Pol J Pathol 2006, 57, 1, 29–33 PL ISSN 1233-9687

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High Thymidylate Synthase Expression is Typical for Sporadic MSI-H Colorectal Carcinoma*

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Colorectal carcinoma is etiopathologically heterogenic. It may develop through a sequence of mutations leading to chromosome instability or be a result of defects in DNA repair mechanisms manifested by microsatellite instability. Carcinomas of this type are supposed to be characterized by a better prognosis and a different response to chemotherapy. The main target of 5-fluorouracil (5-FU) treatment is thymidylate synthase (TS). High TS expression has been identified as promoting resistance to 5-FU. The objective of the present investigation was to determine whether microsatellite instability is associated with thymidylate synthase expression. Ninety-eight cases of colorectal carcinoma were studied. Microsatellite instability was evaluated in frozen material employing the PCR reaction with gel and capillary electrophoresis. TS expression levels were assessed in preparations stained immunohistochemically using a semiquantitative method on a scale with scores from 0 to 3. The MSI-H phenotype was detected in ten cases, MSI-L in 16, and MSS in 72. The mean TS expression score was 1.79. In the MSS group, the mean TS expression score was 1.69, in the MSI-L group the mean TS expression score was 1.73, and in the MSI-H group the mean TS expression score was 2.67. The differences between MSI-H and MSS/MSI-L were statistically significant ($p<0.0002$ and $p<0.004$, respectively). The results may explain the different response of MSI-H carcinomas to 5-FU treatment.

Introduction

According to current opinions, the pathogenesis of colorectal carcinoma (CRC) is not uniform. The majority of cases are associated with chromosome instability, while 10–20% is associated with defects of DNA repair and microsatellite instability (MSI). Carcinomas with microsatellite instability in some loci (MSI-L) have been suggested to constitute the result of the „third route of carcinogenesis" in the colon, yet the issue is still not completely clear [4, 12, 13]. The place of such carcinomas in the classification system continues to be unclear, although the present authors are of the opinion that they constitute a separate category [29]. An important method that improves the prognosis in colorectal carcinoma is chemotherapy. A decreased effectiveness of 5-fluorouracil (5-FU) treatment is supposed to be associated with an increased thymidylate synthase expression [15]. MSI-H carcinomas have been found to show a significantly poorer response to 5-FU treatment. The objective of the present investigation was to determine whether microsatellite instability is associated with thymidylate synthase expression.

Material and Methods

The material consisted of unselected cases of colorectal carcinoma treated at the 1st Department of Surgery, Collegium Medicum, Jagiellonian University. Fresh surgical specimens were transferred to the Chair of Patho-

* The work was supported by the grants No. 3 P05C 034 23 and No. PBZ-KBN-091/PO5/2003/24 of Ministry of Science and Informatics, formerly State Committee of Scientific Research (KBN).

morphology and examined grossly. Tumor samples were selected from the peripheral zone, avoiding the ulcerated and necrotic areas, and from the intestinal wall at the midpoint between the lesion and the margin more distant from the tumor. The collected specimens from the tumor and uninvolved intestinal wall were frozen at –20°C and stored for molecular analysis. The remaining material was fixed in 10% buffered formalin for 24 hours.

Microsatellite analysis was performed according to the previously published protocol [20, 28, 29]. Briefly, DNA was extracted from a fresh-frozen tumor and corresponding non-neoplastic tissue (QIAamp DNA Mini Kit, Qiagen), and PCR-amplified with a screening panel of five microsatellite markers: APC, p53, BAX, BATR II and BAT-26. PCR was performed in 20 l of reaction mixture containing: 2 l DNA template (100 ng), 2 l STR buffer (Promega), 0.5 l of each primer (10nM), 1 U Taq polymerase (Fermentas). 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 further analysis with an extended panel of nine microsatellite markers (Microsatellite Instability RER/LOH Assay Kit, Applied Biosystems) and PCR products were visualized using capillary electrophoresis with an ABI PRISM 310 Analyzer (Applied Biosystems). 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). 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-L group then showing genetic instability at more than one, but not more than 40% of loci. The tumors were classified as MSI-H 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-H phenotype, all tumors with instability at BAT-26 were included in the MSI-H group. The remaining cases were classified as microsatellite-stable (MSS) carcinomas.

The gross assessment and dissection for histology was performed following the standard protocol [26]. The tissue specimens were routinely processed using automatic tissue processors (Shandon) and paraffin embedded. Paraffin blocks were cut into 3 m-sections stained with hematoxylin and eosin for routine histology. The tumor stage was determined according to the TNM system [8].

HE sections were reviewed and in each case a section was selected that would contain representative and well-preserved carcinoma. Selected paraffin blocks served for preparing tissue microarrays using a Tissue MicroArrayer MTA-1 (Beecher Instruments Inc., WI, USA). From each donor block, three 0.6 mm cylinders were cut off. The acceptor paraffin blocks were prepared noting the location of each cylinder, and 3 m-thick sections were cut. Immunohistochemical reactions were performed routinely. Briefly, the slides were dewaxed, rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was performed by microwaving in EDTA solution 3×5 minutes at 750 W. The anti-thymidylate synthase antibody clone MAB4130 (Chemicon, USA) was used in the concentration of 1:50. The ENVISION + (DAKO, Denmark) detection system was employed. It consists of several goat anti-mouse antibody molecules attached to a dextran backbone coupled with horseradish peroxidase, and allows for high signal-low background reactions. 3-amino-9-ethylcarbasole (DAKO, Denmark) was used as the chromogen. The slides were contrastained with Mayer hematoxylin (DAKO, Denmark). The processing was done using the DAKO Autostainter device (DAKO, Denmark). The staining results were assessed semi-quantitatively employing a 0–3 scale, where 0 was no reaction, 1 was a weak cytoplasmic reaction, 2 definitive cytoplasmic reaction, and 3 represented a very intense reaction. From the scores of individual tissue cores (3 per case), arithmetic means were calculated and used as result. The statistical analysis was performed using the STATISTICA 6 PL (StatSoft Inc., USA). To demonstrate inter-group differences, the Kruskall-Wallis ANOVA was used. The significance level was set to 0.05.

Results

The material consisted of 98 cases of colorectal carcinoma; of these, 43 patients were females and 55 males. The mean age of the patients was 64 years (range 34 to 87, SD 10.0). The age showed no sex-associated differences. The tumors were situated as follows: the sigmoid – 34 cases (34.69%), rectum – 38 cases (38.78%), ascending colon – 5 cases (5.1%), cecum – 10 cases (10.2%), transverse colon – 4 cases (4.08%), descending colon – 2 cases (2.04%) and the right flexure – 5 cases (5.1%). The tumors were Astler-Coller stage A in 4 cases (4.1%), B-1 in 27 cases (27.6%), B-2 in 15 cases (15.3%), C-1 in 8 cases (8.2%),

C-2 in 32 cases (32.7%), and D in 12 cases (12.2%). Lymph node metastases were absent (N0) in 47 cases (47.9%), less than 4 (N1) in 18 cases (18.4%), and four or more (N2) in 33 cases (33.7%). In the cases with lymph node metastases, the mean number of the involved nodes was 7.04 (range 1–24, SD 6.2) and the mean percentage of the involved nodes was 39.4% (range 3–100%, SD 28.4). The tumors were well differentiated (G-I) in nine cases (17.6%), moderately differentiated (G-II) in 36 cases (70.6%) and poorly differentiated (G-III) in six cases (11.8%). At the molecular level the MSS phenotype was present in 72 cases (73.5%), MSI-H phenotype in 10 cases (10.2%), and MSI-L phenotype in 16 cases (16.3%).

The mean TS expression score was 1.79, the median 2, SD 0.69. When comparing TS expression in colorectal carcinomas with varying degrees of microsatellite instability, the mean value in the MSS group was 1.69, with the median of 2, in the MSI-L group the mean value was 1.73, with the median of 2, and in the MSI-H group mean value was 2.67, with the median of 3. The differences were statistically significant (Kruskall-Wallis ANOVA $p<0.002$). The post-hoc analysis demonstrated that the differences between MSI-H/MSS and MSI-H/MSI-L were statistically significant ($p<0.0002$ and $p<0.004$, respectively), while the difference between MSI-L and MSS was non-significant.

Discussion

Colorectal carcinomas constitute a heterogenic group of cancers. These include familial cancers, associated with genetic syndromes as familial adenomatous polyposis and hereditary non-polyposis colorectal cancer (HNPCC), as well as sporadic carcinomas, which represent a vast majority of cases [2, 11, 19, 30, 37]. With respect to pathogenesis, two basic mechanisms of colorectal carcinoma development are recognized. One is analogous to the mechanism underlying adenomatous polyps and is associated with significant abnormalities appearing in the genome, what is manifested as chromosome instability (MSS). The other is associated with a more subtle genome instability, which is manifested as changes within short repetitive sequences, especially microsatellite DNA [4, 19]. As compared to tumors with chromosome instability, carcinomas with microsatellite instability (MSI-H) are characterized by their more proximal location, more frequently show a lower degree of differentiation and are mucus producing, as well as manifest numerous lymphocyte infiltrations within the tumor tissue [4, 30, 37]. Apart from these two well-defined categories, there are colorectal carcinomas with a less evident microsatellite instability; such tumors are characterized by changes observable in less than 40% of the investigated microsatellite loci and – what is interesting – they demonstrate defects involving other DNA repair mechanisms than typical cancers with microsatellite instability [35]. This group has been named MSI-L, although its position in the classification continues to be unclear [22, 34, 36].

Apart from unquestionable differences in the pathogenesis of the above-presented types of colorectal carcinomas, there is still the issue of the practical importance of such a classification. Opinions on the subject are not unambiguous [7, 9–11]; however, the currently prevailing view is that the MSI-H tumors are associated with a better prognosis [4]. In spite of the fact that such tumors are supposed to be larger in size [37], at the same time they are characterized by a lower incidence of metastases, both distant [10] and locoregional [4]. The prognostic significance of the MSI-L category is not clear. Although MSI-L cancers may demonstrate lower proliferation indices than other categories, the prognosis was claimed to be worse than MSS [30]. Wright observed that although the overall survival in this group was no different than in patients with colorectal carcinomas developing via the chromosome instability (MSS), yet the mortality rate directly associated with the tumor was significantly higher in the MSI-L group [36]. The molecular basis of the prognostic differences in particular types of colorectal carcinomas is unclear. Phillips et al. seek the reason for a better prognosis in the MSI-H cancers in the higher immunogenicity of this group of tumors [23]. Indeed, the authors noted that tumor infiltrating lymphocytes in cancers of this type are cytotoxic, activated T lymphocytes.

In treating colorectal carcinomas, apart from surgery, a significant place is occupied by chemotherapy. Unfortunately, some of these tumors are characterized by a significant resistance to chemotherapy, which may depend on various molecular mechanisms, such as for example an increased level of glutathione S-transferase [2, 6, 18]. Classic therapeutic agents that are employed here are derivatives of pyrimidine bases, such as 5-fluorouracyl (5-FU). Thymidylate synthase (TS) is an enzyme that converts UMP into TMP, and 5-FU acts just on TS. In colorectal carcinomas resistant to chemotherapy based on 5-FU Johnston et al. found a significant rise of TS levels [15], what was confirmed in numerous analyses [3, 21], yet other authors claimed that patients with colorectal carcinomas with TS overexpression might benefit from 5-FU therapy [1, 34]. High TS expression may not only affect the effectiveness of chemotherapy, but also worsen the prognosis [3, 31, 32], although the issue whether it is an independent prognostic factor remains open [1].

In addition to the prognostic importance of microsatellite instability, a difference has been noted in the response to radiotherapy. Although Rosty et al. found no association

between microsatellite instability and the response to 5-FU therapy [27], the prevalence of MSI-H cases in the series investigated by these authors was low. Numerous authors are of the opinion that also clinical response of MSI-H cancers to chemotherapy is poorer than in the case of MSS tumors [5, 14, 24]. Lawes analyzed reports published by numerous authors and found resistance to chemotherapy to be apparent especially in investigations based on tissue cultures, while clinical trials might have led to contrary conclusions [16]. According to Lievre et al., there is a significant association between the response to 5-FU treatment and mutations in the mitochondrial genome [17].

Results that are analogous to the obtained above were achieved by Ricciardiello et al. [25]. The investigators found significantly higher TS expression in MSI-H colorectal carcinomas as compared to MSI-L and MSS tumors. Therefore, based on the present results and data reported in the literature, it may be postulated that the primary cause of different response to chemotherapy manifested by carcinomas with varying microsatellite instability may lie in differences in thymidylate synthase expression.

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