Multimarker Phenotypes of Colorectal Cancer

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Objective: We tried to correlate the clinico-pathologically features of colorectal cancer (CRC) to expression of eight immunohistochemically (IHC) markers and microsatellite instability (MSI) in order to realyze a molecular subdivision of these tumors.
Methods: 300 CRC, surgical specimens, were statistically and IHC evaluated. MSI status was analyzed in 52 cases, with Real Time PCR, melting point analysis. The following IHC markers have been used: CD8, E-cadherin, HER-2, p53, Ki67, bcl-2, MLH-1, CEA. The molecular phenotypes have been reported to the node status (pN) and MSI.
Results: Based on statistically analyses, we revealed that CEA and Ki67 were not prognostic factors. MLH-1 may indicate the MSI status and the number of tumor inflitrated lymphocytes stained with CD8 seems to be higher in the MSI cases and tumors of the proximal colon. HER-2 expression was correlated to number of the lymph node metastatses and bcl-2 was negative is most of the CRC diagnosed in advanced stages.
Conclusions: The CRC may be subdivided in six molecular prognostic groups, the best prognosis showing the MSI/p53-/bcl-2+/HER-2- and the worst MSS/p53+/bcl-2±/HER-2+. These molecular subdivision may be the basis for targeted therapy in node negative CRC.

Keywords: colorectal cancer, molecular phenotype, prognosis

Introduction

Despite the modern techniques, the antiangiogenic and targeted therapy of colorectal cancer (CRC), it remains the fourth most common cancer diagnosed and the second most common cause of cancer death worldwide [1]. Patients with same features may have different outcome, either in early stages [2]. The undetected lymph node metastases and also the tumor phenotype may be reasons for adverse survival in patients diagnosed with stage II, without lymph node metastases [3,4].

Although significant reduction in mortality was observed in different clinical trials in patients who received 5-fluorouracil/leucovorin chemotherapy in stage II colon cancer versus surgery alone, the adjuvant therapy is not yet approved in non-metastatic cases [5]. Targeted therapy of patients from these stage remains a challenge and multicenter blinded clinical trials should prove the real benefit of its implementation.

Molecular criteria should be used to select the cases with high risk for metastasation. On the other hand, in the metastatic CRC the multimarker phenotypes may also have prognostic and predictive value.

The main purpose of our study was to correlate the classical well-known prognostic factors of CRC with the immunohistochemical expression of eight markers and microsatellite instability (MSI), in order to identify some molecular subgroups. Because the last Staging Manual of the American Joint Committee on Cancer and all recent studies [3,6] agree that lymph node status and MSI remain the key prognostic marker of survival in CRC, the molecular phenotypes have been reported to these two parameters.

Methods

Three-hundred unselected primary CRC, surgical specimens, were retrospectively analysed. All of patients do not received pre-operative radiotherapy. All tissues were formalin-fixed, paraffin-embedded.

For immunohistochemical study, we used the antibodies mentioned in Table 1 and UltraVision system by LabVision (D-Line, USA). Heat antigen retrieval was performed in EDTA, pH9 (p53) or citrate solution, pH6 (all the other antibodies). The development was performed with DAB (Diamino Dihydrochlorid Benzidine), which was applied for 3-5 minutes. Nuclei were counterstained with Mayer's Hematoxylin.

The cut-off value was considered to be 10% for p53 and Ki67 respectively 5 TIL (tumor infiltrate lymphocytes) at high power view for CD8. The expression of CEA (Carcinoembryonic Antigen), bcl-2 and MLH-1 was intracytoplasmatic quantified. The membranar expression of HER-2 in at least 20% of cells was considered positive.

The MSI status was analysed with Real Time PCR, melting point analysis, in 52 from the 300 cases. The mononucleotids BAT25 and BAT26 were used (Fig. 1, 2).

For statistical analysis, GraphPAD In Stat 3 software was used. The two-tails unpaired t-test, chi square test and the contingency tables were performed. A p value less than 0.05 with 95% confidence interval was considered significant.

Results

Clinico-pathological features

From the 300 cases, 74% were located on the distal colon (descending colon + sigma + rectum) and 26% on the proximal colon (ascending + transverse colon). 43.39% of patients were males and 56.61% were females. In both females and males 14.2% respectively 85.8% of cases were diagnosed before and after 50 years old. The youngest patient was a 17 years old male.

Table I. Antibodies used for the immunohistochemical study

Antibody	Clone	Dilution	Provenience
CEA	IL-7	1:50	Dako Denmark
ki67	Ki-S5	1:200	LabVision D-line USA
p53	DO-7	1:50	LabVision D-line USA
bcl-2	100/D5	1:50	LabVision D-line USA
HER-2	polyclonal	1:300	Dako Denmark
E-cadherin	NCH-38	1:40	LabVision D-line USA
CD8	SP16	1:50	LabVision D-line USA
MLH-1	monoclonal	1:10	BD Biosciences

The mucinous carcinomas were commonest in the proximal colon - 46.73% of cases from proximal and 25.66% in the distal segments (p = 0.02).

66.93% of CRC from proximal colon and 80.22% from distal colon were in pT3 stage (p = 0.09). About 60% of cases do not presented lymph node metastases.

From the 52 cases, 9 presented H-MSI status (High-Microsatellite Instability) with both BAT25 and BAT26.

Table II. The correlation between tumor markers, lymph node (pN) and microsatellite status

Tumor marker	pN0 (%)	pN1-2 (%)	p	MSS (%)	MSI (%)	p
CEA	80.23	77.89	0.76	75.43	69.99	0.83
Ki67	22.5	75.32	<0.001	27.14	37.68	0.08
E-cadherin	63.77	42.12	0.02	52.94	25	<0.001
CD8	35.69	48.85	0.24	42.68	93	0.04
MLH-1	56.54	49.49	0.54	62.31	16.45	<0.001
bcl-2+/p53-	70.83	29.17		17.65	37.50	
bcl-2+/p53+	58.06	41.94	0.002	20.59	12.50	<0.001
bcl-2-/p53-	62.86	37.14		23.53	37.50	
bcl-2-/p53+	50	50		38.23	12.5	
bcl-2+/HER-2-	55	45		11.76	25	
bcl-2+/HER-2+	67.39	32.61	0.72	26.47	25	0.01
bcl-2-/HER-2-	53.73	46.27		55.89	37.5	
bcl-2-/HER-2+	53.66	46.34		5.88	12.5	
HER-2+/p53-	71.43	28.57		14.71	25	
HER-2+/p53+	57.90	42.10	0.13	17.65	12.5	<0.001
HER-2-/p53-	62.50	37.50		26.47	50	
HER-2-/p53+	49.06	50.94		41.17	12.5	

MSI = Microsatellite instability; MSS = Microsatellite stable; for the univariate analysis of first five markers the percent of positive cases is shown here

Phenotypes

The correlations between tumor markers and immunophenotypes are revealed by the Table 2. We should mention that the correlations for MSI status have been performed in 52 cases and the other markers and clinico-pathological features were analysed in all 300 cases. For univariate analysis the percent of positive cases was included in table.

If we considered that MSI and pN0 cases present the best prognosis and analyse the statistical relationship between multimarker phenotypes and these parameters, the CRC may be subdivided in prognostically subgroups.

The univariate analysis showed that CEA, Ki67 and MLH-1 were not prognostic factors of CRC but MLH-1 expression may indicate the MSI status. The rate of positivity for CD8 was higher in the tumors of the proximal colon and MSI cases but it was not an independent prognostic factor. E-cadherin was more expressed in the negative lymph-node CRC but its highest positivity in the MSS cases (p<0.001) showed that it did not have prognostic value. HER-2 was correlated in univariate analysis with the number of lymph nodes with metastases (p = 0.02). Most of bcl-2 negative cases were in advanced pT stages and presented lymph node metastases (p = 0.01).

Based on statistical analysis, the combined expression of bcl-2, p53, HER-2 and MSI seems to help us to divide the CRC in six prognostic groups:

1. MSI/p53-/bcl-2+/HER-2- best prognosis
2. MSI/p53-/bcl-2±/HER-2± good prognosis
3. MSI/p53+/bcl-2±/HER-2± intermediary group
4. MSS/p53-/bcl-2+/HER-2- intermediary group
5. MSS/p53+/bcl-2±/HER-2± bad prognosis
6. MSS/p53+/bcl-2±/HER-2+ the worst prognosis

Discussion

The multimarker immunophenotyping of CRC may have a prognostic and predictive value and may help us to identify the cases in stage II, without lymph node metastases (pN0), which present a high risk for recurrences. These cases may be candidate for post-operatory adjuvant che-

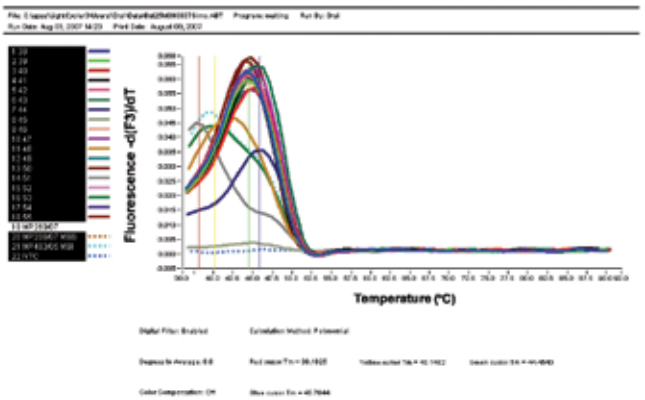

Fig. 1. Analysis of the microsatellite instability with Real-Time PCR and BAT25 mononucleotide. The melting point is 38.5–41.7°C for MSI (microsatellite instable) respectively 43–46°C for MSS (microsatellite stable) cases.

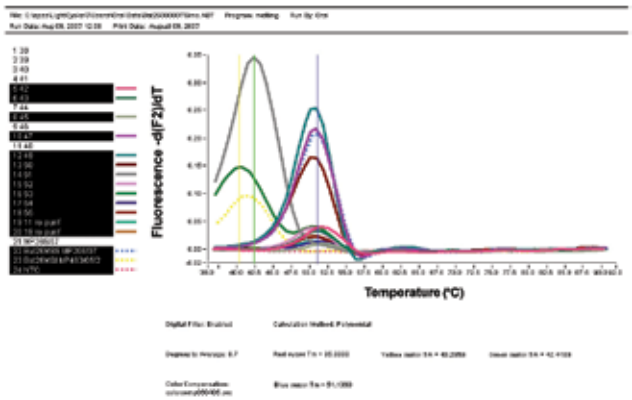

Fig. 2. Analysis of the microsatellite instability with Real-Time PCR and BAT26 mononucleotide. The melting point is 40–50°C for MSI (microsatellite instable) respectively 51–51.5°C for MSS (microsatellite stable) cases.

motherapy. Characterization of the molecular signature of CRC is a transdisciplinary and interdisciplinary field [7] and the data from literature are quite poor for objective conclusions.

In the most recent studies, the molecular profile of CRC has been analysed but more than 50 immunohistochemical antibodies and gene mutations had been taking into consideration [2,8]. The results are either based on the MSI (microsatellite instability) status combined with mutations in the EGFR (Epidermal Growth Factor) pathway [8-10] or sporadic and hereditary cases are compared [11].

Only two complex studies about prognostic impact of immunohistochemically markers were published in the PubMed citated papers [2,4]. One is a review about the cell surface markers [2] and the other is a TMA (tissue microarray) study in which the prognostic value of 13 markers is analysed in lymph-node negative CRC [4].

In the last paper, the authors revealed that lost of E cadherin expression combined to the negativity for CD8 is associated with worse prognosis of non-metastatic CRC [4]. Other authors accept that the molecular classification of CRC is evolving but they mention that it should include the EGFR pathway and the MSI status [9].

Conclusions

Although there are challenges, molecular pathological subdivision of colorectal cancer is a promising area of research. It may be a really help to identify those cases without lymph node metastases which may be candidates for postoperatory adjuvant therapy. This subdivison may improve the targeted therapy in metastatic but especially in node-negative CRC.

New prospective studies are necessary to confirm our data. Other parameters which should be added to obtain more objective results are the aspect of circumferential resection margines, the methylation status of CRC and the EGFR pathway.

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