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ARTICLE

The Intratumoral Microvessel Density and Expression of bFGF and nm23-H1 in Colorectal Cancer

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It has previously been reported that intratumoral microvessel density (IMD), and the expression of bFGF and nm23-H1 are useful prognostic markers in colorectal cancer (CRC). In this study, a total of 100 CRCs were evaluated histopathologically, and IMD, bFGF and nm23-H1 expression were assessed by immunohistochemistry. IMD of patients increased with grade and stage, and this increase was statistically significant ($p<0.05$). A significantly higher incidence of high bFGF expression scores was also associated with increasing grade and stage ($p<0.05$). However, there was no significant difference between the grades in nm23-H1 expression ($p=0.234$). nm23-H1 expression occurred with lower incidence in stages C1, C2 and D than in stages B1 and B2 ($p<0.05$). Thus, a negative correlation was found between nm23-H1 expression and stage or lymph node metastasis (LNM) ($p<0.05$). IMD and bFGF expression were positively correlated with grade, stage, LNM, and lymphovascular invasion. Although positive correlation was found between IMD and bFGF, nm23-H1 expression negatively correlated with both of them. As a result, in clinical practice, increased IMD and bFGF expression and decreased nm23-H1 expression may provide valuable information in characterizing the malignant phenotype. (Pathology Oncology Research Vol 12, No 1, 21–27)

Key words: Colorectal cancer, microvascular density, basic fibroblast growth factor, nm23-H1

Introduction

Colorectal cancer (CRC) is a leading cause of cancer morbidity and mortality in economically advanced regions of the world, such as North America, Western Europe, Scandinavia, New Zealand, and Australia. It is the fourth most frequently diagnosed visceral cancer and the second leading cause of cancer mortality, accounting for about 10% of all cancer deaths in both genders combined. Although there are many prognostic parameters related to CRC, the importance of some of them is unclear.[1]

Angiogenesis is necessary for tumor growth and it facilitates two processes responsible for the malignant phenotype of a growing tumor, i.e. invasion and metastasis. Although the proliferating fraction of a tumor does not depend on the number of microvessels, tumor cells are less prone to undergo apoptosis in well-vascularized tumors.[2] A statistically significant correlation between the incidence of metastasis and microvessel counts has been demonstrated in invasive breast carcinoma,[3] melanoma,[4] cervical cancer,[5] and other tumors. However, the prognostic relevance of tumor-related angiogenesis in CRC remains controversial. In many studies, an association between tumor angiogenesis, reflected by IMD in so called "hot-spots" of angiogenesis in the tumor tissue, and prognosis has been described. IMD has been associated with tumor size, lymphatic and venous blood vessel invasion and metastasis (LNM and distant metastasis). Thus, IMD was regarded as an important prognostic factor by some investigators,[6-9] but not by others.[10-13] Two well-characterized growth factors, VEGF[14] and bFGF[15] are known to induce angiogenesis in rodent tumor models. Tumor growth kinetics correlated with the concentration of angiogenic factors in the blood and tissue of advanced CRC.[2]

The nm23-H1 gene, located at 17q21.3, was first isolated as a metastasis suppressor gene by differential screening of a cDNA library from low- and high metastatic murine melanoma cell lines.[16] Several tumor cohort stud-

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ies have indicated that reduced nm23-H1 expression is associated with aggressive histopathological criteria, tumor behavior, poor patient survival and nodal metastasis in a variety of carcinomas.[17,18] However, other studies have not verified the significance of nm23-H1 in terms of tumor invasion or metastasis in some carcinomas.[19,20] At present, however, the clinical relevance of nm23-H1 as a metastasis suppressor for human cancers remains enigmatic. [21]

In this study, we assessed IMD, bFGF and nm23-H1 expression by immunohistochemistry in 100 CRC patients. The purpose of this study was to evaluate the relationship between IMD, the expression of bFGF and nm23-H1, and conventional histopathological prognostic factors.

Materials and Methods

Patient and tumor characteristics

We examined 100 primary CRCs surgically resected at Cumhuriyet University Hospital, Sivas, Turkey, between 1987 and 2003. The patients' gender, age, stage and tumor size were obtained from surgical and pathological records. All specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and then stained with hematoxylin-eosin for histological examination. Paraffin-embedded tumor tissues were retrieved from the archives of the Institute of Pathology, Medical University of Cumhuriyet, Sivas, Turkey. Histological grading was performed on hematoxylin-eosin-stained sections of the tumors. Grade 1 CRCs are composed mainly of simple tubules, in which the nuclear polarity is easily discerned and the nuclei are of uniform size. Grade 2 CRCs are composed mainly of tubules that may be simple, complex, or slightly irregular, in which the nuclear polarity is barely discernible or is lost. Grade 3 CRCs are characterized by a predominance of the absence of glandular differentiation, as well as by loss of nuclear polarity. Tumors were evaluated according to the modified Astler-Coller clinicopathological classification.[1] Lymphovascular invasion, desmoplasia, and peritumoral lymphocytic infiltration were evaluated as positive or negative. LNM was determined such as negative (N0), 1-3 metastatic lymph nodes (N1) and 4 or more metastatic lymph nodes (N2).

Immunohistochemistry

Immunohistochemical staining was carried out with Avidin Biotin Peroxidase system using monoclonal antibodies against the following antigens: CD31 (CD31/PECAM-1, clone JC/70A, Neomarkers, USA); bFGF (AB-3, rabbit polyclonal antibody, Oncogene; 1:30 dilution); nm23-H1/NDP kinase (rabbit polyclonal antibody, Neomarkers).

Briefly, 4-μm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 /methanol solution, antigen retrieval was conducted using 10 mmol/L citrate buffer (pH 6.0) in a microwave oven for 10 min at 120° C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody, followed by the avidin-horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with AEC chromogen (LabVision). Nuclear counterstaining was accomplished with Mayer's hematoxylin.

Determination of intratumoral microvessel density

In CRC, tumor angiogenesis showed the highest intensity at the invasive front. Most of the CD31-positive microvessels were identified in this area, which thus resembled an "extended hot spot" of angiogenesis. Vessel counts were assessed at the invasive front by light microscopy after staining for CD31. Areas containing the highest numbers of capillaries and small venules were identified by scanning tumor sections at low power (x40 and x100). In each case, the three most vascularized areas were selected, and three fields of these three areas were counted at x200 magnification (x20 objective and x10 ocular, 0.739 mm^2/field). The average counts of the nine x200 fields were recorded for analysis. Based on the criteria of Weidner et al,[3] vessel lumens were not necessary for a structure to be defined as a vessel.

Determination of nm23-H1 and bFGF expression

Immunohistochemical staining of cells was assessed according to both the staining intensity and proportion of positive cells. The intensity of bFGF and nm23-H1 staining was homogeneous within tumors. The slides were scored semi-quantitatively by one pathologist. The scoring was performed as follows: score 0: 0%, score 1: 1-30%, score 2: 31-75%, and score 3: more than 75% cells stained for bFGF or nm23-H1. Intensity of staining was evaluated as score 0 for no staining, 1 for weak, 2 for moderate, and 3 for intense staining. The lymph nodes with metastasis were also examined after immunohistochemical staining for nm23-H1.

Statistical analysis

Associations of IMD, bFGF and nm23-H1 expression with each other, as well as with grade, stage, lymphocytic infiltration, desmoplasia, lymphovascular invasion, LNM and tumor size were analyzed with Pearson and Spearman correlation tests where appropriate. IMD of the tumors of grades 1, 2 and 3, stages B1, B2, C1, C2 and D, and bFGF expression 0, 1, 2 and 3 were compared using ANOVA test

with post hoc Tukey test. The ratio of bFGF and nm23-H1 expression in tumors of grades 1, 2 and 3, and stages B1, B2, C1, C2 and D were compared with χ^2 test.

Results

The age of patients was 57.2 ± 13.7 years, and the male/female ratio was 59/41. There was a lymphocytic infiltration surrounding the tumor in 34 patients, desmoplasia in 51 patients, and lymphovascular vessel invasion in 48 patients. The number of patients with 1-3 LNM was 27 (N1), and the number of patients with 4 or more LNM was 19 (N2). The median tumor size was 5 (1-17) cm. There was no significant correlation between IMD, nm23-H1 and bFGF expression and age, gender, lymphocytic infiltration surrounding tumor or desmoplasia. Of the 100 patients with CRC, 46 had grade 1, 32 grade 2, and 22 grade 3 tumor. The numbers of patients with stage A-C2 CRCs were as follows: none with stage A, 15 with stage B1, 37 with stage B2, 25 with stage C1, 21 with stage C2, and 2 with stage D.

IMD of grade 3 tumors was significantly higher than that of grade 1 or 2 tumors (p=0.001 and p=0.012, respectively). IMD of grade 2 tumors was also significantly higher than that of grade 1 tumors (p=0.001) (*Figure 1*). IMD in patients of stages C2 and D was significantly higher than that in patients of stages B1, B2 and C1 (p<0.05). IMD in patients of stages B2 and C1 was also significantly higher than that in patients of stage B1 (p<0.05) *(Figure 2)*.

The IMD of tumors with bFGF expression score of 2 and 3 was significantly higher than that of tumors with bFGF expression score of 0 and 1 (p=0.001) *(Figures. 3,4)*.

Tumors of grade 2 or 3 had scores 2 or 3 bFGF expression. With increasing grade, the proportion of cases with high bFGF scores also significantly increased (68% vs. 22%) (p<0.05). Also, the ratio of cases with bFGF expression scores 2 and 3 was significantly higher in stages C1, C2 and D than in stages B1 and B2 (69% vs. 31%, p<0.05). There was no significant difference between grades in the proportion of cases with nm23-H1 expression score 2 or 3 (67% vs. 54%, p=0.234) (*Figure 5*). The ratio of cases with nm23-H1 expression score 2 and 3 was significantly higher in stages C1, C2 and D than in stages B1 and B2 (73% vs. 46%, p =0.010).

Table 1 presents associations of IMD, bFGF and nm23-H1 expression with each other and with grade, stage, lymphocytic infiltration, desmoplasia, lymphovascular invasion, LNM and tumor size. Positive correlation was found between IMD and bFGF expression and grade, stage, LNM, and lymphovascular invasion. The median IMD of tumors with lymphovascular invasion and more than three metastatic lymph nodes was higher than that of tumors of no lymphovascular invasion and no metastatic lymph nodes. Negative correlation was found between nm23-H1 expression, and stage or LNM. Positive correlation was found between IMD and bFGF, however, negative correlation of nm23-H1 expression with IMD and bFGF was demonstrated. Moreover, there were 19 patients with nm23-H1 immunoreactivity in the lymph node metastasis *(Figure 5)*.

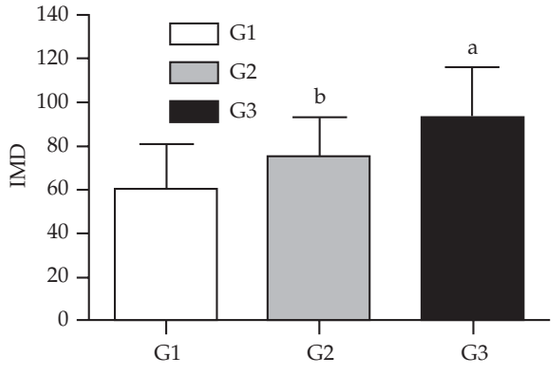

Figure 1. *Intratumoral microvessel density (IMD) in grade 1, 2 and 3 tumors. [a]P<0.05 vs. grade 1 and 2, [b]P<0.05 vs. grade 1*

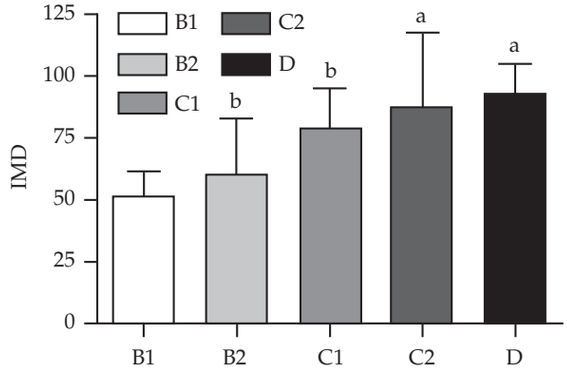

Figure 2. *Intratumoral microvessel density (IMD) in stage B1, B2, C1, C2 and D tumors. [a]P<0.05 vs. stages B1, B2 and C1, [b]P<0.05 vs. stage B1*

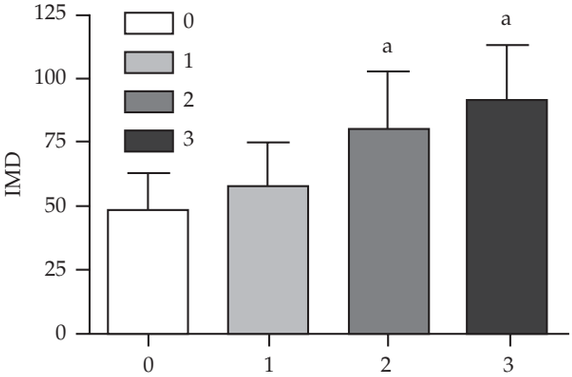

Figure 3. *Intratumoral microvessel density (IMD) in tumors with bFGF expression scores 0, 1, 2 and 3. [a]P=0.001 vs. b-FGF expression scores 0 and 1*

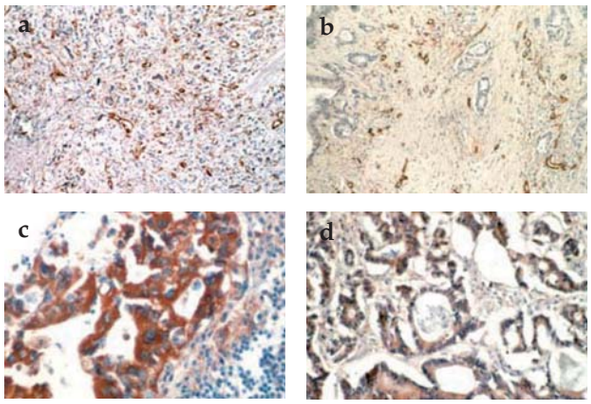

Figure 4. *Immunohistochemical findings of colorectal carcinoma.* ***(a)*** *Intratumoral microvascular density (IMD) in grade 3 tumor with anti-CD31 antibody.* ***(b)*** *IMD in grade 1 tumor with anti-CD31 antibody.* ***(c)*** *Score 3 bFGF expression in grade 2 tumor.* ***(d)*** *Score 2 bFGF expression in grade 1 tumor*

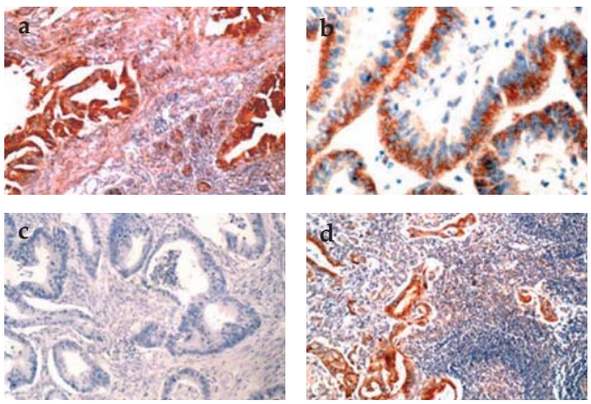

Figure 5. *Immunohistochemical findings of colorectal carcinoma with antibodies to nm23-H1. (a) Score 3 nm23-H1 expression in grade 1 tumor. (b) Score 2 nm23-H1 expression in grade 2 tumor. (c) Score 0 nm23-H1 expression in grade 2 tumor. (d) nm23-H1 expression in metastatic lymph node*

Discussion

The aim of this study was to determine whether IMD, bFGF and nm23-H1 expression were correlated with established conventional histopathologic prognostic variables in CRC. These prognostic parameters were stage, grade, LNM, lymphovascular invasion, tumor size, desmoplasia, and peritumoral lymphocytic infiltration. In this study grade, stage, lymphovascular invasion and LNM were correlated with both IMD and bFGF. In addition, there was a positive correlation between IMD and bFGF expression. However, we found that there was an inverse correlation between nm23-H1 expression and stage, grade, LNM, IMD and bFGF expression. IMD, bFGF and nm23-H1 expressions were not correlated with age, gender, tumor size, desmoplasia, and peritumoral lymphocytic infiltration. In addition, nm23-H1 immunostaining was shown in metastatic lymph nodes as well as in the primary tumor.

Angiogenesis, the development of new blood vessels, is required for tumors to grow larger than 2-3 mm^3 in size, and provides both nutrients and access to the systemic circulation with possible subsequent metastasis.[3-5,22] This angiogenic process depends on the combined action of various cytokines and growth factors (VEGF, bFGF, PDGF and TGF-beta) secreted by tumor cells, cells of the tumor stroma, and infiltrating inflammatory cells. Among inflammatory cells, macrophages are believed to play an important role in tumor-related angiogenesis, and CD8+ dense lymphocytic infiltration of the tumor tissue is associated with a good prognosis.[23] In our study, relationship between IMD, bFGF and nm23-H1 expression and lymphocytic infiltration was not found.

Current methods for assessing tumor angiogenesis could be evaluated with direct or indirect methods. The most frequently used direct methods are microvessel density counts, immunostaining, and reverse transcription polymerase chain reaction (RT-PCR) for angiogenic cytokines. All of these techniques require tumor tissue and they are therefore generally performed on postoperative specimens.[22] This histological IMD technique is the current gold standard to characterize tumor angiogenesis.[24]

Some studies demonstrated that an increase in IMD was found to be associated with the expression of VEGF, and that IMD had a prognostic value in predicting metastasis of various malignant solid tumors.[6-9,25-27] Weidner et al[3] demonstrated a significant correlation between the incidence of metastases and IMD in invasive breast carcinoma. The prognostic role of IMD in CRC was investigated by several authors with controversial results. Takahashi et al[28] reported a correlation of IMD with depth of invasion and LNM. In CRC, IMD was significantly correlated with the presence of liver metastases,[25] hematogenous metastasis,[26] relapse and overall survival.[29] On the contrary, Bossi et al,[12] White et al,[30] and Li et al[31] did not find any association of IMD with metastasis, stage of disease, or patient survival. Several factors may be responsible for obtaining different data on this subject. For example, counting methods for assessment of IMD were not standardized, different antibodies were used for immunohistochemistry (CD31, CD34, vWF-8 and CD105), and different numbers of microscopic fields, ranging from one to five fields per area of hot-spot angiogenesis of the tumors, were evaluated at different magnifications.

bFGF has a potent mitogenic activity for a wide variety of mesoderm-neuroectoderm-derived cells. This growth factor stimulates vascular endothelial cell proliferation, and virtually all these cells either produce or have receptors for bFGF. It has been demonstrated that bFGF is involved in the

neoplastic angiogenesis of several types of tumors such as melanoma, glioblastoma, Kaposi sarcoma, and pancreas, renal, breast, and lung tumors.[32] Landriscina et al[33] reported that no significant correlation was found between bFGF expression and Dukes or TNM stages, grade, or nodal or distant metastases, although there was a significant correlation between VEGF levels and stage of disease. They suggested that bFGF and VEGF were probably involved in CRC angiogenesis with different mechanisms. Zheng et al[34] found significant correlation between the IMD and grade, and there was no significant relationship between the IMD and age, sex, and stage. They reported that VEGF expression was not an independent prognostic factor, although stage, tumor grade and IMD were significant variables. Takahashi et al[35] suggested that patients with node-negative CRC with high vessel counts and/or high expression of VEGF might be good candidates to undergo adjuvant chemotherapy, although they indicated that bFGF was not as important as VEGF in inducing CRC angiogenesis. A relationship between IMD and tumor metastasis has also been demonstrated in CRC, and VEGF expression was associated with advanced disease as well as IMD. By in situ hybridization, some authors have demonstrated "hot spots" for bFGF in patients with human colon cancer, and they have suggested that this method might be more relevant than using immunohistochemistry.[28]

nm23-H1 is a potential metastasis suppressor gene that was originally identified by differential hybridization between low- and high metastatic murine melanoma cells.[16] There are five different nm23-H1 genes including nm23-H1 and nm23-H1-H2 identified in humans. They may play a role in metastatic processes of different tumors.[36] Reduced expression of nm23-H1 mRNA or its protein has been associated with high metastatic potential and poor prognosis in some tumors, such as melanoma and hepatocellular and gastric carcinomas.[37-39] However, increased expression of nm23-H1 was found to be correlated with tumor progression and metastasis in other tumors, such as thyroid carcinoma and squamous carcinoma of the lung.[40,41] The role of this gene in the progression and metastatic potential of CRC is still controversial. Most investigators have found association with reduced nm23-H1 expression in patients with advanced tumor stage and liver metastasis,[17,18,42,43] while others have found no significant association.[44-46] Thus, its role in the prognosis of CRC remains debated.

Similarly to our study, Dursun et al[47] reported that reduced nm23-H1 expression was associated with advanced tumor stages, and there was inverse correlation with vascular invasion, nodal metastasis and liver metastasis. They also found that in well-differentiated CRC there was strong staining for nm23-H1 compared with moderately and poorly differentiated CRC. No significant correlation was found between tumor stage, degree of differentiation, nodal involvement, five-year survival, disease recurrence, age, or sex and nm23-H1 status in some studies.[19,20,38,39] Dusonchet et al[48] reported that nm23-H1 activity was tissue-specific, and that in CRC the expression of the protein was not associated with tumor progression and patient prognosis. These different data may be due to the use of different antibodies and techniques. In addition, neoplasms are heterogeneous and contain subpopulations of cells with varied metastatic potentials and varied staining for nm23-H1. It has been suggested that mutations in the nm23-H1 gene may play a role in metastasis. However, metastasis is a multi-step process, and if nm23-H1 has antimetastatic properties, it is not clear at which stage it acts. It is feasible that the two-subunit types (nm23-H1 and nm23-H2) behave differently in their ability to affect anti-

Table 1. **Associations of intratumoral microvessel density, and bFGF and nm23-H1 expression with each other, and with grade, stage, lymphoid infiltration, desmoplasia, vascular invasion, lymph node metastasis and tumor size**

	bFGF expression	*nm23-H1 expression*	*IMD*
Grade			
r	0.512	–0.206	0.563
Significance	0.001	0.040	0.001
Stage			
r	0.395	–0.330	0.508
Significance	0.001	0.001	0.001
Lymphocytic infiltration			
r	–0.067	0.027	–0.022
Significance	0.509	0.793	0.828
Desmoplasia			
r	0.089	–0.137	–0.002
Significance	0.379	0.176	0.984
Lymphovascular invasion			
r	0.222	–0.177	0.355
Significance	0.027	0.078	0.001
LNM			
r	0.354	–0.354	0.533
Significance	0.001	0.001	0.001
Tumor size			
r	0.139	–0.179	0.122
Significance	0.167	0.433	0.225
IMD			
r	0.633	–0.464	
Significance	0.001	0.001	

IMD: intratumoral microvessel density; LNM: lymph node metastasis; r: correlation coefficient

metastatic control in a tumor. Since the antibody cross-reacts with nm23-H1 and H2, it was not possible in various studies to determine the expression of nm23-H1 alone.[21,49]

We found separate studies dealing with IMD, bFGF or nm23-H1 expressions. However, there is no report investigating all of them in the same study. So, to our knowledge, this is the first study investigating the importance and correlation of IMD and bFGF and nm23-H1 expressions in CRC. Our data showed that increased IMD and bFGF expression and decreased nm23-H1 expression can provide valuable information in characterizing the malignant phenotype. In this study we could not evaluate the prognosis of the patients because of their referring to oncology departments in other cities. Thus, although we found significant data with IMD, bFGF and nm23-H1 expression in CRC, we could not determine their association with prognosis. For this reason, a prospective study including the prognosis of the patients would be essential to evaluate the role of IMD, bFGF and nm23-H1 expression.

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