Significance of tenascin-C, fibronectin, laminin, collagen IV, α5β1 and α9β1 integrins and fibrotic capsule formation around liver metastases originating from cancers of the digestive tract*

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The formation of a fibrotic capsule around liver metastases may functionally act as a barrier to local invasion. However, the prognostic significance of exstracellular matrix (ECM) and of some integrins' deposition around liver metastases remains unclear. An immunohistochemical investigation was carried out on 55 patients with synchronous liver metastases from colorectal and gastric cancers. Encapsulated metastases were detected in 60% of the cases. The "non-capsular" cases showed clear immunostaining for tenascin-C, fibronectin, collagen IV, laminin, αSMA and integrins. On opposite, most of the cases with "capsule" were negative for the studied ECM proteins and the two integrins. The patients with "capsular" pattern had significantly longer median survival after the surgery compared to those with non-encapsulated metastases. The presence of tenascin, fibronectin, fibronectin receptor and laminin, as well as the strong immune signal for αSMA and collagen type IV in the sinusoids attached to the liver metastases was associated with a worse prognosis. The cells, forming ECM in the sinusoids attached to metastases in the "non-capsular" pattern were αSMA-positive myofibroblasts. It was shown ultrastructurally that they were HSCs. The results indicate that fibrotic capsule formation is associated with longer survival after surgery. The appearance of tenascin-C and of its receptor at the periphery of liver metastases could be used as a sign of invasiveness.

Key words: liver metastasis, colorectal cancer, gastric cancer, fibrotic capsule, extracellular matrix

Synchronous liver metastases are found in approximately 10–30% of patients at the time of laparotomy for colorectal cancers [1]. Liver resection of synchronous and metachronous metastases has been accepted as the only option offering long-term survival in patients with colorectal liver metastases [2]. The intestinal type of gastric cancer tends to penetrate blood vessels and to metastasize to the liver [3]. The two types of cancer metastases had a good desmoplastic reaction and tend to form fibrotic capsules around them.

In order to improve survival, it is important to predict possible further local spread from liver metastasis. Many studies have been performed concerning prognostic variables in patients with liver metastases from colorectal [1, 4–7] and gastric [3, 8] cancers. In most of them different immune markers were analyzed in relation to the prognosis. However, there is limited number of reports, devoted to the prognostic significance of fibrotic capsule formation around liver metastases [9–11]. In fact, it is well known that fibrotic capsule formation is an important indicator for better prognosis of human hepatocellular carcinoma (HCC) [9, 12].

It has been already reported that liver extracellular matrix (ECM) modulates cancer invasion and metastasis [13–17]. It was shown that, laminin and fibronectin facilitated cancer cell attachment and migration [18, 19]. Enhanced tenascin-C expression was found in the areas of tissue remodelling and epithelial cell proliferation [20–22]. In order to proliferate tumor cells and endothelial cells must have receptors for tenascin (α9β1 integrin) and in order to migrate these cells must possess receptors for fibronectin (α5β1 integrin) [23, 24]. On the other hand myofibroblasts (MF) from tumor stroma stimulate the invasion of human colon cancer cells and the neoangiogenesis in the tumor [13]. Similarly, trans-

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formed hepatic stellate cells (HSCs) participate in the stroma formation of liver metastases [25, 26].

In the present study we examined immunohistochemically colorectal and gastric liver metastatic tissue and assessed the biologic and prognostic significance of fibrotic capsule formation around hepatic metastases. We have found that tenascin-C and α-smooth muscle actin (αSMA) are markers for the "non-capsule" pattern. We established that patients with "capsular" pattern had better prognosis. The role of liver ECM in the sinusoids attached to metastasis and in the capsule is discussed as a possible mechanism modulating cancer cell invasion.

Material and methods

Patients and liver samples. From 1998 to 2004, 55 patients with synchronous liver metastases from colorectal and/or gastric cancers underwent radical excision of metastases, with a resection margin of at least 1 cm. Excision was done at the time of resection of the primary tumor. The patient population consisted of 34 males and 21 females, between 38 and 87 years of age. We examined 28 liver metastases from primary colonic, 9 from rectal and 18 from primary gastric cancers. The clinical data and the tumor characteristics are given in Table 1. There were no patients with viral hepatitis, cirrhosis, focal nodular hyperplasia or alcohol abuse (<20 g). All patients were followed until 15 December 2005. Informed consent was obtained from all patients. Tissues were taken from liver metastases, including the interface between metastases and adjacent liver parenchyma, and from the non-cancerous liver at some distance from metastases. The surgery were carried out according to the accepted protocols in Bulgaria for surgical interventions and obtaining of human biopsy materials. Liver surgical biopsies approximately 15 x 15 x 8 mm, were used.

Definition of routine histologic evaluation. Paraffin sections were stained with haematoxylin and eosin, and Van Gieson for assessment of capsule formation. The degree of fibrotic tissue layer between the periphery of metastatic nodules and the hepatic parenchyma was estimated based on the thickness of fibrotic bundles. A fibrotic bundle at the margin of the metastatic nodule with approximately 0.5 mm or more regular thickness around the entire surface of liver metastasis was designated as "capsule" pattern. "Non-capsule" pattern was defined as the virtual absence of a fibrous band around the metastasis in which tumor cells faced the hepatic parenchyma directly. A thin fibrous tissue layer was defined as "intermediate" between capsule and non-capsule patterns (Fig. 1a–c). The histologic parameters for assessment of the capsule formation at the periphery of metastases were previously reported [9].

Immunohistochemical staining. Light and electron microscopic immunohistochemistry were carried out on floating sections as described previously [15, 27]. Formalin-fixed paraffin-embedded 10 μm thick sections were deparaffinized and used for light microscope immunohistochemistry and 40–60 μm thick cryostat sections were used for electron microscope immunohistochemistry. Sections were thawed in 10% sucrose in distilled water overnight and the internal peroxidase was inhibited by incubation with 1.2% hydrogen peroxide in methanol for 30 min. Sections were rinsed in 0.1 M phosphate buffered saline (PBS), pH 7.4, for 15 min and then they were then blocked for 30 min with normal mouse

Table 1. The main clinical and histological parameters of the patients with gastrointestinal cancers with liver metastases

Parameters	Number (%)
Clinical data	
Gender	*(n=55)*
Male	34 (61.8)
Female	21 (38.2)
Age (years)	*(n=55)*
Median	64
(range)	(37 – 87)
Location of the primary tumor	*(n=55)*
Stomach	18 (32.7)
Colon	28 (50.9)
Rectum	9 (16.4)
Performance status (clinical group)	*(n=29)*
2	20 (69.0)
4	9 (31.0)
Survivors at the end of follow-up	*(n=41)*
alive	10 (24.4)
dead	31 (75.6)
Chemotherapy	*(n=41)*
yes	15 (36.6)
no	26 (63.4)
Survival after the operation	*(n=41)*
median (months)	7.9
(range)	(0.5 – 149.8)
Histological data	
Differentiation of liver metastases	*(n=55)*
low	8 (14.5)
moderate	32 (58.2)
high	15 (27.3)
No of liver metastases	*(n=46)*
median (range)	1 (1 – 15)
Diameter of liver metastases	*(n=46)*
median (cm) (range)	2 (0,5 – 10)
Sinusoidal inflammatory infiltrate	*(n=55)*
no (–)	2 (3.6)
weak (+)	47 (85.5)
strong (++)	6 (10.9)
Perisinusoidal fibrosis	*(n=55)*
no	40 (72.7)
yes	15 (27.3)
Steatosis	*(n=55)*
no	46 (83.6
yes	9 (16.4)
Capsule	*(n=55)*
absent	22 (40.0)
intermediate	7 (12.7
marked	26 (47.3)

serum (DAKO). After overnight incubation with the primary mouse anti-human antibodies, diluted in PBS in appropriate concentrations, the sections were washed in PBS, and incubated with a secondary anti-mouse biotinylated antibody (DAKO) for 4 h, and subsequently with the streptavidin-HRP complex (DAKO) for 4 h, and rinsed three times in PBS for 10 min each. The reaction was made visible by using a mixture of 3 mg 3,3'-diaminobenzidine (DAB) (DAKO), in 15 ml PBS, and 36 µl 3% hydrogene peroxide for 10–20 min, and rinsed in PBS. The thin sections (10 µm) were mounted on slides, dried overnight at room temperature, soaked in 95° and 100° ethanol, and xylene and then mounted with Entellan®Neu for light microscopy (Merck, Darmstadt, Germany). The sections were not counterstained for better visualization of the DAB reaction product.

The thick cryostat sections (40–60 µm) were post-fixed in PBS containing 2% osmium tetroxide for 30 min at 2 °C, followed by a rinse in PBS. Finally sections were dehydrated in graded concentrations of ethanol and propylene oxide, and flat-embedded with Durcupan, between celophane sheets. Ultrathin sections were cut from the metastatic tissue and from the "liver tissue around metastases". They were counterstained with uranyl acetate only, and examined and photographed with an OPTON EM 109 electron microscope at 50 kV.

Sections incubated with non-immune sera instead of the primary antibodies were used as negative controls. The optimal dilutions for primary antibodies were assessed in a series of stainings performed prior to this study: anti-tenascin-C 1:10 in PBS, anti-fibronectin 1:400 in PBS, anti-collagen type IV 1:40 in PBS, anti-laminin 1:25 in PBS, anti-α9β1 integrin 1:300 in PBS, anti-α5β1 integrin 1:25 in PBS.

Immunochemicals. The antibodies used were mouse anti-human fibronectin (A0245), mouse anti-human laminin (M0638), mouse anti-human α-smooth muscle actin (αSMA) (N1584), and mouse anti-human integrin α5β1 (M0604), obtained from DAKO A/S Denmark, and mouse-anti human tenascin-C mAb 143DB7 (1:10 in PBS), produced and characterized previously [28] (gift from Prof. Ismo Virtanen). The mouse anti-human collagen type IV (MCA 119) and mouse anti-human α9β1 integrin (MCA1585) were obtained from Serotec, Oxford, UK. The detection system used was DAKO LSAB®2 System, HRP (K0675), and DAKO®DAB Chromogen tablets (S3000) (DAKO A/S Denmark).

Immunohistochemical assessment. The deposition of ECM proteins, their receptors and αSMA were studied in the periphery of metastases (the area of capsule, intermediate capsule and "non capsule") and in the remote liver tissue, situated at a distance about 1 cm and more around metastases.

Table 2. Expression of the extracellular matrix proteins and integrins in the periphery of metastases and in remote liver tissue and their associations with the presence of fibrotic capsule around metastases

Parameters	Non-capsulated metastases number (%)	Capsulated metastases number (%)	Significance
In the periphery of metastases			
Tenascin (TN)	(n=22)	(n=33)	p<0.0001 (Fisher Exact test)
no staining (–)	0 (0)	31 (93.9)	
weak staining (+)	22 (100)	2 (6.1)	
Tenascin receptor (TN R)	(n=22)	(n=33)	p<0.0001 (Fisher Exact test)
no staining (–)	2 (9.1)	29 (87.9)	
weak staining (+)	20 (90.9)	4 (12.1)	
a-Smoot muscle actin (α-SMA)	(n=17)	(n=24)	p<0.0001 χ^2 test
no staining (–)	0 (0)	13 (54.2)	
weak staining (+)	4 (23.5)	11 (45.8)	
strong staining (++)	13 (76.5)	0 (0)	
Fibronectin (FN)	(n=18)	(n=25)	p<0.0001 (Fisher Exact test)
no staining (–)	0 (0)	21 (84.0)	
weak staining (+)	18 (100)	4 (16.0)	
Fibronectin receptor (FN R)	(n=18)	(n=25)	p<0.0001 (Fisher Exact test)
no staining (–)	0 (0)	24 (96.0)	
weak staining (+)	18 (100)	1 (4.0)	
Collagen type IV (Coll IV)	(n=17)	(n=24)	p<0.0001 χ2 test
no staining (–)	0 (0)	19 (79.2)	
weak staining (+)	1 (5.9)	5 (20.8)	
strong staining (++)	16 (94.1)	0 (0)	
Laminin (LM)	(n=17)	(n=24)	p<0.0001 (Fisher Exact test)
no staining (–)	0 (0)	19 (79.2)	
weak staining (+)	17 (100)	5 (20.8)	
In remote liver tissue			
Tenascin (TN)	(n=22)	(n=33)	p=0.005 (Fisher Exact test)
no staining (–)	5 (22.7)	21 (63.6)	
weak staining (+)	17 (77.3)	12 (36.4)	
Tenascin receptor (TN R)	(n=22)	(n=33)	NS (Fisher Exact test)
no staining (–)	10 (45.4)	20 (60.6)	
weak staining (+)	12 (54.5)	13 (39.4)	
a-Smoot muscle actin (α-SMA)	(n=17)	(n=24)	NS χ^2 test
no staining (–)	0 (0)	0 (0)	
weak staining (+)	17 (100)	24 (100)	
strong staining (++)	0 (0)	0 (0)	
Fibronectin (FN)	(n=18)	(n=25)	p=0.041 (Fisher Exact test)
no staining (–)	2 (11.1)	11 (44.0)	
weak staining (+)	16 (88.9)	14 (56.0)	
Fibronectin receptor (FN R)	(n=18)	(n=25)	p=0.003 (Fisher Exact test)
no staining (–)	12 (66.7)	25 (100)	
weak staining (+)	6 (33.3)	0 (0)	
Collagen type IV (Coll IV)	(n=17)	(n=24)	NS χ^2 test
no staining (–)	0 (0)	0 (0)	
weak staining (+)	17 (100)	24 (100)	
strong staining (++)	0 (0)	0 (0)	
Laminin (LM)	(n=17)	(n=24)	p=0.008 (Fisher Exact test)
no staining (–)	9 (52.9)	22 (91.7)	
weak staining (+)	8 (47.1)	2 (8.3)	

Intensity of the immune staining was evaluated semi-quantitatively, scored in 3 grades from – to ++ (– = negative; + = low staining; and ++ = strong staining; Tab. 2).

Statistical analysis. The results from immunohistochemistry and clinical data were analyzed using the Stat View™

package for Windows, v.4.53 (Abacus Concepts Inc., Berkeley, California, USA). The descriptive statistical tests, including the mean, median and standard deviation were calculated according to the standard methods. The frequencies of distribution in contingency tables cwere analyzed using chi-square test and Fisher's Exact test. Cumulative survival curves were drawn by the Kaplan-Meier method and the difference between the curves analyzed by the Mantel-Cox (Log-rank) test. Differences and associations with $p<0.05$ were considered to be statistically significant.

Results

Fibrotic capsule and other histological characteristics. The distribution of "capsule", "intermediate capsule" and "non capsule" patterns was 47.3%, 12.7% and 40%, respectively (Tab. 1). Thus, the majority of hepatic metastases was characterized as encapsulated (33 out of 55, 60%). The representative histology of each grade of capsule formation between metastatic foci and liver parenchyma is shown in Figure 1.

No associations were obtained between the frequency of encapsulation and the level of differentiation of metastases, the size and the number of the metastases, the degree of sinusoidal inflammatory infiltrate, and the presence of perisinusoidal fibrosis ($p>0.05$, Fisher exact test). Interestingly, the liver tissue of the patients with steatosis tended to react less intensively to the metastasis, resulting in lack of fibrotic capsule (6 out of 9, 67%) compare to those without steatosis (16 out of 46, 35%, $p=0.134$, Fisher exact test).

Fibrotic capsule and the expression of ECM proteins. Immunohistochemical staining revealed de novo occurrence of tenascin-C (Fig. 2a) and similar expression of αSMA (Fig. 2b) and collagen type IV (Fig. 2c) in the liver attached to metastases in the "non-capsular" cases. The expression of laminin, fibronectin and of the two integrins (α9β1 and α5β1 integrins) was weaker there. αSMA was localized to myofibroblast-like cells, and also distributed to cells in the perisinusoidal space in non-cancerous liver parenchyma adjacent to metastatic tissue. The remote liver tissue was weakly positive for tenascin-C/α9β1, fibronectin/α5β1, laminin, collagen type IV and αSMA.

Weaker immune deposits of tenascin-C and of its receptor α9β1 integrin appeared in the "intermediate capsule". Similar weak immunoreactivity for fibronectin, for its receptor α5β1 integrin, for collagen type IV, laminin and αSMA was visualized in this area. Only the newly formed vessels in the "intermediate capsule" were strongly positive for all studied ECM proteins, the two integrins and αSMA (Fig. 2d). On serial sections it became obvious that tenascin-positive structures coincide with αSMA-positive ones (Fig. 2e, f). The remote liver tissue was negative for tenascin-C/α9β1, fibronectin/α5β1 and laminin and was weakly positive for collagen type IV and αSMA.

In the cases with "capsule" formation around liver metastases, weak αSMA immune reaction, confined to some stromal cells (Fig. 3a) could be observed. All other studied proteins and the two integrins were negative (Fig. 3b) in the

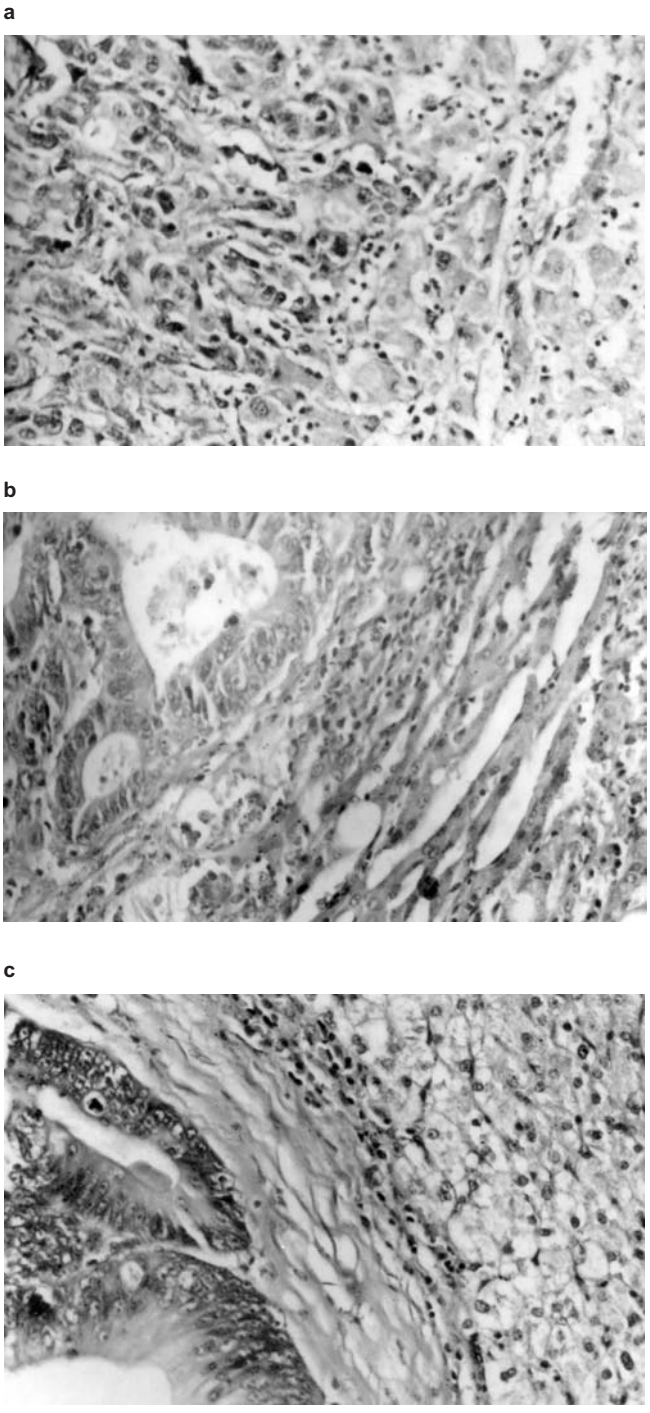

Figure 1. Photomicrographs of fibrotic tissue layer between colorectal and gastric cancer liver metastases and surrounding hepatic parenchyma: a. non-capsule; b. intermediate; c. fibrotic capsule. (H&E) (Magnifications, a, b, c x 250)

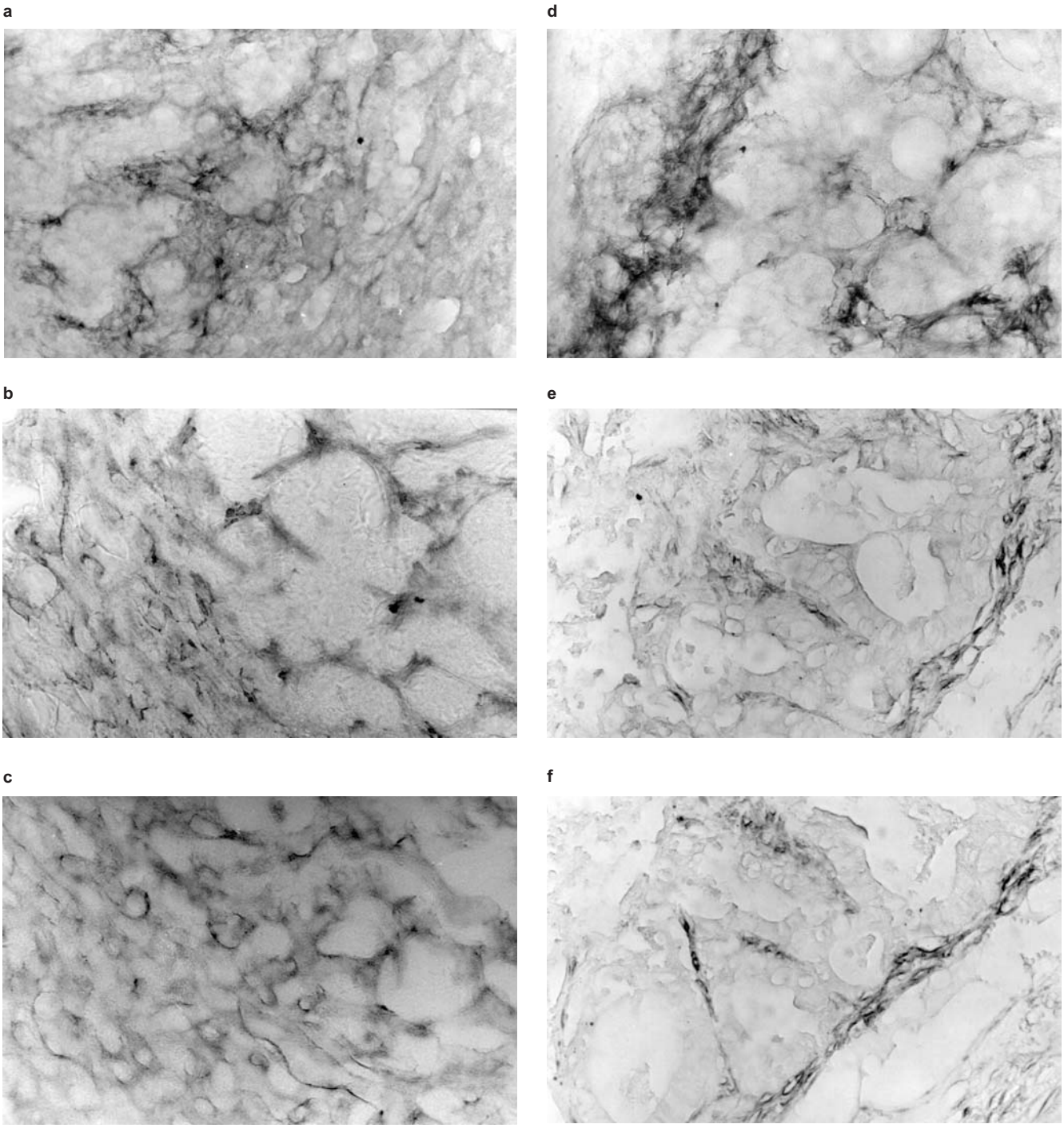

Figure 2. a) Tenascin-C is expressed at the periphery of the "non-capsule" metastatic nodule; b) αSMA is well expressed at the periphery of a "non-capsule" metastasis; c) Collagen type IV is expressed in the sinusoids at the periphery of a "non-capsule" metastasis; d) Tenascin-C is expressed around the newly formed vessels in the "intermediate" capsule; e) Tenascin-C expression at the periphery of a metastasis with an "intermediate" capsule is corresponding to the αSMA-expression (f). (Magnifications a–f x 250).

"capsule". The remote liver tissue was occasionally positive for collagen type IV, αSMA and fibronectin.

Ultrastructural immunohistochemistry revealed amorphous tenascin-C immune deposits in the space of Disse near transformed hepatic stellate cells (HSCs) (Fig. 4a) in the "non-capsule". Tenascin-C immune deposits largely increased at the border between tumor glands and hepatocytes (Fig. 4b). The immune reaction for α9β1 integrin was found

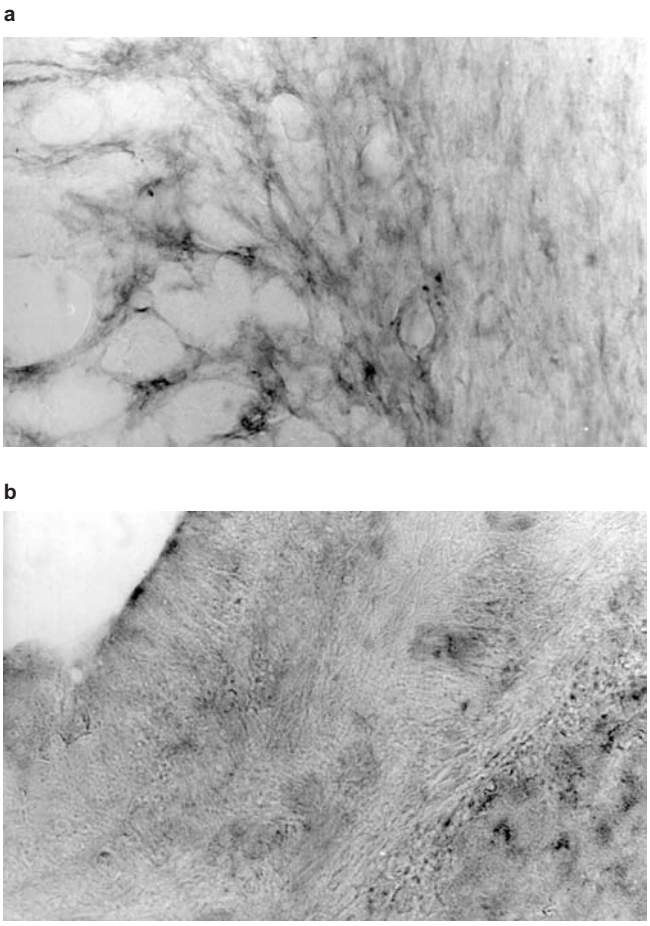

Figure 3. a) A fibrotic capsule around liver metastasis. Weak αSMA immune reaction, confined to some stromal cells could be observed; b) A fibrotic capsule around liver metastasis. Lack of tenascin-C immunoreactivity. (Magnifications, a, b x 250).

in the space of Disse around hepatocyte microvilli and HSC processes. It was enhanced in the space of Disse in liver sinusoids, attached to metastases (Fig. 4c).

The correlations between the encapsulation of liver metastases and the immunohistochemical results of each of the examined ECM proteins and receptors in the different studied areas of the biopsies (periphery of metastases and the remote liver tissue) are presented in Table 2.

Immunohistochemical study revealed highly significant associations between the grades of immune stainings of each of the studied ECM and adhesion proteins in the sinusoids surrounding the liver metastases ($p<0.0001$, Fisher exact test and chi-square test). Tenascin-C and tenascin-C receptor ($\alpha9\beta1$) occurred *de novo* in the liver attached to metastases in the "non-capsular" cases (100% and 90.9%, respectively), whereas these proteins were detected only in 6.1% and 12.1% of the encapsulated cases ($p<0.0001$, Fisher exact test) (Tab. 2). Similarly, all cases with "non-capsule" pattern ($n=17$) were positive (+) for fibronectin and fibronectin receptor ($\alpha5\beta1$), while only 16.0% and 4.0% of those samples with capsule were positive for fibronectin or $\alpha5\beta1$, respec-

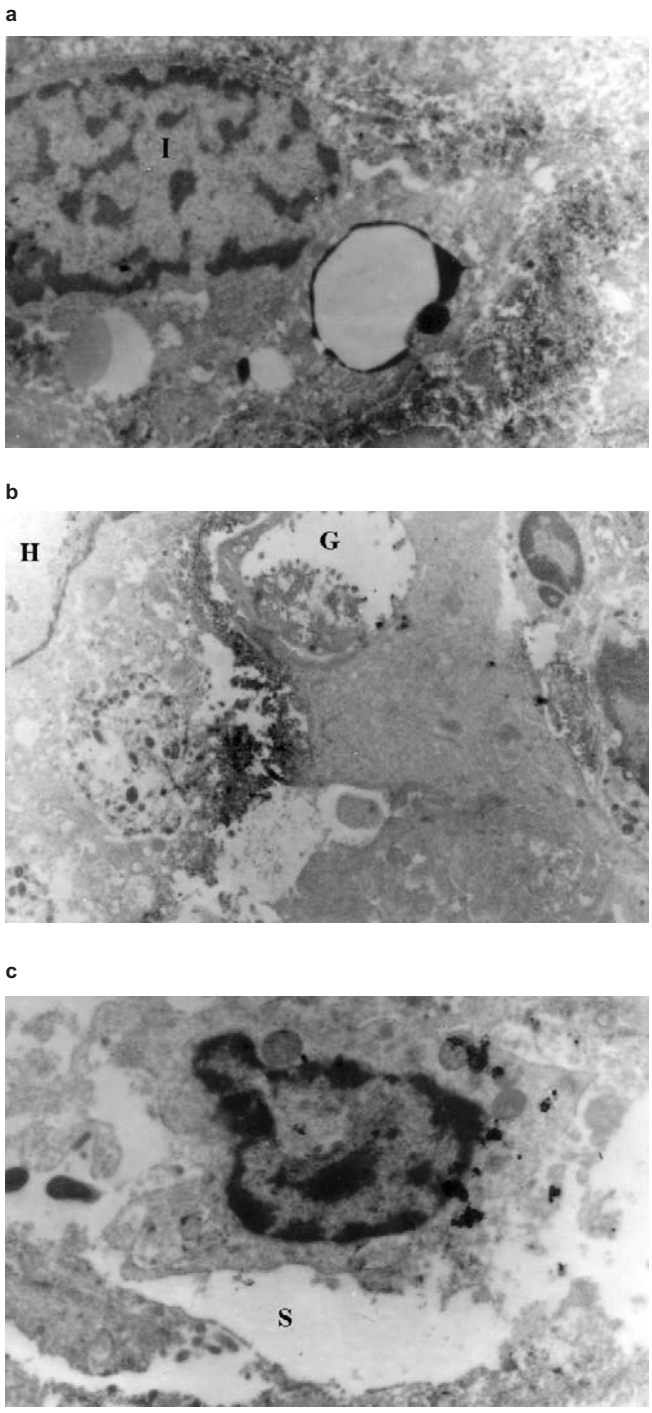

Figure 4. a) Amorphous tenascin-C immune deposits in the space of Disse near transformed Ito cell (I) in a "non-capsule"; b) Increased tenascin immune deposits at the border between tumor glands (G) and hepatocytes (H); c) The immune reaction for α9β1 integrin is found in the space of Disse in liver sinusoids (S), attached to metastasis. (Magnifications, a x 7 000; b x 4 400, c x 7 000).

tively ($p<0.0001$, Fisher exact test). The strong expression levels (++) of αSMA and collagen type IV were also associated with "non-capsule" pattern (76.5% and 94.1%, respectively, $p<0.0001$, chi-square test). αSMA was localized to myofibroblast-like cells, and also distributed to hepatic stellate cells (HSCs) in the perisinusoidal space in non-cancerous liver parenchyma adjacent to metastatic tissue. In the cases with capsule formation around liver metastases, none of the samples showed strong expression of these ECM proteins, and only a weak αSMA and collagen type IV immune reaction, confined to some stromal cells was observed (Tab. 2).

The remote liver tissue was weakly positive for tenascin-C/α9β1, fibronectin/α5β1, laminin, collagen type IV and αSMA. Nevertheless, analogous significant associations were found between the lack of fibrotic capsule and the higher levels of expression of tenascin and fibronectin ($p<0.05$, Fisher exact test) (Tab. 2).

Fibrotic capsule, expression of ECM proteins and survival of the patients. From all 55 patients enrolled in our study, comprehensive clinical informations were available only for 41 (Tab. 1). Among them, a good desmoplastic reaction resulting in formation of fibrotic capsules around the liver metastases was developed in 22 patients (18 with thick and 4 with intermediate capsule). These patients appeared to have significantly longer median survival after the surgery (13.7 mo) compared to those with non-encapsulated metastases (4.6 mo, $p=0.005$, Log-rank test) (Fig. 5). The presence of expression of tenascin, fibronectin, fibronectin receptor and laminin in the sinusoids attached to the liver metastases were significantly associated with a worse prognosis of the patients compared to their absence ($p=0.005$, $p=0.015$, $p=0.001$, and $p=0.018$, respectively, Log-rank test) (Fig. 6a, b, d, e). Analogously, the existence of immune deposits of tenascin receptor also tended to be marker for unfavorable prognosis ($p=0.134$, Log-rank test) (Fig. 6b). Moreover, the strong immune signal of α-SMA and collagen type IV significantly correlated with a shorter survival than their absence or weak expression levels ($p=0.0002$ and $p=0.0004$, respectively, Log-rank test) (Figure 6f, g). No such correlations were observed when the survival of the patients was analyzed according to the expression of the studied ECM proteins and their corresponding receptors in the remote liver tissue ($p>0.05$, Log-rank test).

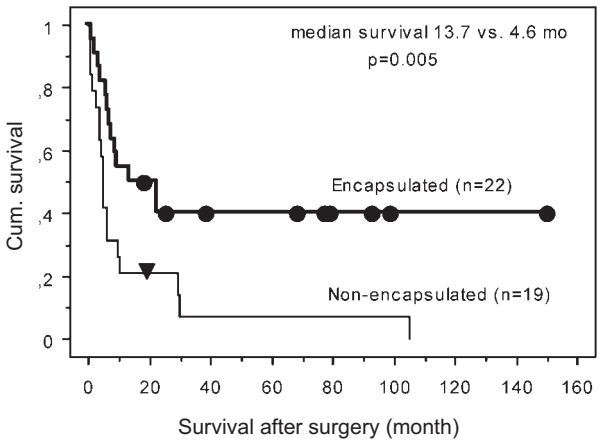

Figure 5. Survival of the patients after the operation according to the presence of fibrotic capsule around liver metastases ($p=0.005$, Logrank test)

Discussion

Desmoplasia consisting of myofibroblasts and ECM is one of the most prominent features of tumor invasion and metastasis. It was shown that progressive tumors were surrounded by a good myofibroblastic network, that isolates them from the host inflammatory response [13, 29]. However, little attention has been paid to the fibrotic stromal response to liver metastases. Some authors classified liver metastases from colorectal cancer into three groups: metastasis without capsule, metastasis with "thin" or "intermediate" capsule, and metastasis with "thick" or "fibrotic" capsule [9, 11]. We used their scheme for assessment of liver metastases periphery, and by the means of immunohistochemistry, revealed the role of liver ECM for tumor progression. Liver metastases from colorectal and gastric tumors, that made a good desmoplastic reaction, when metastasize to the liver, were investigated. The stromal formation and angiogenesis was studied mainly in colorectal cancer liver metastases [26, 30, 31]. In our study synchronous liver metastases with intermediate or thick capsule were the prevalent type (58%), as those cases without fibrotic capsule were 42%. Our results are in accordance with those reported for colorectal carcinoma describing that synchronous and metachronous liver metastases without capsule consisted of 45% [9]. Our results could be explained with the fact that we investigated only synchronous liver metastases originating from two types of primary tumors with a good desmoplastic reaction in metastases.

Cross-talk between cancer cells and liver stromal cells is mediated through the direct heterotypic cell-cell contacts [13] or through the secreted molecules comprising growth factors, cytokines, chemokines, ECM proteins, adhesion proteins etc. In that aspect, we studied the ECM content and the presence of some cellular integrin receptors in the margin of liver metastases. So far, mainly tenascin-C and fibronectin and their receptors have been assessed, because it is well-known that they are important for the metastasis growth and further dissemination [18, 20]. Tenascin-C and fibronectin immunoreactivity at the periphery of liver metastases was compared to the expression of the basement membrane proteins collagen type IV and laminin and to the expression of αSMA – a marker of myofibroblastic cells, the cells synthesizing ECM. The appearance of tenascin-C and its receptor in the invasive margin of liver metastases could be a sign of sinusoidal deterioration and of invasiveness, since normally these proteins are not found in liver sinusoids [32]. The

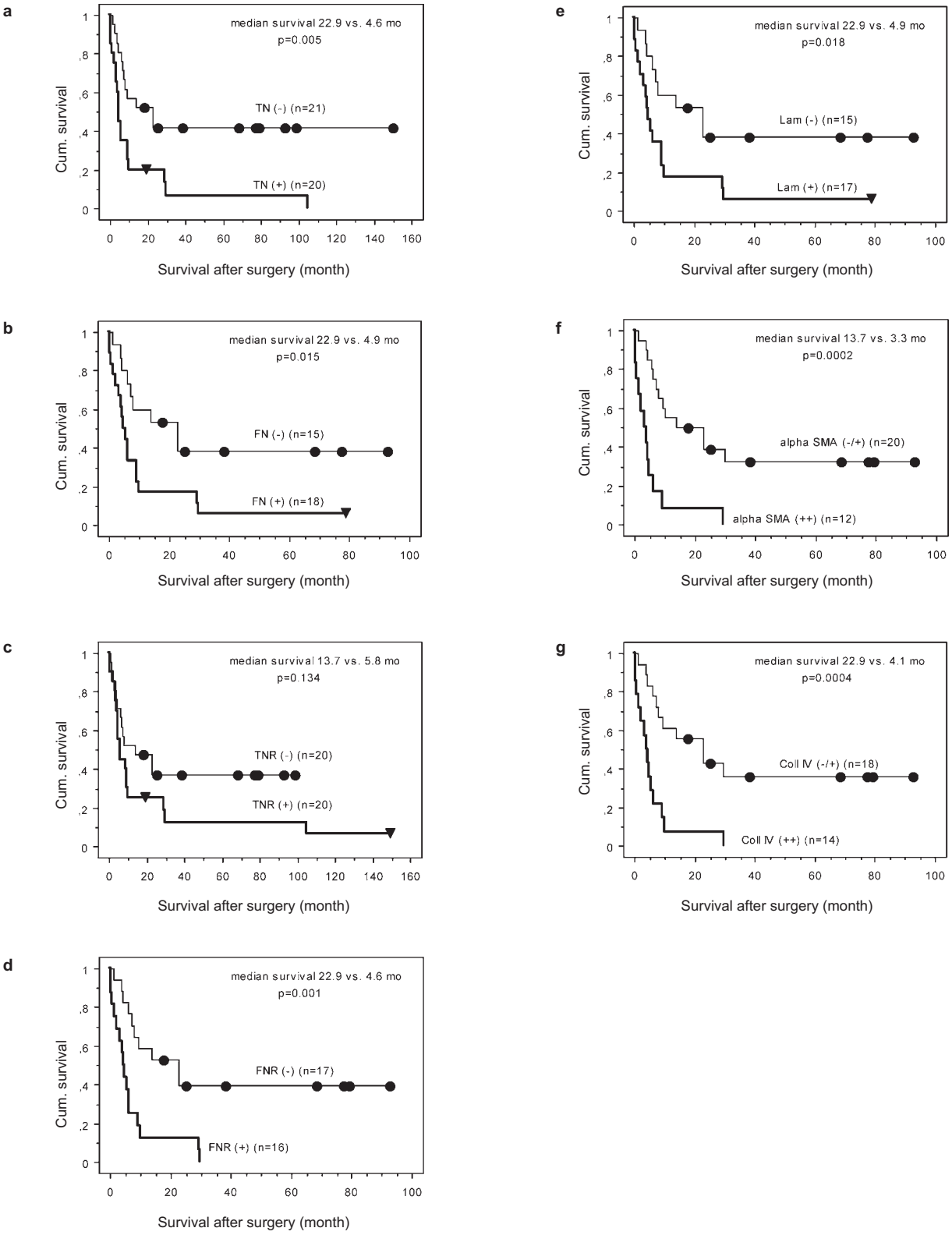

Figure 6. Relationships between the survival of the patients after the operation with the expression of tenascin (TN) (a), fibronectin (FN) (b), tenascin receptor (TNR) (c), fibronectin receptor (FNR) (d), laminin (Lam) (e), α-SMA (alpha SMA) (f), and collagen type IV (Coll IV) (g) in the periphery of liver metastases (Logrank test).

presence of laminin and of increased αSMA expression at the margin of liver metastases could also indicate sinusoidal changes i.e. HSCs activation and initial sinusoidal capillarization [33]. On the contrary collagen type IV and fibronectin/α5β1 integrin were constantly although not so continuously deposited in the normal liver sinusoids [32, 33]. So that, their enhancement could not be a clear marker of ECM deterioration.

We found a strong tenascin immunoreactivity in the sinusoids, surrounding liver metastases with a "non-capsule" pattern. Strongly increased collagen type IV- and αSMA-positive immune deposits were constantly found around these metastases. Weaker but constant immunoreactivity was observed for fibronectin/α5β1, for α9β1integrin and laminin. The remote liver tissue was weakly positive for all studied proteins. The invasive margin of liver metastases with an "intermediate capsule" pattern showed moderate positivity of the studied proteins in the wall of newly formed vessels and a weak positivity in the sinusoids around metastases. The remote liver tissue was weakly positive only for collagen type IV and αSMA. The cases with thick "capsule" showed negative immune reactions in the "capsule" and in the surrounding sinusoids for all studied ECM proteins and for the two integrins. The remote liver tissue showed the ECM profile of the normal liver. Therefore, it could be concluded that the lack of tenascin/α9β1 and of fibronectin/α5β1, as well as of all other studied proteins, could be a sign of tumor limitation and a good prognosis. This last conclusion could be additionally supported by our survival analyses describing a better prognosis for the patients with absent or weak tenascin, α9β1, fibronectin, α5β1, laminin, collagen IV and α-SMA immune deposits in the sinusoids attached to the liver metastases.

It was already shown that tenascin and fibronectin are closely associated with the progression of primary colorectal and gastric cancers [20–22, 34]. Tenascin immunoreactivity was restricted to the invasive gastric tumor cell nests [34]. On the other hand, the expression of fibronectin has been shown to be up-regulated in colon cancers [20] but is not related to their grade, stage or to the development of metastases [35]. It is known that tenascin and fibronectin are in a counterbalance i.e. the first one is counteradhesive and the second is an adhesive protein [20]. We have found an increased expression of tenascin-C and fibronectin in the sinusoids attached to metastases in all cases with "non-capsule" and with an "intermediate capsule" that had poor prognosis. Therefore, these proteins could support the proliferation and motility of cancer cells in liver metastases. Our result is compatible with the results of other authors [20, 36–38].

The integrin receptors are generally thought to be key elements in allowing cells to interact with their environment. Mainly *in vivo* investigations, on the role of integrins α9β1 [39] and α5β1 [40] in colorectal cancer progression, were carried out. We have found a moderate simultaneous expression of fibronectin and tenascin-C with their receptor integrins α9β1 and α5β1 in the invasive margin of liver metastases both with "non-capsular" and any type of "capsular" patterns. This finding indicates that tumor cells have mechanisms for attachment and spreading at their invasive front. The two studied integrins marked well the newly formed vessels in the intermediate capsule of metastases. It could be explained with the fact that the integrins α9β1 [39] and α5β1 [23, 24] are known to support tumor growth and angiogenesis. Therefore, the expression of integrins at the invasive margin of liver metastases could be a sign of active tissue remodeling.

The prevalent presence of tenascin/α9β1 integrin at the periphery of metastases suggests that they may have two roles: to facilitate epithelial tumor cell migration, and to inhibit tumor cell attachment to substrate, possibly via concurrent binding with fibronectin [41]. The weaker expression of α5β1 integrin around tumor metastases with "non-capsular" pattern could reduce the ability of metastatic cells to adhere to fibronectin and allow them to move on tenascin or laminin substrata.

The presence of α9β1 integrin on liver sinusoidal wall attached to metastases, in the "non-capsular" pattern, implied that it can mediate signals for mitogenesis of sinusoidal cells by binding to tenascin-C. The weaker expression of α5β1 integrin there, could be a sign of HSCs activation. It is known that α5β1 integrin is associated with the cytoskeletal changes of HSCs and may diminish their activation [24]. The occurrence of α5β1 integrin in the "intermediate capsule" or in "non-capsule" could also allow a maltitude of cell types (e.g. lymphocytes, platelets and fibroblasts) to interact with the ECM milieu there and to modulate the behavior of cancer cells [24].

The cellular source of the fibrotic capsule around colorectal [9, 33] and gastric [33] cancer liver metastases was shown to be of HSCs origin. In this study we confirm the previous data [9] that fibroblastic stromal cells in the "intermediate capsule" and less in the "capsule" were strongly αSMA-positive. This is the characteristic pattern seen in the capsule around human hepatocellular carcinoma [12] or in the stroma of colorectal and gastric liver metastases, where these cells were shown to be of HSCs origin [33]. These results suggested that the "intermediate capsule" and the "capsule" were composed mainly of transformed HSCs or of vascular smooth muscle cells, both of which were αSMA-positive. In addition, we demonstrated that the elongated cells, lining sinusoidal wall in the liver parenchyma adjacent to metastases are αSMA-positive and tenascin-C-positive and probably are of HSCs origin.

To date, only few investigators have reported on the prognostic significance of capsular formation. The 3-year or 5-year survival rates of patients with severe fibrosis around liver metastases were reported to be significantly higher than that of patients with none or mild fibrosis [10, 42]. Analogously, in our study we found a significantly longer survival of the patients, who had encapsulated liver metastases com-

pared to those without any fibrotic capsule. These results strongly suggest that the capsular formation is a good prognostic and biological indicator that may functionally act as a barrier to local invasion.

Further, the statistical analyses of the survival of the patients according to the expression rates of the assessed proteins in the sinusoids attached to the liver metastases, showed that the positivity for tenascin, fibronectin, fibronectin receptor and laminin, as well as the strong immunohistochemical signal for αSMA and collagen type IV could be considered as markers for worse prognosis. Similarly, the presence of immune deposits of tenascin receptor in vicinity of liver metastases tended to be associated with short survival after the surgery. The review of the literature has shown that the significance of these proteins as prognostic markers in different primary and metastatic cancers is quite contradictory [43–54]. Our results are in consistence with those of the majority of reports describing the importance of the overexpression of tenascin [48, 54], fibronectin [44, 48], different laminin chains [50, 51] and some of the integrins [43, 52] as markers for poor prognosis. On opposite, there are reports describing that high levels of the same ECM proteins [49, 53] are associated with longer disease-free or overall survival. Moreover, in several papers no correlation was depicted between the level of expression of fibronectin, tenascin, laminin or collagen IV and prognosis of the patients with breast cancer, laryngeal carcinoma and cervical carcinoma [45–47]. However, in none of those papers synchronous metastases were examined and expression of the studied proteins analyzed as prognosticators, as it is in our current work.

In conclusion, the fibrotic capsule formed around the liver metastases of gastric and colorectal cancers is suggested to be a barrier to the local invasion and is considered to be a marker for a favorable prognosis of the patients. The cellular source of the fibrotic capsule is proposed to be mainly the transformed HSCs or the vascular smooth muscle cells. The de novo occurrence of tenascin and α9β1 integrin, as well as the constant weak fibronectin, α5β1 and laminin expression, and the strongly increased collagen type IV- and αSMA-positive immune deposits in the sinusoids, surrounding liver metastases with "non-capsule" pattern are though to be signs of sinusoidal deterioration, tumor invasiveness and bad prognosis.

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