REVIEW

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Molecular classification of colorectal cancer: a dream that can become a reality

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Abstract

Despite thousands of studies about colorectal cancer (CRC) as much as extensively usage of prognostic antibodies/genes and clinical trials that include the newest targeted drugs, this tumor still remains in the top of both incidence and cancer-related mortality. In this review, we intended to correlate our experience in field of colorectal cancer with the literature data and to present our vision about the prognostic and predictive role of some of the most used molecular and immunohistochemical examinations in the field. The prognostic and predictive values of parameters such as microsatellite instability, angiogenesis, Maspin gene/protein, K-ras and BRAF mutations are discussed in relationship to the classical antibodies such as Keratin 7/20, p53 or HER2. At the end, we correlated these informations and tried to realize a molecular classification of colorectal cancer, similar to breast carcinomas, in order to establish targeted groups of patients for targeted therapy.

Keywords: colorectal cancer, molecular classification, prognostic, therapy, microsatellite instability, Maspin.

Introduction

Although colorectal cancer (CRC) is one of the malignant tumors that benefits from targeted therapy, it still remains the second cause of cancer death worldwide [1]. Increasing incidence was reported in the last years, above 40% of the cases being still diagnosed in advanced stages [2]. New antibodies and gene mutations are discovered everyday but most of them proved to be only a scientific disappointment. In this review, we intended to present a survey of our results about molecular prognostic and predictive factors of CRC, partially published by our team in the last years, and to confront them with the literature data. The final aim was to establish some prognostic and predictive groups of CRCs.

Prognostic value of molecular examinations in CRC

Microsatellite instability

One of the well-recognized prognostic factors in CRC is the microsatellite instability (MSI). Microsatellites are repetitive DNA sequences those instability reflects a mismatch DNA-repair. The microsatellite status can be determined using the Real-Time PCR technique, according to the Bethesda protocol approved in 1997 that recommend using of two mononucleotides (BAT25, BAT26) and three binucleotides (D2S123, D17S250, D5S346) respectively. Identification of microsatellite instability in at least two of the markers indicates a high-microsatellite instability (MSI-H) but, if it is identified with only one of them, the case is included in the category of low-microsatellite instability (MSI-L) [3]. The other cases are considered to have a micro-satellite-stable status (MSS). However, our experience revealed that BAT25 and BAT26 mononucleotides are quite specifically and enough to predict microsatellite status, presence of instability in at least one of them being usually characteristic for MSI-H status [4]. Turaga K and Shibata D added that D2S123 is the third one regarding the specificity [5]. In order to have a correct assessment of MSI-status, we usually use, instead the binucleotides, a more inexpensive variant, examining the immunohistochemical expression of MLH-1 and MSH-2 mismatch-repair markers (MMR). If both of them are negative, the objectivity about MSI-H-status increases. The other two MMR markers, PMS2 and MSH-6, were rarely negative in our cases. In our laboratory, real-time Light Cycler PCR (Roche GmbH, Mannheim, Germany) and the method of high-resolution melting peak analysis, SYBR Green detection format and specifically primers, previously published [6] are used for BAT25 (forward 5'-TCGCCTCCAAGAATGTAAGT-3' and reverse 5'-TCTGCATTTTAACTATGGCTC-3') and BAT26 (forward 5'-TGACTACTTTTGACTTCAG CC-3' and reverse 5'-AACCATTCAACATTTTTAAC CC-3'). For BAT26, the average melting peak value is 51.2^{0}C for MSS respectively 45.5^{0}C for MSI. For BAT25, the average values are 45.1^{0}C for MSS respectively 42.4^{0}C for MSI. For each laboratory, a proper calibration of the PCR is necessary.

Microsatellite instability seems to associate a more favorable prognosis and a lower metastatic rate compared to the MSS- or MSI-L-cases [5, 7, 8]. MSI-status usually characterize the hereditary cancer or Lynch syndrome but 10–15% of sporadic CRC can also display this status due to hypermethylation of the MMR gene MLH1 [7–9].

MSI-cases are usually localized on the proximal colon to the splenic flexure (>85% of the total MSI-CRC) few of them also involving the distal colon (10%) or rectum (<5%); most of them are mucin-producing-tumors, more poorly differentiated, with a lower intensity of angiogenesis [4, 5, 8]. Compared to MSS-cases they display a high rate of TIL (Tumor Infiltrating Lymphocytes) that can be quantified with CD3/CD4. However, high-TIL-MSS-CRC seems to have a better prognosis than low-TIL-MSI-CRC, the total CD3 score being higher in the cases diagnosed in the II/III stage and also in those with high overall survival rate [7].

The p53 nuclear expression is usually absent or is present in few than 50% of the tumor cells of MSI cases. Some of the recent studies, in line to our results, reported a decreasing intensity or either negativity of MSI-CRC for Keratin 20 and also positivity for Keratin 7 [10, 11] although Keratin 20 positivity/Keratin 7 negativity was usually used in the clinical diagnosis to differentiate metastases of CRC from lung, ovarian or bladder carcinomas [12]. In MSS-CRC with metastases, Keratin 7 can also be positive but diffuse positivity for Keratin 20 is associated [10, 11].

These clinicopathological and immunohistochemical criteria can be used to select the cases that seems to present a better prognosis and also those CRC in which PCR-analyses are necessary to be performed to certify the MSI-status.

Angiogenesis

In case of CRC, the angiogenesis can be quantified immunohistochemically using the Vascular endothelial growth factor A (VEGF-A) that marks the cytoplasm of the tumor cells. For a correct assessment, the micro-vessel density should also be quantified using the markers CD31 and CD105. Although longtime, it was considered that angiogenesis has an important prognostic value, being correlated to the tumor stage, our previous researches revealed that the angiogenesis of CRC presented an oscillating pattern, the VEGF expression being more intense in Stage IV, but the endothelial area quantified with both CD31 and CD105 was smaller than in those cases diagnosed in Stages II and III [13].

Maspin gene/protein

Experimentally studies proved that the serine protease Maspin (Serpin B_5), encoded by Maspin gene, can inhibit tumor proliferation and has antiangiogenic and proapoptotic properties [13]. Few data are known about the prognostic role of Maspin in CRC. It can be immuno-histochemically evaluated but a real problem of its assessment is that both cytoplasm and nuclear expression can be emphasized. Based on the controversial published data, our team proposed a new system of quantification that takes into accounts both cytoplasmic and nuclear expression, with a real prognostic and predictive value [4]. Based on this grouping system, partially in line to the literature data, the cytoplasmic positivity associates the best prognosis but the nuclear ones is correlated with the shortest survival time and high aggressivity, independently by tumor stage [4, 13–16]. Negative cases and those with dual positivity (nuclear + cytoplasmic) presented an intermediary prognosis [4]. Cytoplasmic positivity was more frequently associated with MSI-cases [4, 16]. In line to our results, Dietmaier W *et al.* revealed that increased nuclear Maspin expression indicated shorter survival and local aggressiveness and was correlated with the tumor grade, but not with p53 expression, in CRC with lymph node metastases, diagnosed in stage III [16].

K-ras mutations

Although K-ras status is rather analyzed for the targeted therapy of CRC, it seems that mutations on codon 12 associates unfavorable prognosis, independent by the tumor stage [5]. This aspect is not well defined in the literature.

BRAF mutations

Although V600E-BRAF mutation is detected in about 10% of CRC, the prognostic value of this molecular parameter is still non-elucidated. We observed that the MSI-CRCs that displayed Keratin 7 positivity were BRAF-mutated [10]. This association could indicate a possible serrated pathway in the carcinogenesis of these cases [17–19], the subject being intensely studied. However, the molecular features depend on the tumor location. In one of the most recent studies, we concluded that the serrated pathway adenocarcinomas of the proximal colon that do not display the morphological features of this pattern are more frequent Keratin 7+/p53-/MLH-1-/BRAF-mutated/K-ras-wild type/MSI cases, but those located in the distal colorectal segments seem to be Keratin 7+/Keratin 20+/p53-/MLH-1+/BRAF-wild type/K-ras-mutated/MSS cases [19]. These characteristics could favorize selection of the right-sided cases with high probability for MSI-BRAF mutated status which seem to be sporadic MSI cases with better prognosis and low risk of distant metastases than MSI-BRAF wild type or MSS-BRAF-mut ones [19–21].

Predictive value of molecular examinations in CRC

Basic data about the classical chemotherapy

Despite the several clinical trials that proved the efficacy of the newest molecular-related drugs, there are many patients that are still treated with the classical therapy, one of the reasons being the expensiveness of the new drugs, especially in developing countries. Moreover, the targeted therapy is usually approved to be used as second- or third-line therapy [22].

FOLFOX (Folinic acid/5-Fluorouracil/Oxaliplatin) regimen is the commonest neoadjuvant chemotherapy used in patients with metastatic CRC, associated or not with hepatic metastasectomy. The results are encouraging but more than 50% of patients develop side effects such as steatohepatitis and hepatic perisinusoidal fibrosis, the hepatic tumor nodule being usually replaced by fibrous tissue [23]. Other side effects of Oxaliplatin, a third-generation platinum-based alkylating agent that inhibits DNA synthesis in cancer cells, approved by Food and Drug Administration (*FDA*) in 2002 for the treatment

of Stage III/IV (Dukes' C/D) CRCs, such as sensory peripheral neuropathy, fatigue, stomatitis, nausea, vomiting, diarrhea, pulmonary fibrosis, gastrointestinal toxicity, ototoxicity, pancreatitis and nephrotoxicity were reported [24].

FOLFIRI (Folinic acid/5-Fluorouracil/Irinotecan) regimen is the second option used to treat the metastatic CRC [25].

Microsatellite instability

Beside the well-proven more favorable outcome of MSI-H cases compared to MSI-L/MSS ones, MSI status can also be used as predictive factor, most of studies revealing no response of MSI-H cases at 5-FLU (5-Fluorouracil) [26]. However, this aspect is controversial and not accepted by all oncologists [27, 28].

Because the intensity of CD3 positive-cells in both intra- and peri-tumoral zones assessed a good prognosis compared with those with low-T-lymphocytes-rate, independently by the MSI-status, the immunological moderators seem to be a therapeutically option in CRC [7].

Angiogenesis

Antiangiogenic treatment of metastatic CRC was approved by *FDA* in 2004 but the criteria used to select the patients for this quite expensive therapy are still not well defined. Most of studies revealed that Bevacizumab, the commonest used antiangiogenic drug, increased the survival rate with 3–4 months, one of the most recent ones reporting an increasing from 21.4 to 27.4 months [29]. However, several side effects such as bowel perforation, hypertension and cerebral hemorrhages were reported. The newest antiangiogenic drugs such as Aflibercept and Regorafenib are tested [30]. Regorafenib, a novel oral drug, seems to be an anti-angiogenic and anti-oncogenic kinase inhibitor that acts against KIT, RET, RAF and VEGF genes [31]. Other six anti-angiogenic substances are introduced in clinical trials but their number is still increasing. For feasible results, a proper cost-benefit analysis should be performed before approval of their use in clinical oncology.

Maspin gene/protein

Some experimental data revealed that 5-FLU can inhibit the tumor cells that displayed Maspin nuclear positivity and were p53 negative but Maspin negative/p53 positive CRC are 5-FLU resistant [16]. Quite interesting is that, in our cases, most of MSI-tumors presented a cytoplasmic-Maspin-predominance, the nuclear-Maspin-predominance being associated with MSS status and p53 positivity. Few of MSI-H tumors associated Maspin-nuclear-expression, all of them being p53 negative [4]. Because some but few MSI cases can respond at 5-FLU [23, 26, 27], we conclude that Maspin-nuclear-predominance can be used to select the MSI-cases that can be treated with the classical 5-FLU [4, 13].

We also observed an interesting aspect in cases diagnosed in stage II that usually do not benefit by chemotherapy. In our material, in CRCs diagnosed in stage II, Maspin cytoplasmic expression was associated with p53 negativity and longer overall survival rate but the nuclear predominance was related to p53 positivity and tumor aggressivity. These results show that Maspin expression could be used to identify those patients diagnosed in Stage II for which chemotherapy is mandatory to be performed [4, 6, 13].

The anti-Maspin substances proved experimentally that can have antiangiogenic and antiproliferative effects [32, 33] but the results are still controversial.

K-ras mutations

In the carcinomas located on the distal colon, Keratin 7 positivity could indicate the serrated-pathway carcinomas that are usually K-ras wild type and can respond to the EGFR-targeted antibody Cetuximab or Panitumumab, in Cetuximab-refractory cases [19, 34]. These drugs were approved to be administrated only in K-ras wild-type metastatic CRC as monotherapy or associated to FOLFOX regimen [5, 34]. However, despite of K-ras wild type status, inactivation of the tumor suppressor gene PTEN (Phosphatase and Tensin homologue), identified in about 4% of cases, determines resistance at Cetuximab [35]. In case of wild-type K-ras/Cetuximab-refractory patients, a Cetuximab/Trastuzumab (HER2-targeted antibody) combination was proposed [36]. To increase the sensitivity, simultaneously examination of E-cadherin immunohistochemical expression is proposed, its positivity being a predictor of response at Cetuximab-based regimen [37].

BRAF mutations

Identification of the serrated-pathway carcinomas located on the right colon that seem to be MSI-BRAF mutated cases with loss of MLH-1 immunoexpression and Keratin 7 positivity could be a criteria used to identify patients that could respond to Oxaliplatin-based therapy [19, 20].

Summary and perspectives regarding the molecular classification of colorectal cancer

Prognostic groups of colorectal cancer

Correlation of our experience published in several papers with the literature data [7, 15, 16] can lead to grouping of patients with CRC in the following prognostic groups, the tumor stage also remaining an important prognostic factor; these data were partially mentioned in our previously-published papers [4, 13]:

- Best prognosis: MSI-H/BRAF-mut/p53-negative cases with Maspin cytoplasmic predominance and high CD3 score;
- Intermediate but more favorable prognosis: MSI/MSS cases with Maspin mixed expression/p53 <50%;
- Intermediate but more unfavorable prognosis: MSI/MSS cases/Maspin negative/p53 <50%;
- Worst prognosis: MSS/BRAF-mut/p53 >50% with Maspin nuclear predominance and low CD3 score.

Predictive groups of colorectal cancer

Similar to the prognostic groups, following targeted groups of patients can be identified for targeted therapy of CRC [4, 13, 16, 34, 36, 37]:

▪ MSI-H cases with Maspin-nuclear predominance/ p53-negative: 5-FLU + other drugs;

▪ MSI-H cases with Maspin-cytoplasmic predominance/ p53-negative: chemotherapeutic regimen that excludes 5-FLU;

▪ Maspin negative/p53+ cases: chemotherapeutic regimen that excludes 5-FLU;

▪ pT3N0 cases with Maspin-cytoplasmic predominance/ p53-negative: surgical removal without postoperative chemotherapy or anti-EGFR drugs;

▪ pT3N0 MSS-cases with Maspin-nuclear predominance/ p53+: 5-FLU and antiangiogenic drugs;

▪ Wild type K-ras/E-cadherin positive metastatic cases: Cetuximab-based therapy;

▪ Wild type K-ras/E-cadherin negative metastatic cases: Panitumumab or Cetuximab + Trastuzumab;

▪ Stage IV CRC: antiangiogenic drugs.

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