Colorectal carcinoma molecular classification. A short review

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Abstract

Traditionally, carcinoma classifications have been based on clinical or pathological features. However, with the development of molecular biology in recent decades, more tumors are increasingly being genetically studied and, in several of them, molecular classifications have been created (the most widely studied and used is that for breast cancer). Colon and rectum cancer are no exception. In this short review, the evolution of colon and rectum cancer molecular classification is explained and the consensus conclusions on the subject are addressed.

KEY WORDS: Colon and rectum cancer. Molecular classification. Microsatellite instability.

Introduction

Traditionally, cancers have been classified based on clinical stage and histological characteristics. In colon and rectum cancer (CRC), prognosis has been shown to depend on local tumor involvement, regional lymph node metastasis, lymphovascular invasion, positive surgical margins, carcinoembryonic antigen preoperative elevation and tumor grade. However, treatment response is still difficult to predict in specific scenarios, particularly at the metastatic stage.¹ The International Union of Cancer Control and American Joint Committee on Cancer Tumor, Node, Metastasis (TNM) staging systems are the most commonly used for colorectal cancer.^{2,3} The TNM classification is a dual system and comprises a clinical or pretreatment classification and a pathologic or post-surgical one.⁴ Surgical decisions are based on clinical classification, whereas post-surgical management and prognosis are established using the pathologic classification.

No internationally standardized molecular classification has yet been implemented in CRC; however, there are different pathogenic aspects that might serve as a basis.⁵ With the advent of molecular high technology, numerous genomic, transcriptomic, proteomic and metabolomic data are being generating, which are facilitating cancer

comprehensive molecular characterization. The goal is to identify a molecular signature that can be clinically applied for personalized treatment and care of the patient with cancer. In addition, it would be useful to establish molecular signatures able to identify individuals at higher risk for developing the disease (diagnostic biomarkers), in order to being able to take preventive measures. Molecular signatures might also guide physicians with regard to the prediction of disease recurrence after surgery (surveillance biomarkers).

On the other hand, prognostic biomarkers provide an indication of probable disease progression, and predictive biomarkers guide towards probable responses to treatment. This has been demonstrated in breast cancer,⁶⁻⁹ the diagnosis and treatment of which are in some cases based on the expression of certain molecules, such as estrogen receptors to indicate antiestrogen treatment, or human epidermal growth factor receptor 2 amplification for treatment targeting it.¹⁰ These advances in breast cancer have been driven by its molecular classification, which has not occurred in CRC, where molecular characterization has fallen behind.

Although published CRC molecular classifications appear to be different, there are common factors. In this review, the evolution of different proposed classifications is highlighted.

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Initial molecular classifications

Three main pathways are involved in CRC molecular classification: microsatellite instability (MSI), chromosomal instability (CIN) and CpG islands methylation (CpGIM). MSI is characterized by small insertions or deletions in DNA repetitive sequences (microsatellites);¹¹ when MSI is not present, a tumor is considered to be microsatellite stable (MSS). CIN is defined as MSI high frequency and whole chromosomes segregation; CpGIM is produced when there is CpG islands methylation in the promoter region and it is associated with transcriptional silencing.^{12,13}

In 2007, Jass¹¹ proposed a molecular classification of colorectal cancer including *KRAS*, *BRAF*, defective mismatch repair, CpGIM and *MGMT* gene mutational status. Since then, numerous prognostic and predictive studies on this subject have been published, focusing on a single molecular marker. In 2008, Ogino and Goel proposed a classification based on the six subgroups proposed by Jass:¹¹⁻¹⁴

- Group 1, MSI-H CpGIM-high (10 %).
- Group 2, MSI-H CpGIM-low (5 %).
- Group 3, MSI-L/MSS CpGIM-high (5-10 %).
- Group 4, MSI-L CpGIM-low (5 %).
- Group 5: MSS CpGIM-low (30 to 35 %).
- Group 6: MSI-L/MSS CpGIM-0 (~ 40 %).

This molecular classification depends on common DNA markers and *BRAF* and *KRAS* mutant status.¹⁵

Mesenchymal/epithelial classification

In 2012, Schlicker et al. used a panel of 74 different CRC cell lines.¹⁶ Using iterative grouping, this analysis revealed five subtypes: 1.1, 1.2, 1.3, 2.1 and 2.2, which were successfully validated in more than 1600 CRC tumor samples from publicly-available gene expression datasets. Type 1 tumors showed a mesenchymal expression signature, had a poor prognosis and a similar number of tumors with MSI and MSS. Type 2 tumors had epithelial expression, MSS and had a good prognosis.

Type 1 tumors additional subtyping revealed that subtype 1.1 was strongly mesenchymal, occurred at a late phase and the regulated genes were mainly involved in calcium signaling; in addition, subtype 1.1 was characterized by activation of pathways involved in angiogenesis, inflammation and proliferation. More women had subtype 1.2; for the most part, tumors were MSI and had similar pathways to 1.1, but they also strongly activated JAK-STAT signaling pathway. Subtype 1.3 showed high expression of transporter genes and were mainly MSS.

Type 2 stratification gave rise to subtypes 2.1 and 2.2. In the first one, pathways associated with inflammation, angiogenesis and proliferation were activated. In subtype 2.2, regulated genes were those involved in the cell cycle and synthesis of amino acids.

CRCs classified as subtype 1.2 were highly sensitive to glycogen synthase kinase, *Src* and Wnt signaling pathway inhibitors. On the other hand, the CRC cell lines designated as type 2 were more sensitive to aurora kinase inhibitors.

Classification based on CIN, MSI and KRAS/BRAF mutational status

In 2013, two articles published in *Nature Medicine* revolutionized molecular classification. De Sousa et al. described three molecularly different CRC subtypes: CCS1, CCS2 and CCS3, where CCS3 was characterized for the first time.¹⁷ An integral part of this study was the analysis of gene expression data from 1164 patients with CRC. All CRC samples, xenografts, cell lines and precursor lesions were distributed in all three subtypes using a 146-gene classifier. CCS1 mainly comprises chromosomal instable (CIN) cancers that are mainly found on the left side of the colon. Wnt target genes were highly expressed and metastatic tumors were less common in comparison with CCS3 tumors. CCS2 tumors were MSI/CpGIM-positive and were located in the right side of the colon. There is relatively little information on CCS2.

In turn, tumors with CIN or MSI were not classified in the CCS3 subtype, but there was a relatively large number of patients with mutations in *KRAS* or *BRAF*. Tumors were distributed throughout the colon and tended to be poorly differentiated. Based on the MSS and CpGIM+ status, together with the relative overrepresentation of mutations in *BRAF*, the hypothesis was formulated that these tumors can arise from the serrated pathway. On the other hand, when comparing CCS1 with CCS3 using Gene Set Enrichment Analysis, regulated genes in the CCS3 subtype appeared to be involved in epithelial to mesenchymal transition, matrix remodeling, cell migration and transforming growth factor β signaling. The authors concluded that CCS3 tumors were highly malignant in comparison with CCS1 and CCS2 neoplasms.

In addition to the proposed classification, the authors examined the responses of the different subtypes to targeted treatment. Patients with CCS3 metastatic tumors were resistant to cetuximab, regardless of *KRAS* mutational status. This difference was also observed in vitro.¹⁷

Sadanandam et al. described six subtypes together with their response to cetuximab and to chemotherapy and disease-free survival (DFS). The subtypes were generated by means of a consensus based on gene expression grouping profiles of 1290 samples:¹⁸

- *Stem-like subtype*: it is the least differentiated, associated with the colon-crypt base and with poor DFS. Patients had higher expression of Wnt signaling pathway, stem cells, myoepithelial and mesenchymal genes and lower expression of differentiation markers. It was predicted that this group would benefit from chemotherapy (especially with a regimen based on calcium leucovorin, 5-fluorouracil and irinotecan-FOLFIRI).
- *Inflammatory subtype*: not associated with crypt top or base phenotypes. Main regulated genes in this group were related to interferon and chemokines. According to the above, patients should ideally receive chemotherapy, more specifically with FOLFIRI as adjuvant treatment. On the other hand, in metastatic tumors, chemotherapy and cetuximab might be ineffective. Intermediate DFS was identified in this subtype.
- *Goblet-type subtype*: it was found on the colon crypt top and was associated with good DFS. This subtype exhibited high mRNA expression of goblet-specific MUC2 and TFF3. Patients may not respond to adjuvant treatment and, therefore, implementing close monitoring after surgical resection was suggested. At metastatic stage, chemotherapy should be administered (preferably FOLFIRI).
- *Enterocyte subtype*: characterized by high expression of enterocyte-specific genes. Patients assigned to this group had an intermediate DFS. Based on its gene signature, these tumors were determined to be identified with the colon crypts and ridges phenotype. Compared with the stem or progenitor cell phenotype, it is more differentiated. The authors recommended that patients that were to receive adjuvant treatment should be treated with chemotherapy (preferably FOLFIRI) or with another option (except for cetuximab or c-MET inhibitors). As regards metastatic disease, the authors did not recommend chemotherapy, cetuximab or c-MET inhibitors.
- *Transit-amplifying (TA) subtype*: it was considered heterogeneous, since both stem cell and Wnt target genes were irregularly expressed. This subtype also exhibited a mixed phenotype: 59 % of tumors had a high crypt signature and low Wnt signaling pathway expression. It was significantly

associated with the crypt base, expressed stem and progenitor markers such as LGR5 and ASCL2. The authors recommended avoiding adjuvant chemotherapy and chemoradiotherapy, since they identified that patients with this subtype had poorer DFS.

After a series of proliferation assays to monitor drug responses, the TA subtype was further divided:

- Cetuximab-sensitive transit-amplifying subtype (CS-TA).
- Cetuximab-resistant transit-amplifying subtype (CR-TA).

CS-TA showed higher expression of EREG and AREG in comparison with CR-TA, where higher expression of filamin A, which is involved in c-MET regulation, was observed. The authors concluded that, in the presence of metastasis, patients with CS-TA should be treated with cetuximab, while those with CR-TA should be treated with a c-MET inhibitor.

Classification based on clinicopathological variables

In 2013, Marisa et al. published a gene expression classification of 6 molecular subtypes based on clinicopathological variables commonly used as DNA markers.¹⁵ The relevance of this classification is that the subtypes are associated with different prognoses. The study was carried out with 443 patients and a validation group of 1029 patients. The subtypes were named according to their biological characteristics.

Subtype C1, called CIN-immune-down, shared numerous similarities with C5 CIN-Wnt-up, both in the conventional pathway of the neoplasm precursor, with very low frequency of deficient mismatch repair genes (dMMR) and CpGIM, in contrast to high frequency of CIN. The majority were located in the distal colon. With regard to mutational status, C1-CIN-immune-down and C5 CIN-Wnt-up exhibited *KRAS* and *TP53* mutation intermediate frequency and very low *BRAF* mutation. One of the main differences was that C5-CIN-Wnt-up was associated with an intermediate frequency of the stem cell phenotype similar to the gene expression profiles, while C1-CIN-immune-down exhibited very low frequency.

The other four subtypes, C2 dMMR, C3 KRASm, C4 CSC and C6 CIN-norm-L, were linked to the precursor neoplasm serrated pathway. The C2 dMMR subtype involved mainly dMMR (68 %) and *BRAF* mutations (40%); they were located very often in the proximal colon, with a very high frequency of CpGIM being recorded (59 %). *KRAS* mutant tumors were found with an

intermediate frequency. With regard to the expression of genes the selection of which was monitored, most C2 dMMR neoplasms exhibited a BRAF-type signature and a serrated signature. In addition, both the immune system and cell-growth pathways were found to be positively regulated in this subtype, whereas the Wnt signaling pathway was negatively regulated.

The C3 KRASm subtype was also frequently located at the proximal colon and included mainly KRAS mutant tumors (87 %). Most signaling pathways in C3 KRASm tumors were regulated by inhibition.

The C4-CSC subtype with metastasis (31 %) was the only one with a reproducible association between poor prognosis and stem cell genes expression. This subtype was frequently located in the proximal colon, had an intermediate frequency of CIN+, and 34 % of patients showed CpGIM+. Intermediate frequencies of mutations in *KRAS* and *TP53* were reported. The majority exhibited stem cell phenotype as gene expression signature (91 %).

The final subtype described in this study was C6 CIN-norm-L. Although it was classified within the category of neoplasm serrated precursor, it was mainly CIN+

(86 %), CpGIM-, *TP53* mutant and was located in the distal colon. In contrast to the other CIN+ subtypes, it exhibited very high frequency of normal expression signature and intermediate closed gene expression profile signature.

Comprehensive classification

In July 2013, Budinska et al. described a classification of five subgroups using 1113 CRC gene expression profiles and 720 CRC transcriptomic profiles.¹⁹ The authors used the genome-wide transcriptome analysis and described the biological characteristics, morphology, common clinical variables and molecular markers of each subtype:

- *Surface crypt-like subtype (A)*: it significantly was enriched in *KRAS*, with predominant papillary or serrated histopathology and low percentage of β -catenin-positive nuclei in the invasive front. The authors also observed that it was well differentiated and that it was comparable to normal colon epithelium by gene expression profiles. In this subtype, post-relapse survival was 28.9 months.
- *Lower crypt-like subtype (B)*: well differentiated, it showed complex tubular morphology and had a high percentage of β -catenin positive nuclei in the invasive front. Higher copy number gain/amplification was recorded in chromosome 20q. On the other

hand, top colon crypt genes overexpression was detected, as well as proliferation (mainly EREG) and Wnt signaling pathway. Conversely, the gene expression that regulated the mesenchymal/stroma epithelial transition, the immune system and secretory cells were regulated by inhibition. Tumors were mainly located on the left side and were grade 2. Post-relapse survival was 50.4 months.

- *CpGIM-H-like subtype (C)*: commonly mutant, MSI+ and *BRAF* (87 %), characterized by patterns of solid/trabecular or mucinous growth. Commonly, the tumors are found on the right side and are high-grade. On the invasive front, they did not show β -catenin nuclear immunoreactivity, although, transcriptomically, they had immunity-associated genes, metallothioneins and homeobox gene module high expression. It was associated with poor survival (6.9 months after relapse). It is speculated that once these tumors metastasize they are resistant to chemotherapy.
- *Mesenchymal CRC subtype*: histopathological examination revealed a desmoplastic pattern, and immunochemistry, that only a low percentage of β -catenin-positive nuclei were present on the invasive front. This subtype also had high expression of mesenchymal/stroma epithelial transition genes. Conversely, lipid synthesis gene expression and target signatures Wnt signaling pathway were down-regulated in the mesenchymal subtype. In addition, it showed a high frequency of mutations in *BRAF* and was correlated with poor overall survival (OS), possibly as a result of mesenchymal epithelial transition high expression and associated genes' proliferation low expression. In addition, it was prognostic with regard to relapse-free survival (RFS).

Mixed subtype (E): it was located mainly on the left side of the colon. On histopathological examination, tubular complex was observed, and immunohistochemistry showed a high percentage of β -catenin-positive nuclei in the invasive front. This subtype also had high mesenchymal/stroma epithelial transition gene expression, target signatures Wnt signaling pathways, EREG expression module gene, and homeobox expression module gene.

Classification based on cancer biological characteristics

In 2013, Roepman et al.²⁰ proposed three different intrinsic subtypes based on epithelial-to-mesenchymal

cancer transition biological characteristics, deficiency in mismatch repair genes and cell proliferation. This classification was derived from multi-omic data generated from 188 patients with CRC at phases I to IV. The validation set was comprised by 320 patients at stage II and 223 at stage III and was associated with prognosis and response to chemotherapy; 35 and 22 % of patients were classified as epithelial-like A-type with MMR deficiency in the initial and the validation cohort, respectively. A significantly higher percentage of MSI+ tumors (49 %) and BRAF mutations was observed; 68% of patients showed a MSI/dMMR expression profile; Ki67 higher expression was also observed when compared with the other two subtypes. Prognosis for patients with A-type MSI was very good (93 % with 10-year metastasis-free survival). Chemotherapy response was evaluated in 222 patients at stage III who had better OS from adjuvant chemotherapy, with a hazard ratio (HR) of 0.39 ($p = 0.18$).

Fifty-two percent of patients in the experimental cohort and 62 % in the validation cohort were classified as proliferative epithelium B-subtype. This subtype mainly included tumors with wild-type *BRAF* (98 %), almost entirely MSS and pMMR phenotype (99 %). Mesenchymal markers *CDH2*, *FGFR1*, and *TGFB1* were inhibited, whereas 4 epithelial markers were activated. This subtype was characterized being as highly proliferative. Patients had a relatively poor baseline prognosis, but they were expected to benefit from adjuvant 5-fluorouracil-based adjuvant chemotherapy (HR = 0.42, $p = 0.014$).

Thirteen percent of subjects of the experimental cohort and 17 % of the validation cohort corresponded to the mesenchymal C-subtype. A small proportion showed mutant *BRAF* (16 %) and 36 % dMMR. All mesenchymal markers, with the exception of *FLT1*, were considerably regulated in comparison with epithelial markers *CDH*, *EGFR* and *MET*, which were down-regulated. In addition, low proliferative activity was recorded in this group. Patients had a poor prognosis and were resistant to chemotherapy with 5-fluorouracil.

Consensus molecular classification

In 2015, *The Consensus Molecular Subtypes of Colorectal Cancer* was published,²¹ where four molecular subtypes were identified. The 18 datasets used in the characterization and subtyping studies included 4151 patients. The authors divided the cohort in two equivalent groups for validation

The four consensus subtyping groups included 3104 samples; 858 (13 %) did not correspond to any subtype and, therefore, were described as non-classifiable.

- *Consensus molecular subtype 1 (CMS1)*: 14 % of the cohort corresponded to this subtype, which shows microsatellite instability, MSI+, CpGIM-H, hyper-mutated phenotype with numbering mutations in *BRAF*. The MSI immune subtype was characterized by an increased expression of genes associated with immune infiltrates and strong activation of immune evasion pathways. These tumors were common in women, on the right side, presentation at later stages and with worse survival after relapse.
- *Consensus molecular subtype 2 (CMS2)*: 37 % of the cohort was classified in this canonical-epithelial subtype. CMS2 had the highest copy number gains and losses; in addition, it had high expression of Wnt and MYC downstream targets. These tumors commonly occur on the left side. Patients had better survival rates after relapse in comparison with CMS1.
- *Consensus molecular subtype 3 (CMS3)*: 13 % of the cohort was classified in this subtype. Most tumors displayed mixed MSI, mainly CpGIM-L, and had low frequency of copy number alterations. Mutations in *KRAS* were common.
- *Consensus molecular subtype 4 (CMS4)*: 23 % of the cohort was classified in this mesenchymal subtype, characterized by transforming growth factor- β prominent activity, stromal invasion and angiogenesis. It had high frequency of copy number gains and losses. Tumors tend to be diagnosed at an advanced stage and show the highest relapse and lower overall survival rates

Conclusion

Molecular classification of colorectal cancer is of great importance, since identification of subtypes serves to generate and relate clinicopathological parameters, overall survival, progression-free survival and treatment response and relapse. These correlations can facilitate the understanding of colorectal cancer biology, provide evidence regarding carcinogenesis and help discover new molecular subtypes. A molecular classification is highly important for prognostic and predictive biomarkers discovery and clinical implementation and for making personalized health care a reality.

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