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Correlation of Microsatellite Status, Proliferation, Apoptotic and Selected Immunohistochemical Markers in Colorectal Carcinoma Studied with Tissue Microarray*

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Colorectal carcinoma is a frequent malignant tumor, characterized by varying clinical course and response to treatment. At the molecular level, colorectal carcinomas are divided into tumors with chromosomal instability (microsatellite-stable, MSS), microsatellite instability (MSI-H) and low microsatellite instability (MSI-L). The method of tissue microarrays allows for combining materials originating from multiple patients into a single slide, what makes possible to simultaneously investigate large material for the presence of numerous, diversified markers. The study material consisted of 208 cases of colorectal carcinoma. Microsatellite instability was evaluated in frozen material employing the PCR reaction with gel and capillary electrophoresis. Following a standard histopathological assessment, tissue microarrays were prepared using a MTA-1 microarrayer (Beecher) and standard immunohistochemical reactions were performed to detect the presence of bcl-2, CDX-2, Ki67, MLH1, MSH2, MSH6, p16, p53 and survivin. Apoptotic cells were detected using the TUNEL method. The correlations between the reactions were investigated and differences in the expression of the investigated proteins noted in carcinomas with various degrees of microsatellite instability. The agglomeration analysis showed differences in patterns of expression between MSS, MSI-L and MSI-H carcinomas. The discriminant function analysis demonstrated that the MSI-H carcinomas were best differentiated by MLH1, survivin and Ki67 expression, while the MSI-L tumors differed from the remaining colorectal carcinomas by their apoptotic index, local tumor stage (pT), the presence of angioinvasion and mucin production.

Introduction

Colorectal carcinoma is among the most common malignant tumors in well-developed countries. Its incidence in the United States amounts to 44/100,000/year and in Europe to 35/100,000/year, with a clearly visible increasing tendency [10]. Therefore, in order to improve the prognosis, it is necessary to understand better the biology of the tumor and factors which determine its aggressiveness. The pathogenesis of colorectal carcinoma is not uniform; in the majority of cases, chromosomal instability is observed, while 10–20% of cases are associated with DNA repair defects and microsatellite instability (MSI). A separate group is said to consist of carcinomas with microsatellite instability involving some loci (MSI-low) [13, 22, 26, 39]. The tissue microarrays technique [18] allows for simultaneously performing reactions in tissue specimens originating from numerous patients, what allows for investigating the presence of numerous antigens in a large series of patients.

Material and Methods

The material consisted of unselected cases of colorectal carcinoma treated at First Chair and Department of General

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and Gastrointestinal Surgery, Collegium Medicum and cases taken from the archives of Department of Pathomorphology. Immediately after removal, the specimens were transferred to the Department of Pathomorphology, where gross examination was done. Material from the tumor and uninvolved intestinal wall was snap-frozen and stored at –80°C for molecular studies. The remaining material was fixed overnight in 10% buffered formalin for histopathological examination. Sections were taken following the standard protocol [33]. The sections were processed routinely using automatic tissue processors (Shandon, Astmoor, UK), and embedded in paraffin. Paraffin blocks were then cut into 4 μm-thick sections and stained with hematoxylin-eosin for histopathological assessment. The assessment was performed following the routine protocol, the stage was determined according to the TNM classification [1].

Microsatellite analysis was performed according to the previously published protocol [29]. Briefly, DNA was extracted from fresh-frozen tumor and corresponding non-neoplastic tissue (QIAamp DNA Mini Kit, Qiagen GmbH, Hilden, Germany), and PCR-amplified with a screening panel of five microsatellite markers: APC, p53, BAX, BATR II and BAT–26. PCR was performed in 20 μl of the reaction mixture containing: 2 μl DNA template (100 ng), 2 μl STR buffer (Promega Corp., Madison, USA), 0.5 μl of each primer (10nM), 1 U Taq polymerase (Fermentas Inc., Burlington, Canada). The amplicons were electrophoresed on 6% polyacrylamide gel at 50 W for 1.5 h and visualized using routine silver staining. All the cases demonstrating any, even single, genetic alterations at any marker of the screening panel were subjected to a further analysis with an extended panel of nine microsatellite markers (Microsatellite Instability RER/LOH Assay Kit, Applied Biosystems, Foster City, USA) and PCR products were visualized using capillary electrophoresis with an ABI PRISM 310 Analyzer (Applied Biosystems, Foster City, USA). The kit contains nine primer sets flanking microsatellite loci linked to tumor-suppressor genes: MSH2 (D2S123), DCC (D18S35), APC (D5S346). MLH1 (D3S1611), NM23, HPC1 (D1S2883), MET (D7S501), a dinucleotide marker linked to p53, and a pentanucleotide marker linked to the same gene. The results were analyzed by the Genescan and Genotyper Software (Applied Biosystems, Foster City, USA). A locus was deemed unstable when an electrophoregram of a PCR product derived from the tumor differed from that of normal matching tissue by the presence of at least one new peak with the length corresponding to 2 bp or 5 bp. A case was included into the MSI-low group when showing genetic instability at more than one, but not more than 40% of loci. The tumors were classified as MSI-high when MSI was detected at 40% or more loci analyzed in a given case. Additionally, as the literature strongly supports the high specificity of the BAT-26 marker in respect to the MSI-high phenotype, all tumors with instability at BAT-26 were included in the MSI-high group. The remaining cases were classified as microsatellite-stable (MSS) carcinomas. Tumor allele showing at least a 50% reduction in band/peak intensity in comparison with the corresponding band/peak of normal mucosa was assessed as LOH [23].

Hematoxylin-eosin stained preparations were reviewed and in each case, a section was selected that would contain representative and well-preserved tumor tissue. The site on the preparation that contained such an area was marked with a felt-tip pen. Three tissue cores were then taken from the corresponding paraffin block using a MTA-1 microarrayer (Beecher Instruments Inc., Sun Prairie, USA) and they were placed into the acceptor block. From it, 3 μm-thick sections were prepared, deparaffinized and used for immunohistochemical reactions. These were done by standard method. Briefly, the slides were rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2 N NaOH) 3×5 minutes at 750 W. The primary antibodies are listed in Table 1. The ENVISION + (DAKO, Glostrup, Denmark) detection system was used. It consists of several goat anti-mouse antibody molecules attached to a dextran backbone coupled with horseradish peroxidase molecules, and allows for obtaining high signal-low background reactions. 3-amino-9ethylcarbasole (DAKO, Glostrup, Denmark) was used as chromogen. The slides were contrastained with Mayer hematoxylin (DAKO, Glostrup, Denmark). The processing was done using DAKO Autostainter device (DAKO, Glostrup, Denmark). Apoptotic cells were detected using the ApopTag Peroxidase Kit (S7100, Intergen Company, Norcross, USA), in situ labeling the free 3'-OH DNA termini. The staining was per-

TABLE 1
Antibodies used in the study

Specificity	Type/clone	Manufacturer	Dilution
Bcl-2	100	Immunotech	Stock
CDX-2	CDX2–88	Biogenex	1 : 50
Ki67	MIB–1	DAKO	1 : 50
MLH1	C–20	Santa Cruz Biotechnology	1 : 50
MSH2	N–20	Santa Cruz Biotechnology	1 : 50
MSH6	44	BD Biosciences	1 : 100
p16	ZJ–11	Chemicon	1 : 50
p53	DO–7	DAKO	1 : 50
survivin	4F7	Lab Vision	1 : 50

formed according to the manufacturers' protocols. The assessment was performed independently by two of authors (KO and SD), evaluating the extent and intensity of the reactions. The persons involved in the assessment were unaware of the distribution of particular cases within a block or of any other clinical and pathological data. The values obtained for a given case were averaged.

The statistical analysis was performed using the STATISTICA, v. 6.1 PL software (StatSoft, Inc., Tulsa, USA). The significance level was set to 0.05.

Results

The material consisted of 208 cases of colorectal carcinoma; the group included 111 males and 97 females. The mean age of the patients was 64.8 years, with the range from 23 to 91years and SD of 11.48. One hundred twelve cases were patients operated at First Chair of General Surgery. The remaining 96 cases were taken from the archives of the Chair of Pathomorphology.

The tumors were situated in the cecum in 15 cases, in the ascending colon – in 11 cases, in the hepatic flexure – in eight cases, in the transverse colon – in ten cases, in the splenic flexure – in three cases, in the descending colon – in four cases, in the sigmoid – in 67 cases, and in the rectum in 90 cases. The tumor did not infiltrate the muscularis propria (pT1) in four cases, infiltrated, but not exceeded the muscular layer (pT2) in 59 cases, penetrated through it (pT3) in 136 cases, and involved the adjacent structures (pT4) in nine cases. In 109 cases no metastatic deposits were found in the lymph nodes, 47 cases demonstrated one to three metastatic foci (pN1), and in 52 cases, more than three metastases were noted (pN2). At the time of surgery, 16 patients showed distant metastases. In 142 cases no angioinvasion (pV0L0) was seen, in 29 patients, larger vessels were involved (pV1L0), in 22 cases, the involvement was noted in small vessels only (pV0L1), while 15 individuals revealed both small and larger vessels involved by the tumor (pV1L1). In 22 cases, surgery was not histologically radical (pR1). The carcinoma was low grade (G-I) in 44 cases, showed a medium degree of differentiation (G-II) in 143 patients, and was poorly differentiated (G-III) in 21 cases.

Reaction for p16 was negative in 117 cases, weak in 49 cases, and strong in 42 cases. Reaction for p53 was negative in 193 cases, weak in ten, and strong in five. Reaction for bcl-2 was negative in 168 cases, weak in 27, and strong in 13. Reaction for MLH1 was negative in 35, reduced in 109, and strong in 64 cases. Reaction for MSH2 was negative in 15 cases, reduced in 35, and strong in 158. Reaction for MSH6 was negative in three cases, reduced in 78, and strong in 127. Reaction for survivin was negative in 72 cases, weak in 56, and strong in 80 cases. Reaction for CDX-2 was negative in seven cases, reduced in 26, and strong in 175. The mean apoptotic index amounted to 5.6 (range 0 to 30.5, SD 3.74). The mean proliferative index Ki67 equaled 45.4 (range 0 to 100, SD 28.26).

Reaction for MSH2 was correlated with CDX–2 ($R=0.43$, $p<0.01$), MLH1 ($R=0.39$, $p<0.01$), MSH6 ($R=0.37$, $p<0.01$), survivin ($R=0.35$, $p<0.01$), the Ki67 index ($R=0.33$, $p<0.01$), and the apoptotic index ($R=0.21$, $p<0.01$). Reaction for MLH1 demonstrated correlation with CDX-2 ($R=0.26$, $p<0.01$), the proliferative index Ki67 ($R=0.22$, $p<0.01$), MSH6 ($R=0.18$ $p<0.01$), the apoptotic index ($R=0.14$ $p<0.04$), and bcl–2 ($R=0.12$, $p<0.01$). Reaction for MSH6 was correlated with the apoptotic index ($R=0.23$, $p<0.01$) and the proliferation index Ki67 ($R=0.12$ $p<0.1$). Survivin expression showed correlation with CDX2 ($R=0.32$, $p<0.01$), MSH6 ($R=0.30$, $p<0.01$), p16 ($R=0.29$, $p<0.01$), and MLH1 ($R=0.21$, $p<0.01$), as well as with the number of metastases in the lymph nodes (R-0.14 $p<0.04$). The percentage of involved lymph nodes was correlated with the degree of tumor differentiation ($R=0.26$, $p<0.01$). Reaction for p16 demonstrated a correlation with the proliferative index Ki67 ($R=0.20$ $p<0.01$), MSH6 ($R=0.17$, $p<0.02$), and bcl-2 ($R=0.13$, $p<0.06$). Reaction for bcl-2 was correlated with CDX–2 ($R=0.17$ $p<0.02$). Reaction for CDX-2 showed a correlation with the proliferation index Ki67 ($R=0.15$ $p<0.03$).

MSS and MSI-L carcinomas showed a tendency to being located in the left part of the colon ($p<0.01$). Non-radical procedures were encountered solely among patients with MSS and MSI-L tumors ($p<0.04$). In MSI-H carcinomas, a positive reaction for survivin was somewhat more frequent ($p<0.09$). Only in one MSI-H case strong MLH1 expression was observed ($p<0.02$). Carcinomas with microsatellite instability more often demonstrated the presence of intratumoral lymphocytes ($p<0.0007$). MSI-L carcinomas were slightly more common in male patients ($p<0.08$) as compared to other tumor types.

The agglomeration analysis was performed, and the results are shown in Figure 1. To detect which of the parameters would classify the cases into the microsatellite stable and unstable types, the discriminant function analysis was used. Parameters that best differentiated the MSI-H tumors included MLH1 expression ($p<0.02$), as well as survivin ($p<0.06$) and the proliferative index ($p<0.06$); the MSS colorectal carcinomas were best differentiated by the apoptotic index ($p<0.02$) and MLH1 expression ($p<0.08$), as well as the degree of histological differentiation ($p<0.08$), while the MSI-L group was best described by the apoptotic index ($p<0.005$), pT ($p<0.04$), pL ($p<0.05$), and the mucin production ($p<0.07$).

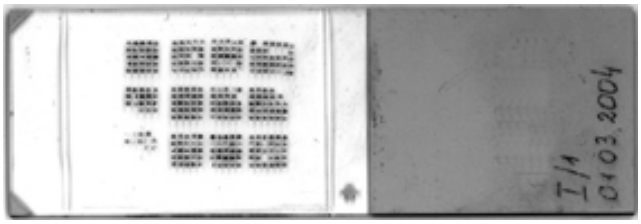

Fig. 1. A typical view of a tissue microarray section.

Discussion

In order to improve the outcome of a malignancy it is necessary to improve the knowledge of tumor biology and to determine factors affecting the prognosis and response to treatment. The most effective prognostic factor is the tumor staging; in the case of colorectal carcinomas the most frequently used include the Dukes, Astler-Coller and TNM systems [1]. An additional, independent information on the clinical course of the disease is provided by the degree of differentiation and histological subtype. Immunohistochemistry is at present a standard and easily available diagnostic method. To improve its performance, tissue microarrays may be employed. The technique consists in constructing a paraffin block that contains material originating from several different cases. Tissue cylinders are prepared from representative sites of original paraffin blocks using a needle 0.6–2 mm in diameter. The resultant cylinders are subsequently placed in a previously prepared new paraffin block and the exact location of each case is recorded. The resulting tissue microarray may serve for preparation of standard sections for special staining, chiefly including immunohistochemical reactions or fluorescent in situ hybridization. The method allows reduce labor and costs, allowing investigation of numerous reactions in large groups of patients [18, 30]. In managing patients with malignant tumors, increasing interest is given to its molecular markers. Analysis of molecular features in colorectal carcinoma shows that the majority of cases develop through the chromosomal instability. In carcinomas of this type, numerous, extensive changes consisting in loss or gains of genetic material occur within the genome. At the molecular level, this phenomenon is manifested as loss of heterozygosity (LOH) in numerous loci. Approximately 10–20% of cancers are associated with DNA repair defects, what is expressed by the microsatellite instability (MSI). A prototype disease in which MSI is observed, is Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome; nevertheless, carcinomas of this type occur also sporadically. Patients with HNPCC demonstrate mutations involving the hMSH2 and hMLH1 genes, while in sporadic cases pathogenesis depends on inactivating methylation of the hMLH1 promoter region [11, 20]. In consequence of DNA repair mechanisms impairment, mutation rate increases. The increased mutation rate may be several hundred times higher as compared to normal tissues. This is particularly true in the case of short repetitive sequences, such as microsatellite DNA [3, 6, 26]. An interesting property of colorectal carcinomas with microsatellite instability is a higher frequency of their multi-focal occurrence [25]. The degree of DNA repair impairment may vary. Cases in which changes involve less than 30–40% of the studied microsatellites are termed "MSI-low" [3]. The classification of this group of tumors is unclear. Their clinical and morphological properties are similar to MSS group. However, the presence of any MSI does not quite fit the accepted concept on the pathogenesis of carcinomas with chromosomal instability. Therefore, Jass et al. proposed that MSI-low cancers should not be treated as the "grey zone", but rather as a separate group with a separate origin and pathogenesis [13]. According to Whitehall et al., in cancers from this group methylation MGMT gene promoter is increased. The product of this gene participates in a DNA repair system separated from MSH/MLH proteins and responsible for correcting the G $\rightarrow$ A transit. Mutations of this type occur within the K-ras gene, what suggests a possible developmental pathway for MSI-low cancers [39].

The prognostic importance of microsatellite instability remains unclear, although evidences are accumulating that it is an independent prognostic factor. This is particularly true for the MSI-H category; these carcinomas were associated with a better prognosis [14, 17, 21], although according to Kakar et al. [16], the MSI status is of a prognostic significance solely in the one-dimensional analysis, while other publications completely fail to confirm such an association [8, 9, 42]. A possible explanation of better prognosis may be the increased apoptotic activity [37]. In the present material, when the apoptotic index was categorized into <5%, 5–10% and >10% groups, it was found that apoptotic activity was increased to some degree in cases showing microsatellite instability, and especially in the MSI-L group ($p<0.01$, Fig. 2). Another possible explanation may be that within such tumors lymphoid cells are characteristically more numerous as compared to other cancer types. These cells are characterized by a cytotoxic phenotype and activated [31]. On the other hand, the response of MSI-H cancers to chemotherapy is supposed to be poorer than in the case of other colorectal carcinomas [4], although some publications do not confirm that [7]. Data on the significance of the MSI-L category are scarcer. Wright et al. observed that tumors of this type were associated with a poorer prognosis as compared to carcinomas without microsatellite instability [40].

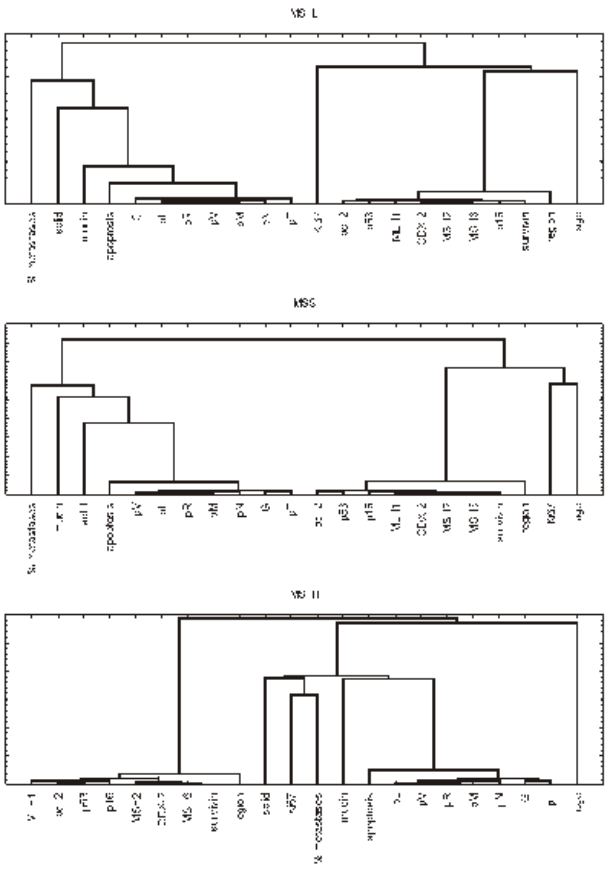

Fig. 2. Agglomeration of the studied variables according to microsatellite status. Note similarity of the plots for MSS and MSI-L but not MSI-H. (Where: age – age of the patient at operation; region – location of the tumor along the large intestine; G – degree of differentiation; solid - percentage of solid areas; mucin – percentage of the tumor with significant mucin production; pT, pN, pM, pV, pL, pR – as defined in TNM classification; % metastases – percentage of lymph nodes containing metastases; Ki67 – Ki67 index; bcl–2, p53, MLH1, CDX–2, MSH2, MSH6, p16, survivin – expression of markers – see Methods section; apoptosis – apoptotic index.

In consequence of the emerging prognostic implications, the assessment of molecular background of colorectal carcinoma is increasingly significant. The classic method of evaluation is based on investigating changes in numerous microsatellite loci [3]. This, nevertheless, is a labor and cost consuming procedure, hence, a search for alternative methods is necessary. To increase performance additional selection of cases is necessary, especially in familial setting [3]. Brenetot et al. [20] believe that molecular assessment of BAT-25 and BAT-26 only was successful in identifying MSI-H cases. Smyrk et al. observed that the presence of intratumoral lymphocytes might constitute a sufficient criterion for differentiating MSI-positive colorectal carcinomas [34]. Immunohistochemistry may help to identify cases for molecular studies [15]. This is based on assessing expression of proteins that constitute elements of DNA repair systems, as MLH1 MSH2 and MSH6. In MSI-H carcinomas, significant reduction in expression of these proteins is expected, regardless whether such a decrease has been triggered by a germ-line mutation in HNPCC, or by promoter methylation in sporadic carcinomas. Marcus et al. achieved promising results by this method [24]. On the other hand, Chapusot et al. noted that immunohistochemistry was of limited value in detecting microsatellite instability and could not replace molecular assessment, because method standardization posed a significant problem [5]. Doubts as to the validity of classifying colorectal carcinomas based on immunohistochemical results were also articulated by Arnold et al. [2]. In the present study it was shown that effective classification of colorectal carcinomas may be immunohistochemistry-based screening followed by assessment of microsatellite instability when even focal reactivity impairment is present.

Survivin is a relatively recently recognized apoptosis inhibitor. Its expression is said to be associated with a poorer prognosis in many tumors, including colorectal carcinoma [36]. At present, trials are in progress on therapeutic methods that act through survivin [38, 41, 43]. The protein product of the p53 gene participates in the mechanisms of proliferation control, DNA repair and DNA damage-associated apoptosis. Mutations in the p53 gene are one of most common phenomena in human cancer, seen in approximately one-half of cases. Since the wild gene product is extremely short-lived, while the product of a mutated gene is accumulated in the cell, the presence of mutation may be evaluated in a relatively easy way. The incidence of the p53 gene damage in colorectal carcinomas approaches 50% [32]. Moreover, p53 mutations may affect the prognosis [35]. Bcl-2 participates in apoptosis regulation and protects tumor cells from death through apoptosis; it also affects telomerase activity [12]. Changes in bcl-2 expression are supposed to be frequent in colorectal carcinomas; what is more, they may affect the prognosis, but this remains not completely understood [19, 27, 28].

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