The features of host immune response with respect to microsatellite status of colorectal cancer

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SUMMARY

Background: *The genetic alterations in colorectal cancer (CRC) progression are determined by two separate pathways, chromosomal and microsatellite instability (MSI). The CRCs with MSI have distinct clinicopathological characteristics with pronounced tumor-associated immune responses. The aim of our study was to investigate the intensity of host immune response in CRC tissue by comparing microsatellite stable (MSS) and instable tumors.*

Methods: *The study was performed on CRC specimens from 28 patients with MSI and compared with 30 MSS tumors. The microsatellite status was evaluated with two markers by PCR and melting point analysis. The immunostaining with anti-CD3 pan-T cell antibody was used to quantify the number of tumor infiltrating lymphocytes. The lymphocytes in peritumoral stromal and the Crohn's-like peritumoral reaction were counted on H&E slides.*

Results: *No significant differences were found in the average number of lymphocytes in peritumoral stroma and in clinicopathological characteristics of CRCs. The conspicuous Crohn's-like lymphoid reactions were present in 67.86% of CRCs with MSI versus 26.66% of MSS cases. The CRCs with MSI cases carried significantly higher numbers of tumor infiltrating T-lymphocytes (13.21 versus 7.47) ($p<0.0001$).*

Conclusion: *The presences of peritumoral Crohn's-like lymphoid and intraepithelial lymphocytic reaction were intensive markers for MSI in colorectal carcinomas in our study. The peculiar genetic instability in MSI tumors may lead to a continuous production of abnormal peptides, which act as neoantigens. They could induce specific antitumor immune responses effective in limiting tumor growth and spread. Abnormal peptides are potentially promising in immunotherapy advancing and in the design of a vaccine against colorectal tumors with MSI.*

Key words: *Colorectal Neoplasms; Microsatellite Instability; Lymphocytes, Tumor-Infiltrating*

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INTRODUCTION

The genetic alterations in colorectal cancer progression are determined by one of two separate and distinct underlying pathways of genomic instability. The first pathway, chromosomal instability which involves tumor suppressor genes and oncogenes, is characterized by allelic losses and aneuploidy. The second pathway, microsatellite instability (MSI), is characterized by an abundance of subtle DNA mutations and diploidy. Microsatellites (also called simple sequence repeats) are small tandem repeat DNA sequences that occur randomly distributed in noncoding genomic regions and in regions coding for proteins and structural nucleic acids. The MSI is defined as any change of length of these loci due to either insertion or deletion of repeated units (1). The DNA mismatch repair (MMR) system is necessary for the maintenance of genomic stability by eliminating single-base mismatches and insertion-deletion loops that may arise during DNA replication and results from gains or losses of short repeat units within microsatellite sequences. The replication machinery slips more frequently on repetitive sequences, so microsatellite sequences accumulate mutations in MMR-deficient cells, which results in MSI (or replication error). Defective mismatch repair presumably facilitates malignant transformation by allowing the rapid accumulation of mutations that inactivate genes that ordinarily have key function in the cells, involved in growth suppression, apoptosis, or signal transduction (2). In 1997, a National Cancer Institute of U.S.A. on meeting held in Bethesda proposed a panel of five microsatellite markers (two mononucleotide; BAT25 and BAT26, and three dinucleotide; D2S123, D5S346, D17S250) for the uniform detection of tumors with MSI. Tumors with instability at two or more of these markers were defined as being high-level instability (MSI-H). Tumors with instability in one marker are nominated as low-level instability (MSI-L), or showing no instability was designated as microsatellite stable (MSS) tumors (3). MSI was first seen in hereditary nonpolyposis colorectal carcinoma (HNPCC), often referred to by eponym of Lynch syndrome, and is caused by germ-line mutation of MMR genes (4,5), however, mutations in genes mlh1 and msh2 account for approximately 90% of HNPCC (5,6). In 1997, methylation of the promoter region and transcriptional silencing of mlh1 gene was shown to occur in CRCs (7), and caused MSI seen in 15% unselected sporadic cancers of the large bowel (1,2). Since the discovery of MSI, many investigators have shown that CRCs with this feature have distinct clinicopathological characteristics; predominantly right-sided anatomic location, lack of dirty necrosis (9), medullary histological type of carcinoma (10), mucinous differentiation (9,11,12) and more poor histological differentiation (8). Almost every description of MSI in CRCs noted a sign of pronounced tumor-associated immune responses in form of explicit peritumoral inflammatory reaction and lymphoid nodules (the so-called Crohn's-like lymphoid reaction) and high number of intraepithelial activated cytotoxic lymphocytes (13,14). One attractive hypothesis to explain this phenomenon is constituted by the possibility that, besides favoring the occurrence of mutations at genes critical for oncogenesis, the mutator phenotype may also increase the production of abnormal peptides able to elicit cytotoxic immune responses against tumor cells (13). A number of studies have investigated the relationship between MSI and CRCs prognosis. CRC with MSI have a significantly better prognosis compared to those with intact mismatch repair (15). The last updated evidence-based clinical practice guidelines of American Society of Clinical Oncology for the use of tumor

markers in colorectal cancer in 2006 is not recommended MSI to determine the prognosis of operable CRC nor to predict the effectiveness of fluorouracil-based adjuvant chemotherapy (16). There is supposition that induction of clinically relevant cytotoxic local antitumor immune responses is probably one of the major determinants of the more favorable course observed in CRCs patients with MSI (17). In addition to prognostic significance of microsatellite status, specific molecular characteristics of colorectal carcinoma have predictive significance to identify tumor with a best respond to a particular chemotherapy (8,18–20).

The aim of our study was to investigate the intensity of host immune response in colorectal cancer tissue by comparing microsatellite stable (MSS) and instable tumors.

MATERIALS AND METHODS

Tissue specimens

This study was performed on a selected group of 28 patients with sporadic CRC with MSI-H and compared with 30 MSS CRC patients who underwent curative surgical resection of CRC at General hospital in Senta. Histological analysis was performed on hematoxylin-eosin (H&E) stained slides prepared using routine histological methods. Colorectal adenocarcinomas were histological classified, typed and graded according to the World Health Organization (WHO) criteria (21). The tumors were classified in the pathologic TNM staging system of American Joint Committee on Cancer and Union Internationale Controle le Cancer (AJCC/UICC) (21,22). The number of peritumoral stromal infiltrating lymphocytes (SIL) was assessed by counting lymphocytes within the area of five high-power fields (Olympus S-Plan x40 objective, total area of five fields, 0.98 mm^2) (23). The Crohn's-like peritumoral reaction was assessed positive in case of two or more large lymphoid aggregates with germinal centers in a section at the tumor edge (10) (Figure 1).

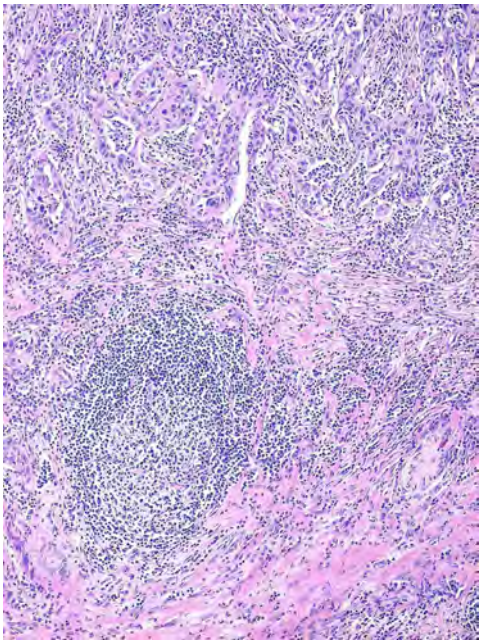

Figure 1. Peritumoral stromal infiltrating lymphocytes and Crohn's-like lymphoid aggregat with germinal center at the tumor edge, H&E x100

Microsatellite instability testing

The microsatellite status was evaluated in the Department of Molecular Pathology of Hungarian National Institute of Oncology, with two microsatellite markers (BAT25, BAT26) selected from the international guidelines suggested by the National Cancer Institute (3). Tumor tissues were dissected from 10 μm sections of formalin-fixed, paraffin embedded tissue. DNA preparation was done using the High Pure PCR Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) (24). Quality of DNA was checked by electrophoresis. Detection of microsatellite instability was done by real time PCR and melting point analysis using sequence-specific hybridization probes labeled with LightCycler dyes, LCRed640 and LCRed705 described by Dietmaier. Amplification of microsatellites by real-time PCR was followed by melting point analysis to display alterations in the length of repetitive sequences. For BAT25 melting temperature (Tm) of MSI-H tumors is from 42 to 50° C, due to shortenings of 3 to 12 nucleotides in a stretch of 26 repetitive adenosine nucleotides within BAT26 marker (Figure 2). Tm of BAT25 for MSI-H tumors is from 42.5 to 43.7° C corresponding to a shortening of 5 to 7 nucleotides within 25 repetitive thymine nucleotides of BAT25 marker (25). When both markers were positive tumors were defined as MSI-H. Colorectal tumor specimens with stabile both examined markers were defined as MSS. We did not distinguish low-frequency MSI from stable tumors. Tumors with only one positive marker were excluded from study group.

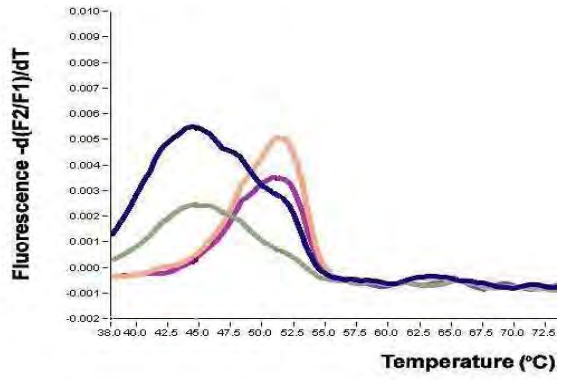

Figure 2. The BAT26 mononucleotid microsatellite marker. Examples of the melting temperature of microsatellite instable tumors are 44.2° C (green line) and 44.9° C (black line). The melting temperatures of microsatellite stable tumors are between 51.0 and 51.5 ° C (orange and violet line)

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded material. The sections were deparaffinized in xylene, rehydrated, washed in phosphate-buffered saline, and immersed in 0.01 mol/l citrate buffer, pH 6, or 0.1 mmol/l EGTA and microwaved for 5 minutes at 750 W three times. The immunostaining using anti-CD3 pan-T cell antibody (clone: NCL-CD3-PS1; Novocastra, Newcastle upon Tyne, England), was performed in working dilution 1:200, in the TechMate 1000 automated system (Ventana, BioTek Solutions, Tucson, AZ), with diaminobenzidine as the chromogen. The number of tumor infiltrating lymphocytes T cells (TIL) within the tumor epithelium was assessed by counting within the area of five high-power fields (Olympus S-Plan x40 objective, total area of five fields 0.98 mm^2) (Figure 3).

Statistical analyses

The statistical analyses were performed using statistical program for biomedical researchers MedCalc, MedCalc Software, Mariakerke, Belgium, Version 9.3.0.0. The comparisons between groups were performed using unpaired t test for continuous variables, and frequency table and χ^2 tests for categorical

variables. A p value of < 0.05 was considered to be statistically significant.

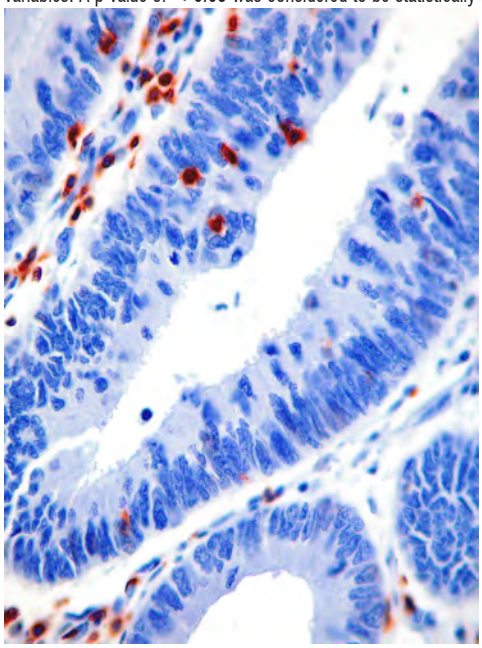

Figure 3. Immunohistochemistry for anti-CD3 pan-T cell antibody, tumor-infiltrating T lymphocytes within neoplastic epithelial structures, x400

RESULTS

This study was performed on a selected group of 58 CRC patients. Thirty-one female and 27 male patients with average age 62.10(16.02) and 56.4(13.56) years, res pectively underwent curative resection of tumors. Twenty-three carcinomas were located in the proximal colon (cecum, ascending colon, hepatic flexure, and transverse colon), whereas 27 had distal location (splenic flexure, descending colon, sigmoid, and rectum). Six patients had multiple tumors in the moment of surgery. Of 58 CRC patients 2 (3.45%) were stage I, and 30 (51.72%) were stage II, and 26 (44.83%) were stage III of disease at surgery. In respect of histological type, classic adenocarcinoma was dominant (84.48%) with 6 (10.35%) mucinous and 3 (5.17%) medullary type of CRC. Among colorectal tumors 17 (29.31%) had low-grade and 41 (70.69%) high-grade differentiation. The intensity of immune host reaction in context of clinicopathological characteristics and microsatellite status of colorectal tumors is presented in Table 1.

The average number of lymphocytes in peritumoral stroma at the invasive front of CRCs in 5 high power fields ($\approx$1 mm^2) was 37.04(10.46). There were no significant differences in the average number of lymphocytes in peritumoral stroma and clinicopathological characteristic of CRC. Differences in the intensity of peritumoral lymphocytic infiltration according to the gender of patients and microsatellite status of tumor tissue almost reach statistical

Table 1. The comparison between host immune features and several clinicopathological characteristics and microsatellite status of colorectal carcinomas

	Peritumoral lymphocytes*	p value	Crohn's-like lymphoid reaction§	p value	Intraepithelial lymphocytes*	p value
Sex						
Female (n=31)	35.45		54.84%		10.39	
Male (n=27)	30.37	0.053	37.04%	0.275	10.07	0.777
Age						
<50 years (n=15)	35.00		40.00%		10.93	
≥50 years (n=43)	32.42	0.395	48.84%	0.656	10.00	0.458
Site of tumor						
Right (n=23)	34.26		52.17%		11.65	
Left (n=29)	29.71		37.93%		9.41	
Multiple (n=6)	39.56	0.079	66.67%	0.292	8.83	0.063
Stage of disease						
I. (n=2)	28.50		50.00%		9.50	
II. (n=30)	29.23		53.33%		10.51	
III. (n=26)	29.71	0.810	38.46%	0.396	9.69	0.436
Histologic type						
Adeno (n=49)	33.08		44,90%		9.80	
Mucinous (n=6)	36.16		50.00%			13.00
Medullary (n=3)	27.00	0,756	66.67%	0.822	12.00	0.079
Histologic grade						
High-grade (n=41)	34.33		53.38%		11.10	
Low-grade (n=17)	32.49	0.5114	43.24%	0.692	9.76	0.241
Microsatellite status						
Stable (n=30)	29.60		36.67%		7.47	
Instable (n=28)	34.82	0.052	67.86%	0.034	13.21	0.0001

* - average number; § - presence in percentage

significance. The conspicuous Crohn's-like lymphoid reactions were present in 19/28 (67.86%) of MSI CRCs, versus 11/30 (36.67%) of MSS cases (χ^2=4.463, p=0.034). The statistically nonsignificantly explicit Crohn's-like lymphoid reactions were found in the multiple and right side localized CRC and in female patients. The average number of TIL between colorectal tumor cells in 5 high power filed (≈1 mm^2) was 10.24(4.1520), (range: 0-21 lymphocytes). In colorectal tumor without replication error the average number of TIL was 7.4 (2.69), (range: 0-12 lymphocytes). In the tumor with microsatellite instable molecular features number of T-lymphocytes ranged from 6 to 21, with average number of 13.21(3.30). The differences between TIL according microsatellite status of CRCs tumors were statistically significant (p<0.0001). Statistically nonsignificant trend toward highest number of TIL was recorded in right side CRC, and in the mucinous and medullary histological type of tumors.

DISCUSSION

The cutaneous malignant melanoma is the most extensively investigated human malignancy in tumor immunology, and is considered a highly immunogenic tumor, with clinical and experimental evidence for some level of immunological control of tumor growth (26). CRC with MSI represents a subtype of CRC tumors that is associated with pronounced host immune responses with the possible influence on the apparently favorable clinical outcome displayed by these patients. Characteristic histological features exist for CRCs with defective DNA MMR; among them are the marks of explicit immune host response: SIL, Crohn's-like lymphoid reaction, and TIL by cytotoxic T lymphocytes. HNPCC cancers were more like traditional CRCs apart from higher frequency of lymphocytic infiltration (1,5,11). In our work, CRCs with MSI carried significantly higher numbers of T lymphocytes infiltrating within neoplastic epithelial structures, as shown by immunostaining for CD3 (13.21 versus 7.47, p<0.0001). In the most of our MSS cases, the infiltration of mononuclear cells was generally restricted to the stromal areas with only few lymphocytes infiltrating between neoplastic cells. A statistically significant conspicuous Crohn's-like lymphoid reaction was found in the 67.86% in tumors with MSI, versus 36.67% MSS CRC cases (p<0.05). Statistically nonsignificant trend toward highest intensity of peritumoral lymphoid infiltration was also registered in the CRCs with replication errors and microsatellite instability. The presence of peritumoral Crohn's-like lymphoid and intraepithelial lymphocytosis reaction was an intensive marker for MSI-H tumors in the results of by Alexander et al. (10). MSI was the major predictor of the amount of activated cytotoxic CD8$^+$ T lymphocytes infiltrating within neoplastic epithelial structures in the study of Dolcetti et al., and content of activated cytotoxic TILs in CRCs with MSI is strictly associated with higher numbers of apoptotic neoplastic cells. The peritumor lymphoid nodules and increased number of activated cytotoxic TILs probably represent different aspects of a complex host immune response against the tumor (13). Tumor-associated lymphocytes (Crohn's-like, peritumoral and tumor infiltrating) are more frequently seen in HNPCC cancers, but differences between familial and nonfamilial microsatellite unstable CRCs had no high statistical significance (27). Another study confirmed that HNPCC group and the sporadic MSI group exhibited similar lymphocytic infiltration findings (28). The peculiar genetic instability in MSI tumors may lead to a continuous production of abnormal peptides that, by acting as neoantigens, could elicit specific antitumor immune responses potentially effective in limiting tumor growth and/or spread (17). There was identified a number of peptides, novel class of tumor-associated antigens in tumors with replication errors, with possible immunogenic function, derived from a frameshift mutation in TGFRII (29,30), OGT (31), Caspase-5, TAF-1b, and HT001 genes (32). Applying the microarray technology which allows rapid gene expression profiling of tissue-derived RNA to give an mRNA expression signature, upregulation of a large number of pro-inflammatory genes was found in microsatellite unstable CRCs, as a strong indicator that immune response is activated in these tumors. Significantly, many key immunomodulatory genes are upregulated in cancers with MSI. They include antigen chaperone molecules (HSP-70, HSP-110, Calreticulin, gp96), pro-inflammatory cytokines (Interleukin (IL)-18, IL-15, IL-8, IL-24, IL-7), and cytotoxic mediators (Granulysin, Granzyme A) (33). The specific antitumor immune responses are certainly not single factor responsible for limiting tumor growth and/or spread. One possible explanation for the favorable outcome of these patients is that the enhanced mutation rate associated with MSI, besides favoring mutations at genes critical for oncogenesis, may also induce a mutation burden no longer compatible with tumor cell survival (17). On the other hand it is supposed that the process of cancer immunoediting holds that the immune system not only protects the host against tumor development but can also promote tumor development by selecting for tumor escape variants with reduced immunogenicity (34). Distinct group of CRCs may have inherent immunogenic properties and that further elucidation of these may be invaluable to the development of successful immunotherapy. There is a belief that MSI-H may be a natural paradigm of host-tumor interactions in immunogenic CRC and as such further studies are required to clarify the nature of immune responses in these tumors. The use of heat shock proteins and abnormal peptides, derived from frameshift mutations in coding microsatellites, is already promising in advancing the field of immunotherapy and in the design of a vaccine against colorectal tumors with MSI (32,34).

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Conflict of interest

We declare no conflicts of interest.

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