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ORIGINAL ARTICLE

Quantitative evaluation of stromal myofibroblasts and their significance for the metastatic capacity of colorectal carcinoma

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Summary

Purpose: *To quantify the myofibroblasts in the tumor stroma of colorectal carcinomas using immunostaining with anti smooth muscle actin (SMA) as a marker for myofibroblasts.*

Methods: *The study was carried out on 46 surgically resected primary colorectal adenocarcinomas from the archive of the Centre for Pathology and Forensic Medicine of the Military Medical Academy in Belgrade, from 2008-2010. All samples were analysed by the scientific software "Image J". Myofibroblasts were visualized using anti-SMA antibody and quantified in order to predict tumor capacity for invasion and metastasis. Receiver Operator Characteristic (ROC) analysis was carried out, and a score of 5.72 was suggested as the score of SMA that is significant for the clinical outcome with lymph node involvement.*

Results: *Overall, the average SMA was 7.29 (range 0.39-16.84). Further analysis showed correlation of SMA with clinical and pathological tumor characteristics, i.e. SMA was significantly higher in tumors with more advanced stage, higher histological grade, greater amount of desmoplasia, smaller amount of inflammatory infiltrate, lymph node involvement, vascular and perineural invasion and infiltrative tumor growth.*

Conclusion: *Our study suggests that it is possible to define the tumor capacity for invasion and metastasis by quantifying the myofibroblasts in the tumor stroma of colorectal carcinomas. Therefore, further investigations are needed to determine targeted therapies to signaling pathways in myofibroblasts.*

Key words: carcinoma, colon, image analysis, myofibroblasts

Introduction

Tumor invasion involves complex interactions between tumor and the stromal cells. The stromal component of colonic carcinomas varies from little or no stroma to frankly scirrhous tumors. Because the dynamic changes in the cancer-associated stroma resemble a wound-healing reaction [1], they are termed "desmoplastic reaction". The desmoplastic reaction is thought to be supported mainly by the activation of host fibroblasts referred to as ''myofibroblasts'' [2-4]. Intestinal subepithelial myofibroblasts are presented immediately subjacent to the basement membrane and close to the basal surface of the epithelial cells. Early ultrastructural studies have shown that these cells have characteristics of fibroblasts [5,6]. In subsequent studies, they were also shown to share ultrastructural and immunochemical characteristics with smooth muscle cells and have therefore been designated as myofibroblasts [7-9]. Myofibroblasts produce an extracellular matrix enriched in type III and V collagen, which is considered to be responsible for the hard consistency of many carcinomas [10]. A variety of clinical studies of human lung, breast and squamous cell carcinomas of the oral cavity and skin suggested an association between myofibroblasts and desmoplasia with tumor invasion, development of metastases, or tumor recurrence [11-14]. In other experiments it was confirmed that myofibroblasts might have a supportive or facilitating role in tumorigenesis and progression of carcinomas of the prostate, breast, and

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keratinocytes [15-17]. The number of myofibroblasts in the stroma of colorectal carcinomas has been correlated with the capacity of tumor invasion, as previously reported [18,19]. Using computer-assisted image analysis myofibroblasts were quantified and the results suggested that they could be a useful indicator of disease recurrence after surgery [20].

In the present study, we quantified myofibroblasts in tumor stroma of colorectal carcinomas using computer assisted image analysis, with anti-SMA as a marker for myofibroblasts. We tried to elucidate any role of myofibroblasts in relation with tumors' capacity for invasion and metastasis and their ability to predict the metastatic capacity of carcinoma.

Methods

In this study we used specimens from 46 surgically resected primary colorectal adenocarcinomas during 2008 through 2010 from the archives of the Center for Pathology and Forensic Medicine, Military Medical Academy, Belgrade. The tissues were first fixed in 5% formaldehyde, treated in routine way and embedded in paraffin. Four μm thick tissue sections were prepared and stained with routine H&E. For visualization of desmoplasia, the special histochemical method Van Gieson was used. For myofibroblasts, immunostaining was carried out using the En Vision system (K5007) and chromogen (DAB Liquid K3466, Dako) with primary anti-SMA monoclonal antibody (Dako M0851,1: 50). H&E stained sections were reviewed and conventional histopathological prognostic parameters for colorectal carcinoma, such as tumor grade, depth of invasion, lymph node involvement, lymphatic, venous and perineural invasion, were registered. Conventional semiquantitative grading for desmoplasia was also carried out [21]. The tumors were graded according to the World Health Organization criteria and staged according to the AJCC/UICC TNM staging system [22]. The clinical stage of disease was determined according to the modified Astler-Coller staging system [23]. Tumors' growth characteristics were classified as expanding or infiltrating [24].

The sections were photographed using digital microscope (COOLSCOPE-Nikon) at ×20 magnification for SMA. In each case 10 digital images were randomly selected. All images were analysed using the free scientific software "Image J", available on http://rsb.info.nih.gov/ij. This software is intended to visualize parameters of interest in red color and allows manual selection and separation of desired parameters. For each captured area, which was 1280×960 μm (1.2 mm^2), the percentage of the red-stained area was determined automatically (area %). Ten photos for each section were used and for further analysis a mean value of 10 fields was determined.

Statistical analysis

Graph Normal Q-Q Plot Histogram, Kolgomorov's-Smirnoff's test and Shapiro-Wilk's test were used for exploring any association between the distribution of variables and their normal distribution. For description of variables depending on their nature, we used descriptive statistical methods: frequency, percent, mean value (average), standard deviation (SD) and range. For the level of statistical significance the value of α=0.05 was accepted. Bonferroni test was used to compare multiple pairs (α_1=0.05/3=0.0167). For testing differences between groups of interest depending on their nature we used Pearson's x^2 test, Fisher's exact test, Kruskal-Wallis test, Wilcoxon rank sum test, and Exact Wilcoxon rank sum test. The ROC curve method and areas under the curve (AUC) were used to establish the critical SMA value which could predict if lymph nodes would be involved [25]. For testing the AUC significance, Z-test was used. All data were analysed using the statistical program R (version 2.8.1/ 2008-12-22; Copyright [C] 2008; The R Foundation for Statistical Computing, ISBN3-900051-07-0). The Microsoft Office Excel 2003 was used to create graphs.

The study protocol was approved by the Human Ethics Committee of the Military Medical Academy, Belgrade, acknowledged by the Declaration of Helsinki, 2000.

Results

The clinical and pathological characteristics of the patients and tumors are listed in Table 1. Overall the SMA mean score was 7.29 and varied widely from 0.39 to 16.84. In each section, myofibroblasts were stained for SMA (brown color, Figure 1), then selected in color (red color, Figure 2) and determined by computerized image analysis program (Table 2). The SMA scores were analysed and compared with the various clinical and pathological variables (Table 3). There was no association between female and male sex, tumor subtypes and SMA expression. The SMA score was significantly higher in tumors with higher stage (T3 vs. T2), with lymph node involvement, lymphatic and venous invasion, perineural invasion, and tumors with infiltrative growth. The SMA score results were compared with the histological grade, desmoplasia and inflammatory infiltrate (Table 4). High SMA expression was significantly lower in grade (G) 1 tumors compared with G2 and G3. Tumors with slight desmoplasia had significantly lower SMA scores compared with those with moderate and extensive desmoplasia. Tumors with slight inflammatory infiltrate had significantly higher SMA scores than tumors with moderate and extensive inflammatory infiltrate.

For our research it was important to investigate the SMA score in association with lymph node involvement. The surface under the ROC curve (AUC ROC) with 95% CI (confidence interval) was 0.73 (range 0.66-0.93), which was statistically significant (H_0: AUC ROC=0.5; Z-test; p=0.001; Figure 3). Due to confirmed SMA significance in association with lymph node involvement, based on the ROC curve the cut off value was $SMA_{cut.off}$= 5.72, with maximal sensitivity and specificity (88 and 66.7% respectively; Figure 3). To further analyse the situation we created 2 groups: the first with high SMA score (>5.72) and the second with low SMA score (< 5.72) according to SMA expression

Table 1. Clinical data of the investigated patient groups

Characteristics	*N (%)*
Total number	46
Sex	
Male	33 (71.7)
Female	13 (28.3)
Age (years)	
Mean	67.2
Range	22-87
MAC stage	
B1	11 (23.9)
B2	10 (21.7)
C1	1 (2.1)
C2	21 (45.6)
D	3 (6.5)
Tumor grade	
1	13 (28.3)
2	26 (56.5)
3	7 (15.2)
Tumor type	
Tubular	40 (86.9)
Mucinous	6 (13)
T stage	
T2	13 (28.2)
T3	33 (71.7)
Lymph node involvement	
Negative	21 (45.6)
Positive	25 (54.3)
Lymphatic invasion	
Negative	19 (41.3)
Positive	27 (58.7)
Venous invasion	
Negative	33 (71.7)
Positive	13 (28.3)
Perineural invasion	
Negative	21 (45.6)
Positive	25 (54.3)
Desmoplasia	
Slight	13 (28.3)
Moderate	19 (41.3)
Extensive	14 (30.4)
Inflammatory infiltrate	
Slight	32 (69.6)
Moderate	9 (19.6)
Extensive	5 (10.9)
Tumor growth	
Expanding	14 (30.4)
Infiltrating	32 (69.6)

MAC: Modified Astler-Coller

at a cut off point 5.72. The results are presented in Table 5. There was no statistically significant difference between the 2 groups in relation to tumor subtype and venous invasion. On the other hand, there was statistically significant difference between groups of tumors with higher stage (T3 vs. T2), lymphatic and perineural invasion and infiltrative growth. There was also statistically significant difference between G1 and G2 and G3. Statistically significant difference was found between

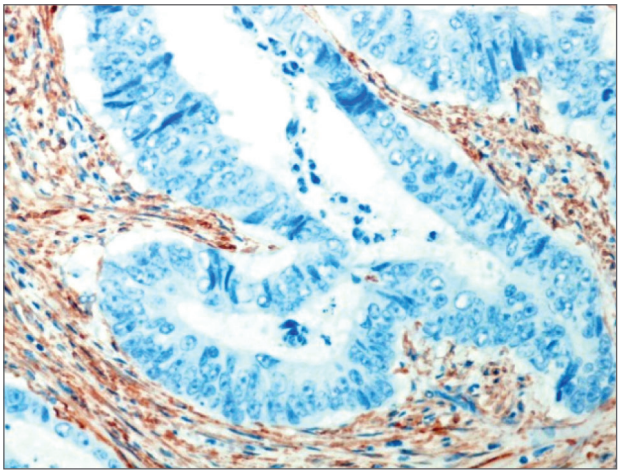

Figure 1. Myofibroblasts before selection of red color (SMA staining ×20).

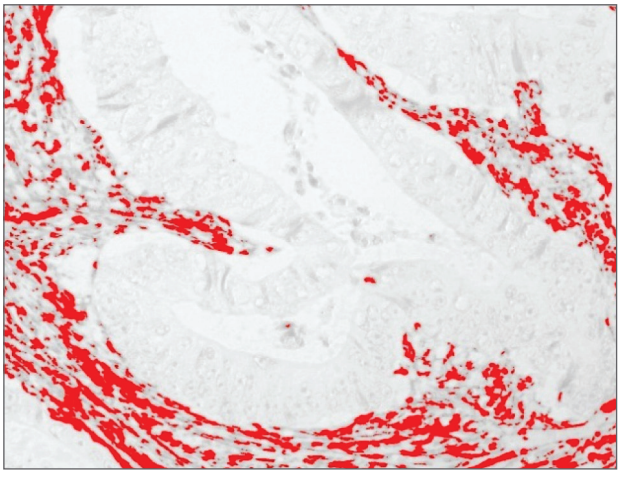

Figure 2. Myofibroblasts after selection of red color ("Image J").

tumors with slight desmoplasia compared to tumors with moderate and extensive desmoplasia. The results between groups with different inflammatory infiltrate showed that there was statistically significant difference between tumors with slight compared to those with moderate and extensive infiltrate.

Table 2. Presentation of SMA ("Image J")

Label	*Area*	*Area %*
1.111.jpg: red	41979.8	3.4
2.112.jpg: red	23924.9	1.9
3.113.jpg: red	26040	2.1
4.114.jpg: red	91007	7.4
5.115.jpg: red	84117.4	6.8
6.116.jpg: red	86894.7	7.2
7.117.jpg: red	31185	2.5
8.118.jpg: red	51969.4	4.3
9.119.jpg: red	65816.8	5.4
10.1110.jpg: red	56378.8	4.6

Table 3. Correlation of SMA with clinical and pathological tumor characteristics

Characteristics	*N*	*SMA (SD)*	*Wilcoxon rank sum test*
Sex			
Male	33	7.9 (4)	W=256; p =0.1
Female	13	5.6 (4.2)	
Tumor type			
Mucinous	6	6.3 (2.4)	W=101; p =0.5
Tubular	40	7.4 (4.8)	
T stage			
T2	13	2.7 (1.7)	W=22; p =8.5×10^{-8}
T3	33	9.1 (3.4)	
Lymph node involvement			
Negative	21	5.9 (4.2)	W=109; p =0.4×10^{-4}
Positive	25	9.1 (3.3)	
Lymphatic invasion			
Negative	19	4.1 (3.9)	W=54; p =1×10^{-6}
Positive	27	9.5 (2.7)	
Venous invasion			
Negative	33	6.2 (4)	W=93; p =0.02
Positive	13	9.9 (3.2)	
Perineural invasion			
Negative	21	4.3 (3)	W=54; p =6.4×10^{-7}
Positive	25	9.8 (3.2)	
Tumor growth			
Expanding	14	2.5 (1.6)	W=6; p =2.5×10^{-10}
Infiltrating	32	9.4 (3)	

SD: standard deviation, SMA: smooth muscle actin

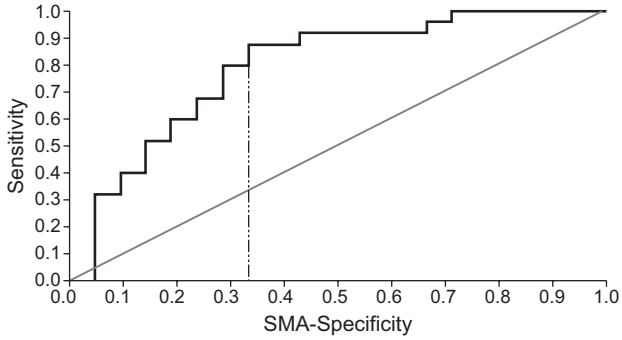

Figure 3. Receiver Operator Characteristic (ROC) curve for smooth muscle actin (SMA) in association with lymph node involvement. SMA cut off value 5.72; area 95%.

Discussion

The stromal tumor microenvironment plays a crucial role in tumor progression [1]. Tissue stroma consists of a variety of matrix substances such as interstitial collagen, elastin, fibronectin, glycoaminoglycans and a variety of cell types including inflammatory cells, immune cells, fibroblasts, muscle cells, and vascular cells [1]. In some reports myofibroblasts were suggested to have the most important role in producing the tumors' stroma [4,10,15-17,26], while the precise mechanism of influencing on tumor progression is complex [15-17]. Myofibroblasts are activated by cytokines, such as trans-

Table 4. Correlation of SMA with histological grade, desmoplasia and inflammatory tumor infiltrate

Characteristics	*N (%)*	*SMA (SD)*	*Kruskal Wallis test*
Histological grade			
1	13 (28.3)	2.7 (1.5)	
2	26 (56.5)	8.7 (3.2)	$x^2 = 23.8$; $p = 6.6\times10^{-6}$
3	7 (15.2)	10.6 (3.7)	
1 vs. 2		Wilcoxon rank sum test W = 19; p = 5×10^{-7}	
1 vs. 3		Wilcoxon rank sum test W = 0; p = 2.6×10^{-5}	
2 vs. 3		Wilcoxon rank sum test W = 62; p = 0.2	
Desmoplasia			
Slight 1	13 (28.3)	2.7 (1.7)	
Moderate 2	19 (41.3)	8,25 (3.9)	x^2=25.3; p=3.3×10^{-6}
Extensive 3	14 (30.4)	10.2 (2.1)	
1 vs. 2		Wilcoxon rank sum test W = 20; p = 1.5×10^{-5}	
1 vs. 3		Wilcoxon rank sum test W = 2; p = 4×10^{-7}	
2 vs. 3		Wilcoxon rank sum test W = 72; p = 0.03	
Inflammatory infiltrate			
Slight 1	32 (69.6)	8.9 (3.4)	
Moderate 2	9 (19.6)	3.9 (3.5)	$x^2 = 25.3$; $p = 3.3\times10^{-6}$
Extensive 3	5 (10.9)	2.6 (1.3)	
1 vs. 2		Wilcoxon rank sum test W = 246; p = 0.7×10^{-3}	
1 vs. 3		Wilcoxon rank sum test W = 152 p = 0.3×10^{-3}	
2 vs. 3		Wilcoxon rank sum test W = 26; p = 0.7	

SD: standard deviation, SMA: smooth muscle actin

Table 5. Overall tumor characteristics with SMA cut off value <5.72 vs. >5.72

Characteristics	*SMA≤5.72 N (%)*	*SMA>5.72 N (%)*	*N (%)*	*Fisher's Exact test*
	17 (36.9)	29 (63)		
Tumor type				
Mucinous	3 (17.6)	3 (10.3)	6 (13)	p = 0.6
Tubular	14 (82.3)	26 (89.7)	40 (86.9)	
T stage				
T2	13 (76.5)	0 (0)	13 (28.3)	$p = 2.3\times10^{-8}$
T3	4 (23.5)	29 (100)	33 (71.7)	
Lymphatic invasion				
Negative	16 (94.1)	3 (10.3)	19 (41.3)	$p = 1.5\times10^{-8}$
Positive	1 (5.88)	26 (89.7)	27 (58.7)	
Venous invasion				
Negative	16 (94.1)	17 (58.6)	33 (71.7)	p= 0.01
Positive	1 (5.9)	12 (41.4)	13 (28.3)	
Perineural invasion				
Negative	16 (94.1)	5 (17.2)	21 (45.6)	$p = 2.9\times10^{-7}$
Positive	1 (5.9)	24 (82.7)	25 (54.3)	
Tumor growth				
Expanding	14 (82.3)	0 (0)	14 (30.4)	$p = 2.8\times10^{-9}$
Infiltrating	3 (17.6)	29 (100)	32 (69.6)	
Histological grade				
1	13 (76.5)	0 (0)	13 (28.3)	
2	4 (23.5)	22 (75.9)	26 (56.5)	$p = 2\times10^{-8}$
3	0 (0)	7 (24.1)	7 (15.2)	
1 vs. 2	Fisher's Exact test			$p=2.9\times10^{-7}$
1 vs. 3	Fisher's Exact test			$p=1.3\times10^{-5}$
2 vs. 3	Fisher's Exact test			p=0.5
Desmoplasia				
Slight 1	13 (76.5)	0 (0)	13 (28.3)	
Moderate 2	3 (17.65)	16 (55.2)	19 (41.3)	$p = 3.8\times10^{-8}$
Extensive 3	1 (5.8)	13 (44.8)	14 (30.4)	
1 vs. 2	Fisher's Exact test			$p=3.3\times10^{-6}$
1 vs. 3	Fisher's Exact test			$p=7.4\times10^{-7}$
2 vs. 3	Fisher's Exact test			p=0.6
Inflammatory infiltrate				
Slight 1	4 (23.5)	28 (96.5)	32 (69.6)	
Moderate 2	8 (47.1)	1 (3.45)	9 (19.6)	$p =2.9\times10^{-7}$
Extensive 3	5 (29.4)	0 (0)	5 (10.9)	
1 vs. 2	Fisher's Exact test			$p=4.2\times10^{-5}$
1 vs. 3	Fisher's Exact test			$p=0.2\times10^{-4}$
2 vs. 3	Fisher's Exact test			p=1

SMA: smooth muscle actin

forming growth factor β (TGF-β) produced by tumor cells, which in turn produce cytokines or growth factors that stimulates tumor cell proliferation and angiogenesis [15,17]. Myofibroblasts produce lytic enzymes able to degrade the basement membrane around tumor cells, and participate in the production of extracellular tumor's stroma matrix which in turn affects the tumor's adhesion and migration. All this suggests that myofibroblasts have a significant role in tumors' invasive and metastatic potential [17, 26, 27]. On the other hand, myofibroblasts prevent the physical contact between cancer cells and the macrophages and T-lymphocytes, which are part of the immune response against cancer [28]. In earlier investigations myofibroblasts in tumors' stroma were quantified using the semiquantitative grading [18]. In some reports, myofibroblasts were also found in adenomas, indicating that they might have a role in cancer initiation [29]. In one study of breast cancer, myofibroblasts around *in situ* carcinoma participated in the stabilization of the peritumoral matrix by producing lysyl oxidase and preventing invasion of cancer cells [30]. In hepatocellular carcinomas SMA-positive cell populations were detected not only within but also around the tumor [31]. In addition, it is suggested that these cells are responsible for tumor capsule formation around liver metastases from colorectal cancer, and that the capsule may serve

as a mechanical and/or chemical barrier to local invasion by metastatic tumor cells [32].

The difference in quantity of myofibroblasts in the intra-and peritumoral stroma in human colon cancer had shown that myofibroblasts were more abundant in the intratumoral than in the peritumoral area. In this study [33] it was suggested that the quantity of the peritumoral myofibroblasts, but not the intratumoral ones, is connected with more aggressive biological behavior, lymphatic invasion and lymph node involvement. Myofibroblasts in the invasive front of carcinoma are usually most abundant [14], and less abundant in a tumor that invades the serosa, but also less abundant in a higher stage (T3), compared with a lower stage (T2) tumor [18]. In order to avoid subjectivity in the quantification of myofibroblasts we used a software system, which is a more complicated method than the semiquantitative method, but gives more precise results. Further analysis of SMA and comparison to clinical and pathological tumor characteristics had shown that higher stage tumors, with vascular and perineural invasion, with more abundant desmoplasia, slight inflammatory infiltrate, infiltrative tumor growth, higher histological grade and lymph node metastasis have a greater number of myofibroblasts. In previously reported studies, the software systems were used to quantify the myofibroblasts in tumors with results similar to ours. Also, it was suggested that the myofibroblasts could be an useful indicator for the lymph node involvement and disease recurrence after the surgical treatment [20]. Similar results were found in breast cancer [34]. For therapeutic manipulations and disease outcome, the most important prognostic factors are lymph node involvement and liver metastasis [35,36]. In our study we tried to investigate whether any score of myofibroblasts could predict lymph node involvement with considerable sensitivity and specificity by determining the SMA cut off value. We found that the optimal cut off value for SMA might be 5.72 with satisfactory sensitivity and specificity. One of the disadvantages in our study was that we did not evaluate the correlation between patients with liver metastases, number of metastases, the time elapsed between operative treatment and occurrence of metastasis, and SMA score. Further investigations could provide a precise SMA score as a prognostic parameter for the tumor's metastatic potential, indicating adequate and timely treatment.

Conclusion

This study has shown the importance of the quantification of the myofibroblasts in the stroma of colorectal carcinomas and the correlation between stromal myofibroblasts and tumors' potential for invasion and metastasis. These results might suggest that targeted therapies against the myofibroblasts' signaling pathways may open new perspectives in the treatment of colorectal carcinoma.

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