in a constitutive activation of the EGFR pathway and induces a malignant transformation of the cell. Since *KRAS* mutations are observed in both early and metastatic stages of CRC, *KRAS* is a candidate driver gene in the malignant transformation of colonic polyps. Some authors suggest that *KRAS* mutations drive the formation of aberrant crypt foci that lead to the formation of colonic polyps. Due to the heterogeneity of genetic alterations observed in CRC, *KRAS* mutations can only provide a partial explanation for tumor development.

In a metastatic setting, patients are often treated with anti-EGFR therapeutics (e.g., cetuximab and panitumumab). Since *KRAS* and *NRAS* are downstream actors of the EGFR pathway, mutations can diminish therapeutic efficacy. Multiple research groups observed that *KRAS*-mutated tumors respond poorly to anti-EGFR treatment and therefore anti-EGFR therapy is not indi-

cated in these patients [42, 43]. However, results should be interpreted cautiously since the addition of cetuximab to first-line chemotherapy seems to benefit patients with *KRAS* G13D-mutant tumors. There is increasing evidence that *KRAS* mutations form a heterogeneous group of mutations with distinct biological behaviors, and individual genotypes should therefore be considered separately [44, 45]. Furthermore, not all *KRAS* wild-type tumors benefit from anti-EGFR therapy since *NRAS* mutations also cause anti-EGFR resistance.

BRAF is another downstream actor of the EGFR pathway that might predict the response to anti-EGFR treatment. *BRAF* mutations are detected in 10% of all CRC and are mutually exclusive with *KRAS* mutations. Literature concerning the effect of *BRAF* mutation status on anti-EGFR therapy is conflicting, but a recent meta-analysis concluded that *BRAF*-mutated CRC do not benefit from anti-EGFR therapy. Therefore, the National Comprehensive Cancer Network recommends *BRAF* mutation analysis in *KRAS* wild-type tumors before starting anti-EGFR therapy in the metastatic setting [46, 47].

Molecular Profiling Studies

In 2013, multiple gene expression profiling studies on CRC were published to improve tumor classification and to select patients that benefit most from adjuvant therapy.

Sadanandam et al. [48, 49] analyzed the gene expression profiles of 1,290 colorectal tumors and classified them into the following five subtypes: (1) goblet-like, with a high expression of MUC2 and TFF3; (2) enterocyte-like; (3) stem-like, defined by active Wnt-signaling and a high expression of Wnt-target genes and mesenchymal genes; (4) inflammatory-type, defined by a high expression of chemokines, interferon-related genes, and a correlation to MSI status; and (5) transit-amplifying, a heterogeneous group with variable expression of stem cell markers and Wnt target genes. These subtypes showed a significant association with disease-free survival in untreated patients. Goblet-like and transit-amplifying type tumors had the best prognosis and inflammatory and enterocyte subtypes had an intermediate prognosis, whereas stem-like tumors had a significantly shorter disease-free survival [48, 49].

Simultaneously, De Sousa et al. [50] analyzed 1,100 CRC and identified 3 molecular subtypes. The first molecular subtype (CCS1) included CIN tumors, the second subtype (CCS2) correlated to the genetic group of MSI tumors, and a heterogeneous third subtype (CCS3) consisted of MSI-, MSS-, and CIMP-positive tumors. The CCS1-CIN group was characterized by chromosomal ab-

errations (e.g., loss of 18q) and mutations (e.g., *TP53*) and these tumors were predominantly situated in the left colon. CCS2-MSI tumors were mainly located in the right colon and they were characterized by high levels of CIMP. CCS3 tumors were evenly distributed throughout the colon and they were associated with *KRAS* or *BRAF* mutations. Furthermore, an association between CCS3 and sessile-serrated adenomas was reported, characterized by a high expression of genes involved in the process of epithelial-to-mesenchymal (EMT) transition. These subgroups significantly correlated with prognosis. CCS1 tumors showed the best survival rates, followed by CCS2, in contrast to CCS3 tumors, which had a significantly poorer survival with resistance to anti-EGFR therapy [50].

In May 2013, Marisa et al. [51] published data on 750 CRC, identifying 5 distinct molecular subtypes (C1, C2, C3, C4, C5, and C6) with unsupervised clustering. Tumors characterized by MSI and CIMP were categorized as C2, whereas CIN tumors were categorized as C1, C3, C4, C5, or C6. C1 tumors had a downregulated immune response; C3 tumors were characterized by *KRAS* mutations and CIMP positivity; C4 tumors were considered to have a stem cell phenotype; C5 tumors had active signaling through the Wnt-pathway; and C6 tumors were regular CIN tumors. These subtypes correlated to survival rates, with C4 and C6 both associated with a shorter relapse-free survival [51].

In July 2013, Roepman et al. [52] clustered the gene expression profiles of 188 CRC into 3 major subtypes (A, B, and C types), which were subsequently confirmed in a validation series of 543 colorectal carcinomas. A-type tumors were associated with a high-MSI status, *BRAF* mutations, and better overall survival. B-type tumors were CIN tumors with low mutation frequencies, and C-type tumors were correlated to an EMT-like gene expression profile and poor overall survival. A- and B-type tumors were highly sensitive to adjuvant chemotherapy in contrast to C-type tumors which showed resistance [52].

The study of Budinska et al. [53] (July 2013) on applying unsupervised clustering in 1,113 colorectal carcinomas identified 5 subgroups (surface crypt-like, lower crypt-like, CIMP-H-like, mesenchymal, and mixed) with prognostic and therapeutic relevance. The CIMP-H-like subtype (enriched for MSI tumors) and mesenchymal tumors had the poorest overall survival. For mesenchymal tumors this is due to early relapse and a high expression of EMT-associated genes. However, for CIMP-like tumors, which consist of a high percentage of MSI tumors, poor overall survival was an unexpected observation. Ac-

cording to the authors, this could be explained by short survival rates after relapse [53].

These different studies addressing multiple gene expression profiling in CRC all clustered CRC into 3–6 subtypes. Each research group defined a specific subgroup which consisted of MSI tumors characterized by high mutation frequencies, *BRAF* mutations, increased inflammation, and mostly a favorable outcome. Furthermore, they all defined a subgroup associated with poor overall survival and stem-like/EMT-like gene expression profiles. However, the existence of multiple molecular classifications, derived from different data sources and clustering approaches, hampered its integration into the routine clinical setting.

In 2015, all of these data sets were analyzed together and clustered by the CRC subtyping consortium, which resulted in 4 consensus molecular subtypes (CMS) with unique features and 1 subgroup lacking specific molecular characteristics [54]. MSI tumors, characterized by CD8 cytotoxic immune cell infiltration and high mutation frequencies, were grouped into CMS1. CMS2 tumors are CIN tumors with expression of epithelial markers and activation of the Wnt pathway. CMS3 tumors represent a heterogeneous subtype containing both MSI and MSS status, a high metabolic activity, and *KRAS* mutations. CMS4 tumors have high levels of copy number alterations and a mesenchymal-like expression profile, and they have the worst overall survival rates. Molecular subtyping of colorectal tumors represents a major step forward in CRC management. However, 13% of all CRC cases cannot be classified, resulting in a fifth subtype without clear molecular characteristics. This indicates the need for further refinement of the CMS classifiers. Further analysis of the colorectal genome and epigenome is required to increase the understanding of CRC biology and allow better disease stratification.

Inflammatory Heterogeneity

As described above, all of the currently used clinical stratification methods primarily focus on the tumor cell component. However, accumulating evidence suggests that CRC progression and recurrence are determined not only by genetic changes intrinsic to the tumor cells but also by a complex interplay between the cancer cells and the tumor microenvironment. The tumor microenvironment is composed of epithelial cells, blood and lymphatic vessels, stromal cells, and immune cells. The latter can have a tumor-suppressive effect and control tumor growth as defined by the first 2 steps in the immune editing theory or they can induce tumor progression by over-

ruling adaptive immune responses, stimulating the proliferation and survival of cancerous cells, and promoting angiogenesis and metastasis. The exact type of immune cells and the intratumoral localization are important factors for determining the effect on clinical outcome [55].

Different research groups have already demonstrated that MSI tumors are highly infiltrated by cytotoxic T cells, and it was suggested that the presence of tumor-infiltrating T cells might be a beneficial prognostic factor. Deschoolmeester et al. [55] found that intraepithelial infiltration of CD3+ and CD8+ T lymphocytes and stromal infiltration of CD3+ lymphocytes had a major impact on the patients' overall survival, independently of their association with MSI status [56, 57].

Galon et al. [58] found immunological data (type, density, and location of tumor cells within the tumor samples) to be a better predictor of patient survival than the histopathological methods currently used to stage CRC. Galon et al. [58] designed Immunoscore based on the quantification of T lymphocytes (especially cytotoxic and memory T cells) in the center and periphery of the tumor. In that study and additional studies conducted during the following years, Immunoscore proved to be a clinical useful prognostic marker in CRC patients [59, 60]. An international task force has been assembled to promote the implementation of Immunoscore [61]. More recently Galon et al. [61] found Immunoscore to be superior even to MSI in predicting patients' disease-specific recurrence and survival. In that paper he also suggested that the high mutational burden in MSI most probably influences the clinical outcome by increasing the presence and functionality of tumor-specific effector memory T cells. Additionally it was demonstrated that strong and effective antitumor immunity might naturally be elicited against true tumor-specific antigens resulting from somatic mutations. Taken together, this data suggests a role for mutation-specific vaccination, especially in MSI tumors high in frameshift mutations and as such high in neoantigens [62].

Becht et al. [63] correlated immune and angiogenic signatures to the CMS and found that the tumor microenvironment is associated with the type of tumor cells. The CMS1 subtype (linked to MSI status, *BRAF* mutations, and immune infiltration) is correlated to high levels of class 1 MHC genes, genes specific for cytotoxic T-cell activation, and genes that are involved in immune cell attraction and activation. Another feature of MSI tumors is that they have low myeloid and angiogenic signatures. In contrast, CMS4 tumors are characterized by a poor prognosis, TGF- β activation, stromal infiltration, and angio-

genesis. Stromal classification according to the MPC counter method identified a stromal gene signature for this CMS4 subgroup with high levels of cancer-associated fibroblasts, angiogenesis, and myeloid cells. This signature, already described in other tumor types, is expected to be prometastatic, explaining the poor survival rates. CMS2 and CMS3 tumors, described to be canonical and metabolic, respectively, are characterized by low levels of immune and myeloid signatures and a low MHC class 1 expression according to the MPC counter method, and they are not very immunogenic. This data supports testing of adoptive T-cell therapies in CMS2 and CMS3 colorectal tumors [63].

Recent advancements in the understanding of immune-oncology have also led to great improvements regarding immunotherapy. Checkpoint blockade therapy is a hot topic. These drugs are designed to counteract the tumor's ability to escape the immune system by inducing tolerance and suppressing immune responses directly and indirectly through physiological pathways that serve to maintain tissue homeostasis and prevent normal tissue damage and autoimmunity. The main targets of this strategy are CTLA4 and PD1 and its ligand, i.e., PD-L1. A first study on immune checkpoint blockade therapy in CRC was disappointing since a limited or no survival benefit was observed [64]. However, immune checkpoint blockade therapy is promising for MSI tumors [65], which can be explained by the immune signatures for CMS1/MSI tumors. Results from the MPC counter method suggest testing for antiangiogenic, anti-inflammatory, and immune checkpoint blockade therapies in CMS4 tumors [63].

Intratumor Heterogeneity

Intratumor heterogeneity is described as the presence of various clones within one tumor, each with its own unique features on the level of morphology, inflammation, genetics, epigenetics, or transcriptomics [63, 66, 67]. One study investigated the extent and consequences of intratumor heterogeneity in 12 cancer types, albeit without the inclusion of CRC. Across all cancer types studied, different subpopulations, each containing unique mutation profiles, were found. The presence of intratumor heterogeneity was linked to a favorable outcome, which has also been described in other papers concerning head and neck cancer and leukemia. This unexpected finding can be explained by the fact that the presence of numerous clones might boost the immune system and thus attract

more antitumoral immune cells that combat the cancer cells [68].

Intratumoral heterogeneity can develop via different evolutionary patterns. Fearon and Vogelstein [69] were the firsts to describe the adenoma-carcinoma sequence. This is a stepwise progression system in which more and more mutations accumulate, resulting in colorectal neoplasia that has the ability to metastasize. The stepwise progression system, based on a continuous gain of function, represents a linear or clonal evolution model. With the development of high-throughput sequencing systems, other evolutionary models were proposed, with more and more data supporting a neutral evolution model. This model entails that a tumor grows predominantly as a single expansion populated by numerous heterogeneous clones. Public mutations already present in the first transformed tumor cell will persist and thus be present in all tumor cells (clonal). Private mutations acquired early will become pervasive in the final tumor while remaining nondominant and thus generating subclones. Late arising mutations are only present in small regions of the tumor. The timing of a mutation, rather than clonal selection for that mutation, is the primary determinant of its pervasiveness. This could be explained due to the rapid expansion and spatial constraints. Intratumoral heterogeneity results from private mutations continuously accumulating due to replication errors [70].

Histopathologic and Inflammatory Intratumor Heterogeneity

Presence of intratumor heterogeneity on the level of histopathology has been documented to be more extensive in MSI compared to MSS tumors [55, 71]. In a study by De Smedt et al. [72], more than 50% of MSI tumors revealed a mixed morphology, defined as a combination of morphologic growth patterns, such as glandular, mucinous, or medullar, within one tumor. This feature of mixed morphology was only seen in 10% of the MSS tumors. Besides the presence of a morphologic growth pattern, variations in inflammatory infiltrate (T lymphocytes, T-helper lymphocytes, cytotoxic T lymphocytes, B lymphocytes, and macrophages) were also observed within one tumor. Again, these variations were more frequent in MSI compared to MSS tumors and are highly correlated with the morphologic growth pattern of the tumor. Briefly, glandular components (or clones) within MSI tumors were infiltrated by both intratumoral (immune cells that infiltrate the stroma surrounding the neoplastic glands) and intraepithelial (immune cells that infiltrate into the neoplastic glands) inflammatory cells. Medullar clones presented with high levels of intraepithelial in-

flammatory cells while the mucinous components were only infiltrated by few inflammatory cells [72]. Analysis by another research group validated these heterogeneous inflammatory signatures (including cytotoxic T cells, macrophages, and granzyme B granules) in MSI tumors.

Molecular Intratumor Heterogeneity

The data on molecular intratumor heterogeneity remains sparse. However, a few publications have investigated the mutation profile in different parts of the primary tumor and associated metastases. As already mentioned, Sottoriva et al. [70] investigated the evolutionary model of CRC development and emphasized the presence of intracryptal heterogeneity. Kim et al. [66] took multi-region biopsies from primary colorectal tumors and their associated metastases and performed whole-exome sequencing and copy number profiling. They demonstrated that most, if not all, driver mutations were present in all biopsies from the primary tumor and the associated metastases. This universal spread of driver mutations indicates that those mutations are early events during carcinogenesis and that they are required for cancer initiation. These results are positive for the administration of targeted therapies directed against the EGF receptor since the mutation status of *KRAS* and *BRAF* is mostly universal in primary tumors and metastases [66].

However, one should be careful not to overinterpret the findings of Kim et al. [66] since Losi et al. [73] found intratumor heterogeneity for the *KRAS* and *P53* mutations and a loss of 18q in $\pm 50\%$ of the samples tested. Furthermore, in all of the tumors tested a series of metastasis clonal mutations were found. Metastasis clonal mutations are mutations that are present in all of the biopsies of the metastasis and in at least one but not all of the biopsies derived from the primary tumor. These mutations are considered to occur later in the evolution of the colorectal tumor and give that clone a specific advantage for metastasis. Targeting these mutations might be a new strategy to prevent the formation of metastatic clones. However, it remains difficult to assess and compare percentages of intratumor heterogeneity in CRC since the level of intratumor heterogeneity decreases during cancer progression [73].

Heterogeneity in the Primary Tumor versus Metastases

High percentages of genomic similarity are observed between primary colorectal tumors and their associated metastases [66]. Brannon et al. [67] demonstrated that mutation analyses of *KRAS*, *BRAF*, and *NRAS* – which is now performed in daily clinical practice to determine ben-

efits from anti-EGFR therapy – revealed a 100% match of the results found in primary tumor and metastasis tissues. This finding is also applicable to other driver genes such as *APC*, *TP53*, and *PIK3CA*. However, passenger mutations are more often clonal, generating heterogeneity between the primary tumor and associated metastases [67]. With regard to microsatellite status, a high concordance in MSI status between primary tumors and their metastases was also observed [57]. Only in a limited number of cases was an MSS metastasis observed with an MSI primary tumor. Since MSI in a sporadic context is the consequence of epigenetic silencing of one of the MMR genes, it is highly plausible that differences in MSI status are possible for primary tumors and metastases. It is generally accepted that MSI is important for tumor initiation rather than for tumor progression. Therefore, MSI already manifests in early stages of cancer progression. This might also explain why a loss of MSI is seen in the metastasis of an MSI primary tumor, but a gain of MSI in a metastasis of an MSS tumor has not been observed so far [74].

Finally, it has to be emphasized that higher levels of heterogeneity between the primary tumor and its metastasis were observed for patients who did not undergo chemotherapy prior to surgery compared to patients who underwent a chemotherapy cycle before surgery. These results suggest that chemotherapy might decrease the levels of genetic heterogeneity [67].

Conclusion

CRC is a highly heterogeneous disease, and therefore extensive disease stratification that accounts for inter- and intratumor heterogeneity is highly required. It is important that the assessment of MSI status, TNM stage, *BRAF*, *KRAS*, and *NRAS* mutation status, lymph vascular invasion, tumor budding, and tumor differentiation be summarized in the pathology report. Together they give an indication of the disease behavior, disease progression, and the prognosis and guide therapeutic decisions. It is expected that the spread of the inflammatory infiltrate and the molecular subtype will be added to pathology reports in the future since this might hold a lot of information concerning the prognosis and the response to targeted and immune therapy. Unfortunately, big differences in outcome and therapeutic guidance are still observed in patients with the same disease stage and the same MSI status. This might be explained by the presence of intratumor heterogeneity. Intratumor heterogeneity implies the presence of distinct morphologic, inflammatory, genetic, or transcriptomic subclones within one tumor and this impacts the disease outcome and the therapy response.

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