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The Relationship between MSI Status and Vessel Density in Colorectal Carcinoma*

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The development of new blood vessels is a prerequisite for progression of malignant neoplasms. Factors that induce neoangiogenesis include VEGF, VEGF-C, VEGF-D, PD-ECG, ANG-2, TSP-1, HIF-1 and HIF-2. From the etiopathogenetic viewpoint, colorectal carcinoma is heterogenic. It may develop *via* a sequence of mutations leading to chromosome instability or else result from DNA repair defects, which are manifested as microsatellite instability. The objective of the present investigations was the comparison of neoangiogenesis in microsatellite-stable colorectal carcinomas, as well as in tumors with low and high instability levels. The material included 71 surgical cases of colorectal carcinoma. Vessel density was assessed by immunohistochemical reactions to CD34 and vWf, calculating the number of vessel sections within the invasion margin, in visual fields selected at random, and within hot spots. Microsatellite instability was evaluated in frozen materials employing the PCR reaction with gel and capillary electrophoresis. In all the cases, the authors detected CD34+ and less numerous vWf+ vessels within the tumor and in its vicinity. In 45 cases, no microsatellite instability was found, in 13 cases, low level instability (MSI-L) was observed, and in another 13 – high microsatellite instability (MSI-H). Some differences in vessel density were noted between the above groups, yet they were not statistically significant. On the other hand, the authors observed more numerous CD34+ vessels in cases with metastases to the regional lymph nodes. In conclusion, it is suggested that neoangiogenesis in sporadic colorectal carcinoma is directly related to metastatic potential, but not to MSI status.

Introduction

Angiogenesis (AG), i.e. the formation of small blood vessels, occurs in the course of ontogenesis, but under normal conditions does not take place in an adult human. On the other hand, AG participates in various pathological processes, such as wound healing or organization of inflammatory exudate. AG is indispensable for the development of cancers. Only very early tumors, few millimeters in size, may be supplemented by diffusion from their neighborhood. Acquiring the ability to induce AG is necessary for tumor progression. In the case of some diseases, such as breast carcinoma, the density of the vascular network may be an independent prognostic factor; in other entities, such as clear cell carcinoma of the kidney, no such relation has been observed. In recent years, the interest in angiogenesis investigations has increased [11].

According to contemporary views, the pathogenesis of colorectal carcinoma (CRC) is not uniform. The majority of cases are associated with chromosome instability, while 10–20% is related to DNA repair defects and microsatellite instability (MSI-H). There is also a separate group, where instability is observed in a small number of loci (MSI-L). The significance of this group and its position in the classification are not clear. Clinical properties of the MSI-L cancers

* 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).

are similar to these characteristic of the MSS group; however, it seems that subtle but significant differences appear on the molecular level. The MSI-H cancers have been suggested to develop *via* a separate carcinogenetic route [15, 8].

Material and Methods

The material consisted of colorectal carcinoma cases treated at the 1st Chair of General Surgery, Collegium Medicum, Jagiellonian University. Fresh surgical material was referred to the Chair of Pathomorphology. After the gross assessment, sections were selected from the tumor and the intestine wall situated equidistant from the lesion and margin. Tumor sections were collected from the peripheral zone, bypassing ulcerated and necrotic areas. These specimens were placed in the Eppendorf test tubes and frozen at the temperature of –40°C. The remaining material was fixed in 10% buffered formalin for 24 hours. The fixed material served for section collection according to the standard protocol [25]. The sections were processed in a routine manner using automatic tissue processors manufactured by Shandon, embedded in paraffin, cut into 3- m thick sections and stained with hematoxylin and eosin for histopathological evaluation. This was done routinely, and tumor stage was defined in concordance to the TNM classification [7].

Microsatellite analysis was done according to the previously published protocol [21]. Briefly, DNA was extracted from 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 the reaction mixture containing: 2 l DNA template (100 ng), 2 l STR buffer (Promega), 0.5 l of each primer (10 nM), 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 when 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 [16].

For immunohistochemistry, HE stained sections were reviewed, and sections containing representative and well-preserved carcinoma together with the tumor margin were chosen. Three- m thick sections were cut from the selected paraffin blocks and used for standard immunohistochemical reactions. Briefly, the slides were dewaxed, 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) for 3×5 minutes at 750 W. The primary antibodies for vWf (clone F8/86, DAKO, Denmark) diluted to 1:25 and for CD34 (clone QBEnd10, DAKO, Denmark) diluted to 1:25 were used. 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 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). Positivity was defined as discernible, positive vessel profiles, cell groups and in the case of vWf reactions also single cells that would show distinct, granular cytoplasmic reaction. In the case of CD34, slightly positive reaction in the stromal spindled cells (“fibrocytes”) was disregarded.

The assessment of vessel density was performed under an Axioskop microscope (Zeiss, Germany) with a Plan-Neofluar 40× lens (diameter of view field 0.49 mm). The number of profiles of CD34+ vessels was counted in ten fields of view selected at random (CD34 random). Subsequently, the section was assessed under a lower magnification, selecting an area with the highest vessel density and calculating the number of profiles of CD34+ vessels in ten fields of view (CD34 hot spot). Finally, the number of CD34+ vessels within the interface between the tumor and the surroundings (CD34 border)

was counted. The same was done for vWf stained slides. Firstly, the number of profiles of vWf+ vessels was counted in ten fields of view selected at random (vWf random). Subsequently, the section was assessed under a lower magnification, selecting an area with the highest vessel density and calculating the number of profiles of vWf+ vessels in ten field of view (vWf hot spot). Finally, the number of vWf+ vessels was counted within the interface between the tumor and the surroundings (vWf border).

The statistical analysis was performed with STATISTICA 6 PL package (StatSoft, Inc., USA). To demonstrate inter-group differences, the U Mann-Whitney test and Kruskall-Wallis ANOVA were used, when appropriate; the frequencies were compared with the Pearson's ^{2} test. The correlations between the variables were assessed by the Spearman's and gamma correlation coefficients. The significance level was set to 0.05.

Results

The material consisted of 71 cases. The F:M ratio was 32 to 39; the mean age of the patients was 64.2 years, range 34 to 87, SD 10.0.

The tumors were located in the cecum and ascending colon in eight cases (11.2%), transverse colon in 14 cases (19.7%), descending colon in two cases (2.8%), in sigmoid in 24 cases (33.8%), and in rectum in 23 cases (32.5%). The mean diameter of the tumor was 5.2 cm (range 1.5 to 15 cm, SD 2.48). Twenty-three cases (32.4%) were Astler-Coller stage B-1, seven (9.8%) stage B-2, six (8.5%) stage C-1, 29 (40.8%) stage C-2 and six (8.5%) stage D. According to TNM system, 31 cases (47.7%) were stage pT2, 36 (50.7%) – stage pT3 and four (5.6%) – stage pT4. In 30 cases (42.3%), there were no lymph node metastases (pN0), whereas 13 cases (18.3%) were stage pN1 and 28 cases (39.4%) – stage pN2. In the cases with lymph node metastases, the mean percentage of positive nodes was 33.5% (range 3–100%, SD 24.17). In 60 cases (84.5%), the surgical margins were tumor-free (pR0), and in 11 (15.5%), the margins were microscopically positive (pR1).

In 40 cases (56.3%), no vessel invasion was seen, in eight cases (11.3%), there was larger vessel invasion (pV1) only, in 12 instances (16.9%) there was smaller vessel invasion (pL1) only, and 11 cases (15.5%) were both pL1 and pV1. The grade was G-I in 23 (32.4%) cases, G-II in 38 (53.5%) cases, and G-III in ten (14.1%) cases. The tumor margins were infiltrating in 29 cases (40.8%), mixed in 32 cases (45.1%), and pushing in ten (14.1%) cases. The MSI status was stable (MSS) in 45 cases (63.4%), MSI-L in 13 cases (18.3%), and MSI-H in 13 cases (18.3%).

In all the cases, immunohistochemistry demonstrated the presence of small vessels within the tumor and its vicinity. CD34-positive vessels were numerous and formed easily discernible hot spots. These foci manifested a tendency towards being situated in the luminal part of the tumor. The results of vessel counting are shown in Table 1. The counts performed using CD34 and vWf stains were positively correlated, though only the count done at the infiltrating border significantly ($R=0.33$, $p<0.006$). No statistically significant differences were found in vessel density as related to tumor location. There were some differences in vessel count depending on the MSI status (Table 2), but they were statistically non significant. The size of the tumor was positively correlated with the vWf border parameter ($R=0.23$, $p<0.05$), and weakly with vWf random ($R=0.13$) and vWf hot spot ($R=0.16$). CD34 random and CD34 hot spot were negatively correlated with tumor size ($R=–0.14$ and $R=–0.09$, respectively), and

TABLE 1
Values of vessels count obtained by four methods

	mean	(min – max)	SD
CD34 random	112.61	(14 – 211)	40.12
CD34 hot spot	168.96	(14 – 333)	53.95
CD34 border	125.62	(15 – 296)	43.21
vWf random	16.28	(1 – 74)	14.25
vWf hot spot	34.41	(1 – 102)	27.04
vWf border	26.31	(0 – 63)	16.89

TABLE 2
Relationship between vessel counts and MSI status

	CD34 random		CD34 hot spot		CD34 border		vWf random		vWf hot spot		vWf border	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
MSS	112.84	39.00	170.47	55.43	127.67	45.15	15.29	13.80	34.22	29.64	24.13	16.69
MSI-L	103.85	44.51	171.92	45.25	126.54	40.49	14.38	9.98	28.85	17.61	30.54	17.68
MSI-H	120.54	41.01	160.77	59.81	117.62	41.11	21.62	18.67	40.62	25.67	29.62	16.81

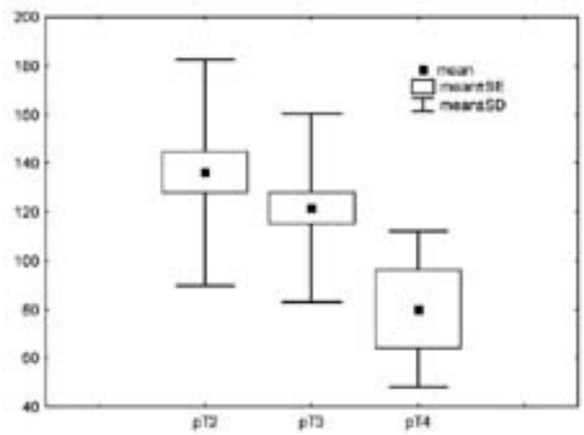

Fig. 1. Relationship between CD34 border count and pT stage.

CD34 border showed a marginal correlation only (R=0.05). There was no significant relationship between vessel count results and the age or sex of patients. There was a marginally significant (p=0.05) difference in CD34 border pT stages (Fig. 1). Lymph node-positive cases had significantly lower CD34 border as compared to negative cases (mean 138.1, SD 52.57 vs. mean 116.5, SD 32.58, $p<0.05$). CD34 border also demonstrated a tendency towards a negative correlation with the percentage of metastatic lymph nodes (R=–0.20). There was a tendency towards a positive correlation between CD34 random and the degree of histological differentiation (gamma 0.17), as well as a tendency towards a positive correlation between vWf border and the degree of differentiation (gamma –0.20), but none of these correlations was statistically significant.

Discussion

The formation of blood vessel network (angiogenesis, AG) is an indispensable step in cancer development and progression. In the initial phase of their growth, tumors are supplied with oxygen and nutrients through simple diffusion from the surrounding tissues. However, the process is spatially limited and, therefore, tumors that do not possess the capacity of AG induction cannot grow to a size huger than few millimeters. Cancer acquiring the ability to induce angiogenesis is also indispensable for metastasis formation. An interesting contribution to the topic of AG in cancer progression is the report by Pavlopoulos et al. [22]. These investigators found significant differences in the density of blood vessel branching in adenomas vs. colorectal carcinomas. Such a difference might have been evoked by VEGF. However, no differences in AG related to tumor stage were seen once the carcinoma arose.

The formation of vessels within the tumor interstitium depends on numerous factors. The exact role of various angiogenesis-regulating factors in colorectal carcinomas has not been fully elucidated [26]. VEGF is the chief mediator of angiogenesis, while bFGF appears to be less potent. On the other hand, in the course of the lesion progressing from an adenoma to various stages of colorectal carcinoma, a parallel increase is noted in microvessel density (MVD) and bFGF expression, whereas the expression of VEGF does not change [26]. VEGF production in cancer has been suggested to depend on the presence of mutated p53, although this mechanism is most likely not the only one in operation [11]. Of AG-inducing mediators, VEGF may show the strongest association with the prognosis, at least in higher stage cases. Kaio et al. identified as prognostic factors metastatic involvement of the lymph nodes and VEGF expression [9]. Beside VEGF-A also VEGF-D and C might be involved in AG. White et al. found that VEGF-D was present in colorectal carcinomas, but not in adenomas. VEGF-D might be associated with the formation of new lymphatic rather than blood vessels, although the authors did not observe any relation between VEGF-D expression and VEGFR-3-positive vessel density. One should bear in mind, however, that the latter marker has a limited value in differentiating lymph and blood vessels. In the above- mentioned report, an association was noted between VEGF-D and the density of all small vessels [29]. VEGF-C expression may be related to the risk of invasion of intratumor vessels [6]. In the publication by Kaio et al., PD-ECGF, VEGF and VEGF-C expression in the invasion front was demonstrated to be associated with vessel density [9]. A significant factor that regulates angiogenesis is agiongiopoietin–2 (ANG–2). According to Ochiumi et al., VEFG and ANG-2 co-expression in the invasion front shows a significant association with new vessel formation. The presence of ANG-2 alone is not sufficient to induce AG [20]. Also, COX-2 expression may be related to the AG initiation. The effect might be partly responsible for the prognostic value of COX-2 expression [19]. A fundamental factor that induces the formation of new blood vessels is hypoxia, whose effect depends on the production of hypoxia-inducible factors (HIFs). According to Yoshimura et al., HIF–2 is the chief substance operating in AG, while both HIF-1 and HIF-2 affect the progression of colorectal carcinomas and the prognosis [31]. HIF-1 might act through inducing the production of VEGF. Kuwai et al. observed that HIF-1 expression was associated with vessel density in the tumor, as well as with the risk of vessel invasion. Nevertheless, HIF-1 does not demonstrate a significant correlation with the prognosis [12]. An important correlation with MVD has been demonstrated for Ets-1, possibly through its associations with

VEGF activation pathways [27]. On the other hand, thrombospondin-1 (TSP-1) may constitute a factor that prevents new vessel formation [18]. Apart from their paracrine activity, both VEGF and TSP-1 may be also detected systemically. Serum VEGF has been found to affect the prognosis. VEGF and other angiogenesis-promoting factors may be produced by tumor cells but also inflammatory cells recruited by the tumor [23, 24, 28]. In the opinion of Acikalin et al. such a source of angiogenic factors might be mast cells [1].

The prognostic importance of AG intensity and its association to other clinico-pathological parameters in colorectal carcinomas are not completely clear. Tabara et al. observed that in small tumors of the colon there was a positive correlation between vessel density and tumor size [26], whereas Pavlopoulos et al. noted a decrease in vessel density in higher stage tumors [22]. Li et al. [13] detected a decline in vessel density and tumor perfusion in higher stage disease, but failed to find any association with metastases. ANG-2 expression is related to the degree of tumor differentiation, lymph and blood vessel invasion, metastasizing disease and tumor stage [20]. Barozzi et al. [2] discovered that colorectal carcinomas with remote metastases were characterized by significantly higher MVD values. Nevertheless, the results of the multivariate analysis did not confirm MVD to be an independent factor in prognosticating the presence of metastases. In the opinion of Ochiumi et al., independent prognostic factors in colorectal carcinomas include – apart from metastatic lymph node involvement – also VEGF and ANG–2 expression in the invasion front [20]. Kaio et al. stress the prognostic importance of VEGF-C and PD-ECGF in the invasion front in addition to VEGF-A [9].

The pathogenesis of colorectal carcinoma is not uniform. In the majority of cases, numerous and extensive DNA abnormalities are observed as chromosome instability. Approximately 10–20% of carcinomas are associated with DNA repair defects. The effect of impaired DNA repair mechanisms is manifested as an accelerated mutation rate, with mutations occurring even several hundred times faster as compared to normal tissues. This is particularly true for short, repetitive sequences, such as microsatellite DNA (microsatellite instability – MSI) [3, 5]. The degree of DNA repair impairment may be diversified. Cases with changes involving less than 30–40% of the investigated microsatellite loci are termed "MSI-L" [3]. The position of this group of cancers in the classification is unclear. The clinical and morphological properties of these tumors make them similar to MSS carcinomas, yet Jass et al. proposed MSI-L cancers as a group characterized by a separate origin and pathogenesis [8].

The prognostic importance of microsatellite instability remains unclear, although there is an increasing body of evidence that it is an independent prognostic factor. This is particularly true for the MSI-H category, with cases belonging here being supposed to have a better prognosis [14], although according to Kakar et al. [10], the MSI status is prognostically significant in the univariate analysis only, whereas other publications do not support such an association [32]. On the other hand, the response of MSI-H carcinomas to therapy is believed to be poorer than it is observed in the remaining colorectal carcinomas [4]. Data on the significance of the MSI-L category are much scarcer. Wright et al. observed that tumors of this group have a poorer prognosis as compared to carcinomas without microsatellite instability [30]. Colorectal carcinomas associated with various routes of carcinogenesis might differ in their ability to induce the growth of vascular network. Thus, Losi et al. investigated colorectal carcinomas in patients with the Lynch II syndrome and noted that such tumors were characterized by lower density of the vascular network than sporadic cancers [17]. The present results suggest that the differences in the biology and prognosis between colorectal carcinomas with varying degrees of microsatellite instability may not be determined by differences in AG.

The methods for AG assessing are not fully standardized. The most commonly employed method is histology based with immunohistochemical staining. To detect small vessels, immunohistochemical reactions with primary antibodies against antigens specifically present on endothelial cells are used. Many such antibodies are commercially available, and may mark different subsets of vessels. Li et al. [13] compared histological assessment of vessel density and tumor perfusion in colorectal carcinomas measured by radiological method. They found no association between these parameters. The cited-above authors are of opinion that evaluating perfusion may be of a greater clinical benefit than the assessment of vascular network density. There is evidence supporting the notion that the assessment of angiogenesis-regulating factors as VEGF, VEGF-C, VEGF-D, Ets-1 and TSP-1 might also be of prognostic significance [6, 18, 29].

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