Originals

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CDX-2 Expression Is Reduced in Colorectal Carcinomas with Solid Growth Pattern and Proximal Location, but Is Largely Independent of MSI Status*

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The homeobox genes are transcription factors that control the development of tissues and organs. In the colon one of such genes is CDX-2. In colorectal carcinomas, the CDX-2 expression is reduced. The aim of the present study was to investigate the presence of CDX-2 in colorectal carcinomas and to relate it to the histological features and microsatellite stability status. The material consisted of 20 carcinomas without microsatellite instability, 19 cases with low microsatellite instability and 19 cases with high microsatellite instability. CDX-2 expression was investigated using immunohistochemistry with CDX2-88 monoclonal antibody and assessed semiquantitatively. In 10 cases no expression of CDX-2 was observed, while in 6 the protein was present in less than 25% of tumor cells. It was noted that reduced expression of CDX-2 was more frequent in carcinomas situated proximally to the splenic flexure ($p<0.015$) and in tumors with solid growth pattern ($p<0.03$). On the other hand, no significant differences were encountered between groups differing in microsatellite stability. The results suggest that the major factors that determine the presence of CDX-2 in colorectal carcinomas at the protein product level may include cancer location and the solid phenotype of the tumor.

Introduction

The homeobox genes and their products are an interesting subject of investigations. These genes constitute the elements that control the differentiation of tissues and organs. Their expression may be highly specific for a given organ. The homeobox genes significant for the differentiation and maintenance of colonic structure include CDX-1 and CDX-2 [5]. Recently, the assessment of the CDX-2 product presence has been proposed as a specific and sensitive marker of colorectal carcinoma [4, 7, 16, 19].

The pathogenesis of colorectal carcinoma (CRC) is not uniform. The majority of cases are associated with chromosomal instability, while 10–20% is related to DNA repair defects and microsatellite instability (MSI) [9, 11]. Apart from cases with no microsatellite instability (MSS) and cases with a clear-cut microsatellite instability (MSI-high), there is also a group where instability is noted in a small number of loci (MSI-low). The role of this phenomenon and the place of MSI-low colorectal carcinomas in the classification are presently unclear. Clinically, MSI-low carcinomas are similar to the MSS group, yet it seems that subtle, but significant differences appear at the molecular level. Therefore, MSI-low carcinomas have been suggested to result from the "third path" of carcinogenesis within the colon [8, 10, 11, 15].

The aim of the present investigations was to determine CDX-2 expression in colorectal carcinomas and to compare the results with the MSI-status and morphological features.

Material and Methods

From the material studied in our previous reports [13–15], 20 MSS, 19 MSI-low and 19 MSI-high cases were randomly selected.

Microsatellite analysis was performed according to the previously published protocol [14, 15]. Briefly, DNA was extracted from dewaxed tumor and corresponding non-neoplastic tissue, and PCR-amplified with a screening panel of five microsatellite markers (APC, p53, BAX, BATR II and BAT-26). The amplicons were visualized using polyacryla-

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mide gel electrophoresis and routine silver staining. All the cases demonstrating any, even single, generic 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 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 determined 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 length corresponding to 2bp or 5bp. According to our preliminary experience, the capillary electrophoresis method, when applied to the archival DNA, has its background, equal to roughly one unstable locus. Thus, we decided to define the MSI-low group as 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, we included all the tumors with instability at BAT-26 into the MSI-high group. The remaining cases were classified as microsatellite-stable (MSS) carcinomas.

For CDX-2 detection, a primary antibody, purchased from Biogenex San Ramon, CA, USA (clone CDX2-88) was used. The procedure followed the manufacturers' instructions. 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 2N NaOH) 3x5 minutes at 750W. The primary antibody was diluted 1:50. The ENVISION+ (DAKO, Denmark) detection system was used. It consists of several goat anti-mouse antibody molecules attached to a dextran backbone coupled with horseradish peroxidase, and allows 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).

Sections of disease free colonic wall from surgical resection margins served as positive controls. The fragments of non-neoplastic intestinal mucosa present in the tumor sections were used as the internal controls. The strength of reaction was expressed semiquantitatively in a scale of 0, +, ++, +++. The percentage of positive cells was also semiquantitatively assessed. The measurements were done in a blinded manner, i.e. without the knowledge of clinical, histological and molecular data. For the purpose of statistical analysis, the group with marked reaction reduction was defined as that containing less than 40% of positive cells or characterized by the strength of reaction of + at the most.

The statistical analysis was carried out using the Statistica v.5.5A PL software (StatSoft Inc. Tulsa, OK, USA). U Mann-Whitney, Kruskall-Wallis ANOVA, ^{2} tests and non-linear regression analysis by LOGIT method were used, when appropriate. The significance level was set to p=0.05.

Results

The mean age of the patients was 64.4 years, with the range of 19–91 years and the standard deviation (SD) of 12.7. In the MSS group the age was 64.7, in the MSI-low group was 68.9, while in the MSI-high group was 59.8. The cohort included 29 females and 29 males. The male-to-female ratio was 1:1 in MSS, 0.9:1 in MSI-low and 1:0.9 in MSI-high group. The location of tumors was as follows: the cecum – 2, the ascending colon – 3, the hepatic flexure – 1, the transverse colon – 4, the splenic flexure – 1, the descending colon – 2, the sigmoid – 22 and the rectum – 20. The stage according to Astler and Coller was: A – 2 cases, B1 – 2 cases, B2 – 24 cases, C1 – 1 case, C2 – 28 cases, D – 1 cases.

Within the non-neoplastic colonic mucosa a strongly positive nuclear reaction was detected in all or almost all intestinal epithelial cells. In colorectal carcinoma specimens the nuclear reaction was CDX-2 negative in 10 cases. In 6 subsequent cases the reaction was present in less than 25% of cells. The majority of carcinomas showed a strong or very strong CDX-2 expression in the majority of cells (Fig. 1). These results are presented in Tables 1 and 2. While comparing the strength of reaction and extent in MSI groups (Table 3), no significant differences were detected in CDX-2 expression. CDX-2 expression showed no differences related to sex or age of the patients, tumor stage and grade of differentiation. A tendency was, nevertheless, observed towards CDX-2 expression present in less cells in carcinomas situated in the proximal segment of the colon ($p<0.1$). When the material was divided into carcinomas proximal and distal to the splenic flexure, an evident difference was seen ($p<0.008$ for strength of reaction, and $p<0.03$ for percentage of positive cells) (Fig. 2). Reduced number of CDX-2 expressing cells was also more pronounced in solid tumors ($p<0.03$).

To evaluate the simultaneous effect of the analyzed factors on CDX-2 expression, the regression analysis was

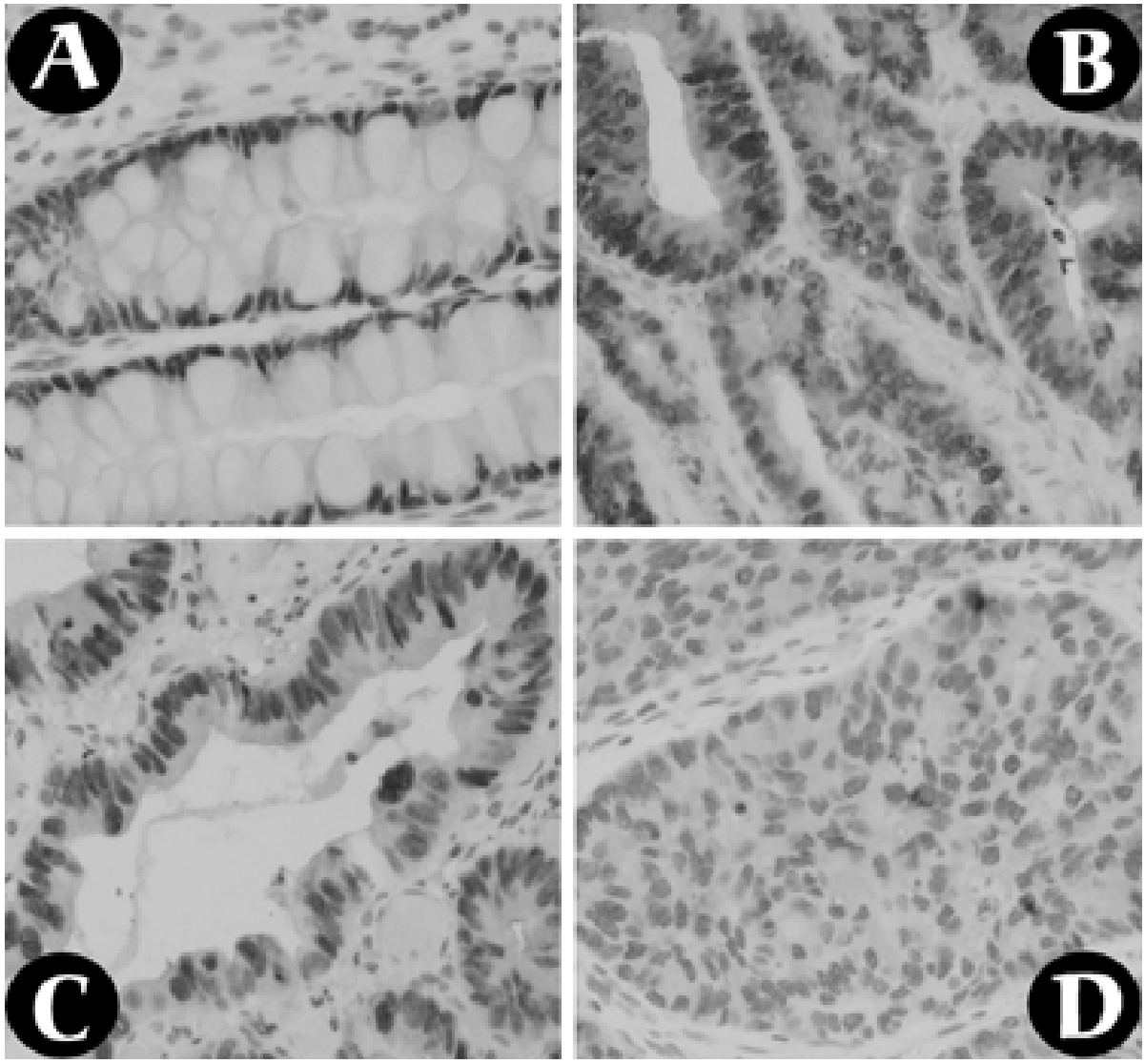

Fig. 1. CDX-2 expression in neoplastic tissues. Immunohistochemistry, lens magnification 60x. A – strong nuclear reaction in normal glandular epithelial cells of the colon; B – well differentiated adenocarcinoma with nearly all cells strongly positive; C – adenocarcinoma with reduced expression in some cells; D – tumor with a more solid growth pattern is largely negative.

TABLE 1
The strength of CDX-2 reaction

	N of cases
0	10
+	1
++	25
+++	22

TABLE 2
The extent of CDX-2 reaction

	N of cases
0%	10
< 25%	6
25–50%	11
50–75%	6
75–100%	25

TABLE 3
The strength of reaction and MSI status

	0	+	++	+++
MSS	4	1	6	9
MSI-low	2	0	12	5
MSI-high	4	0	7	8

performed using the LOGIT method. The univariate analysis demonstrated the significant role played by the location of carcinomas ($p<0.018$ in the above model), as well as by the solid growth pattern of the tumor ($p<0.046$). A model based on the two above variables also showed a high predictive value of CDX-2 assessment ($p<0.036$). When other variables, in particular the MSI status, were added to the model, the goodness of fit was not improved.

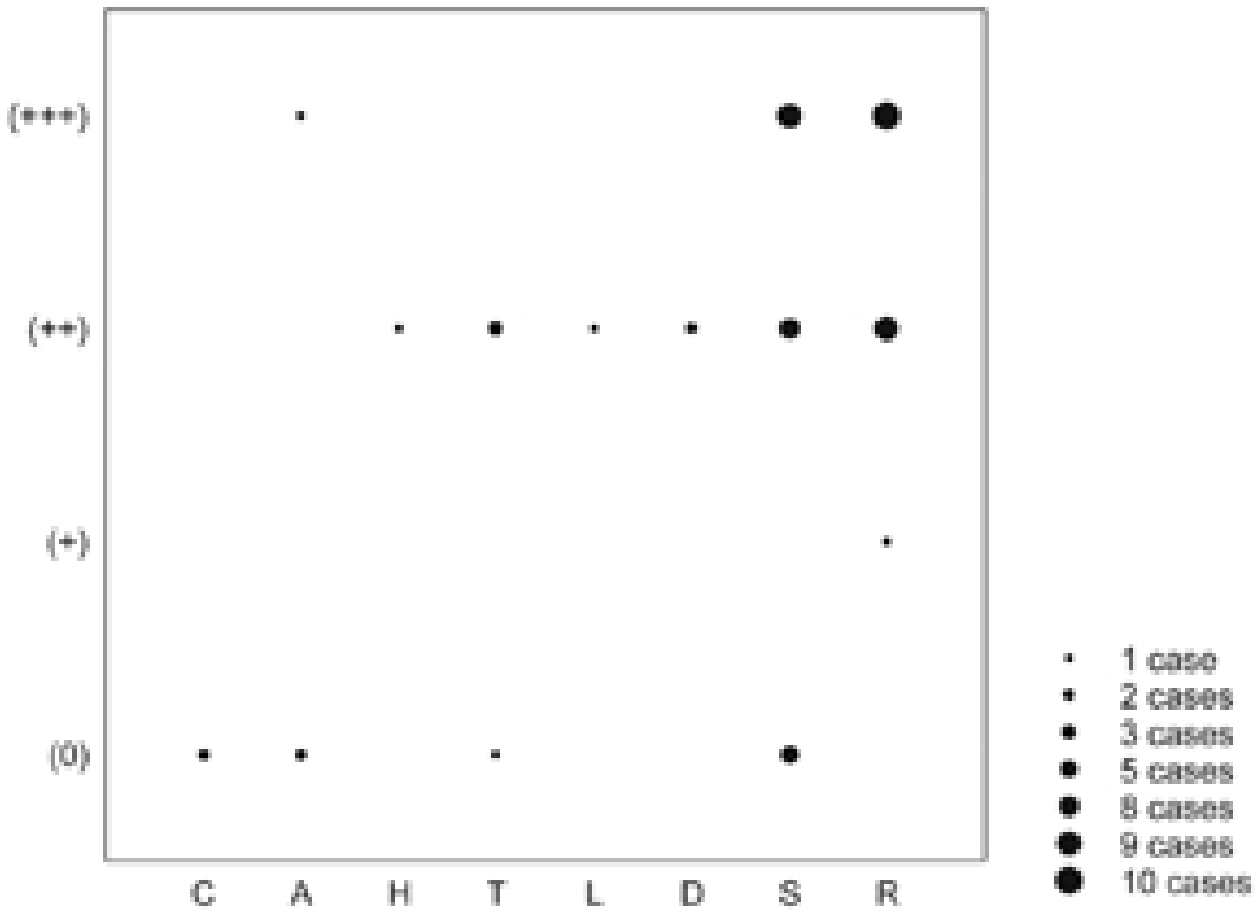

Fig. 2. Distribution of CDX-2 expression along the large bowel depending on the MSI status. C – cecum, A – ascending colon, H – hepatic flexure, T – transverse colon, L – splenic flexure, D – descending colon, S – sigmoid, R- rectum.

Discussion

The genesis of colorectal carcinoma is believed not to be uniform. The majority of cases originate from chromosome instability, where the primary phenomenon seems to be the APC gene inactivation. This group of tumors includes both sporadic cases and carcinomas developing in patients with familial adenomatous polyposis. In this type of cancer numerous and extensive genetic changes are observed, such as loss or addition of genetic material, manifested as loss of heterozygosity (LOH) in numerous genes [9]. In approximately 10–20% of carcinomas microsatellite instability (MSI) is present. This phenomenon is seen in both Hereditary Non-Polyposis Colorectal Cancer (HNPCC), and a subset of sporadic cancers. In consequence of impaired DNA repair mechanisms, the mutation rate is preferentially increased within short repeat sequences. Such changes are best detectable within microsatellite DNA [2, 3, 9, 11]. Yet the degree of DNA repair mechanism impairment may be variable. Cases with changes involving less than 30–40% of the investigated microsatellites are termed “MSI-low” [2]. The position of this group of carcinomas in the classification is unclear. Their clinical and morphological features make them similar to MSS carcinomas. However, Jass et al. postulated that MSI-low tumors did not fall into the gray zone, but rather constituted a separate group with a different origin and pathogenesis [8].

Homeobox genes are characterized by a common sequence, the ability to bind DNA and thus govern other gene expression, and their primary role in organ development. The expression of genes belonging to this group is often limited to a specific organ or tissue and this is why they may play the role of an organ-specific or tissue-specific marker. Such a use has been found for prox-1, which is a factor governing lymph vessel endothelial differentiation [12].

Caudal type homeobox gene 2 (CDX-2) encodes an intestinal-specific transcription factor. It is a homologue of *Drosophila* "caudal" homeobox gene. In humans CDX-2 is a homeobox gene necessary for ontogenesis and differentiation of colonic tissues and for maintaining their structure in

the adult. Therefore, immunohistochemical reaction for CDX-2 has been proposed as a mean of confirming the colonic origin of metastatic adenocarcinoma with unknown primary [11].

Ee et al. analyzed the presence of CDX-2 in human large bowel and in colonic carcinomas. The authors observed that under normal conditions, all epithelial cells are CDX-2-positive along the segment from the Bauhin's valve to the splenic flexure, while further distally along the colon the reaction is reduced within the deeper parts of the crypts. A similar drop in CDX-2 expression was also observed in proximal and distal colonic adenomas. On the other hand, the above investigators found a positive reaction solely in the minority of carcinomas; they found no clear association with the location of the lesions [6]. In our material we have noted a tendency towards a weaker CDX-2 expression in carcinomas situated proximally to the splenic flexure. Hinoi et al. [7] observed a clear reduction in CDX-2 expression in large cell minimally differentiated carcinoma of the colon. No such tumors have been included in our material. On the other hand, in conventional CRC we have found a reduced CDX-2 expression as compared to data presented by the aforementioned authors. These differences may depend on the use of a different monoclonal antibody.

The interest in the role of CDX-2 in the development of CRC has been triggered by experiments performed in CDX-2 knockout mice, which develop multiple adenomatous polyps of the colon. Interestingly, the polyps are supposed to develop mainly in the proximal colon. On the other hand, Wicking et al. found a mutation of this gene in one of a series of 85 CRC. The authors associated inactivation of CDX-2 gene with the MSI-high phenotype [18]. Presently, the frequency of CDX-2 mutations in CRC is estimated as approximately 10% of cases. Changes within the gene are believed to play a role in the progression of the disease in some CRC patients, but CDX-2 itself does not participate in triggering carcinogenesis [7, 19]. In our opinion these data are not discordant with our findings, as gene mutation can not be the sole mechanism of reduced expression at the protein level.

Conclusion

The present results confirm the reduction of CDX-2 expression in CRC, at the same time suggesting that differences in the gene expression may be chiefly associated with the location of the primary tumor.

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