Integrin $\alpha_v\beta_3$ Expression in Colon Carcinoma Correlates with Survival

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Integrin $\alpha_v\beta_3$ is expressed by newly formed blood vessels in diseased and neoplastic tissue and can therefore be used as a marker for angiogenesis. We investigated its expression on the vasculature of 40 colon carcinomas using the anti-$\alpha_v\beta_3$-specific monoclonal antibody LM609. The average relapse-free interval and overall survival in patients suffering from colon carcinomas with high vascular expression of $\alpha_v\beta_3$ integrin was significantly reduced compared with that in patients with low $\alpha_v\beta_3$ integrin expressing tumor vasculature. Moreover, the expression level of $\alpha_v\beta_3$ integrin correlated with the presence of liver metastases. In conclusion, we propose vascular expression of $\alpha_v\beta_3$ integrin as a prognostic indicator for colon carcinoma.

KEY WORDS: $\alpha_v\beta_3$ integrin, Carcinoma, Endothelium, Survival.

Angiogenesis, the sprouting of new vessels from preexisting capillaries and postcapillary venules, is a crucial step in normal physiological processes such as embryonic development, the menstrual cycle, wound healing, and inflammation. It also contributes to pathological conditions like diabetic retinopathy, macular degeneration, psoriasis, rheumatoid arthritis, and childhood hemangiomas. Moreover, solid tumors are highly dependent on the formation of new blood vessels to grow beyond the critical size of a few millimeters (1).

The angiogenic process consists of three distinct steps. First, it is initiated by the release of cytokines, such as VEGF, bFGF, IL-8, and TNFα. In a second step, one or more of these substances stimulate the proliferation and migration of endothelial cells and smooth muscle cells. This step is accompanied by the secretion of proteolytic enzymes by endothelial cells, facilitating the vascular invasion process by degradation of the extracellular matrix. Eventually, the invasive vascular sprout reaches the hypoxic source (*e.g.*, a tumor or an ischemic region in wounded or diseased tissue), where it differentiates. In the case of tumoral angiogenesis, the newly formed vessels not only serve for nutrition and waste removal but also as a conduit for tumor cell dissemination (2–3).

In recent years, interest has been focused on $\alpha_v\beta_3$, a member of the integrin family that was identified as a marker of angiogenic vascular tissue. The $\alpha_v\beta_3$ is also expressed by malignant melanoma cells (4), by certain activated leukocytes, by macrophages, osteoclasts, and platelets, and at low levels by some vascular, intestinal, and uterine smooth muscle cells (5–6).

During neovascularization, integrin $\alpha_v\beta_3$ is actively involved in the second step of angiogenesis, which includes the maturation of blood vessels during embryogenesis, and injection of an anti-$\alpha_v\beta_3$ antibody (LM609) into embryos generated a disruption of the normal pattern of vascular development by perturbing vessel lumen formation (7). The same anti-integrin antibody was also reported to inhibit human breast cancer growth and angiogenesis in human skin (8).

The aim of this study was to investigate a possible correlation between vascular $\alpha_v\beta_3$ integrin expression in colon carcinomas and clinical parameters such as survival and relapse-free interval.

MATERIAL AND METHODS

Cases

Frozen tissue samples of both normal mucosa and tumor were obtained from 40 patients having undergone surgery for colon cancer between 1990
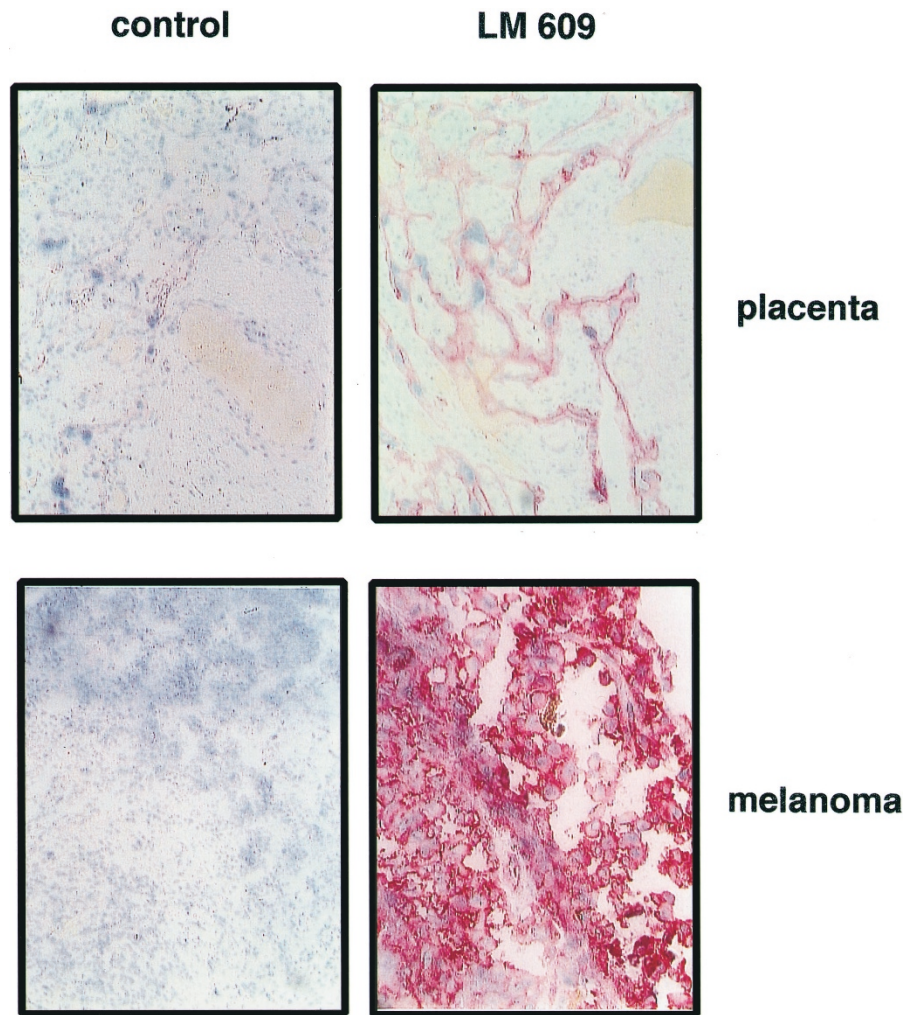
FIGURE 1. Detection of $\alpha_{v\beta3}$ integrin on frozen sections, placenta and melanoma metastases. Expression of $\alpha_{v\beta3}$ integrin in third-trimester placenta predominates on the surface of the syncytiotrophoblast, whereas it is only weakly expressed on endothelial cells of fetal blood vessels. In melanoma metastases, melanoma cells and endothelia of the tumor vasculature exhibited intense $\alpha_{v\beta3}$ integrin reactivity. Control sections were not incubated with the primary antibody but were included in all following staining steps.

and 1995 at the Tiefenauspital and the Sonnenhofklinik in Bern. Tumors were classified at the Institute of Pathology, University of Bern, according to the TNM classification of the International Union Against Cancer. Tumor sizes were pT_1 (10 cases), pT_2 (6 cases), pT_3 (32 cases), and pT_4 (1 case). 18 patients had lymph node involvement; the remaining 22 patients were node negative. In 4 cases, distant organ metastases were reported. There were 5 Dukes A adenocarcinomas, 16 Dukes B, 17 Dukes C, and 2 Dukes D. Carcinomas of 33 patients were located at the left hemicolon, 6 at the right hemicolon, and 1 at the colon transversum. The median age of the patients was 65.5 years. There were 19 female and 21 male patients. The median follow-up time of the patients was almost 48 months (range, 4–82 months).

Tissue Processing

From each specimen, several samples of tumor and of noninvolved adjacent mucosa were embedded in OCT compound medium (Tissue Tek, Bayer, Switzerland), snap-frozen, and kept at $-80°$ C. For each case, one sample of neoplastic and normal tissue was selected, and serial cryostat sections, 4–5 μm thick, were cut, air-dried for 1 hour, and kept in airtight boxes at $-20°$ C until use.

Immunohistochemistry

Tissue sections were immunostained using a biotin/streptavidin technique with alkaline phosphatase. All incubations were done at room temperature. Staining with anti-$\alpha_{v\beta3}$ integrin mAb LM609 was done as follows: the sections were dried at room temperature for 30 minutes, fixed in ethanol for 1 minute, rinsed in distilled water, and immersed in PBS for 5 minutes. After blocking for 15 minutes with 10% normal rabbit serum, the sections were incubated for 1 hour with the anti-$\alpha v\beta_3$ monoclonal antibody LM609, washed with PBS, and incubated for 30 minutes with biotinylated
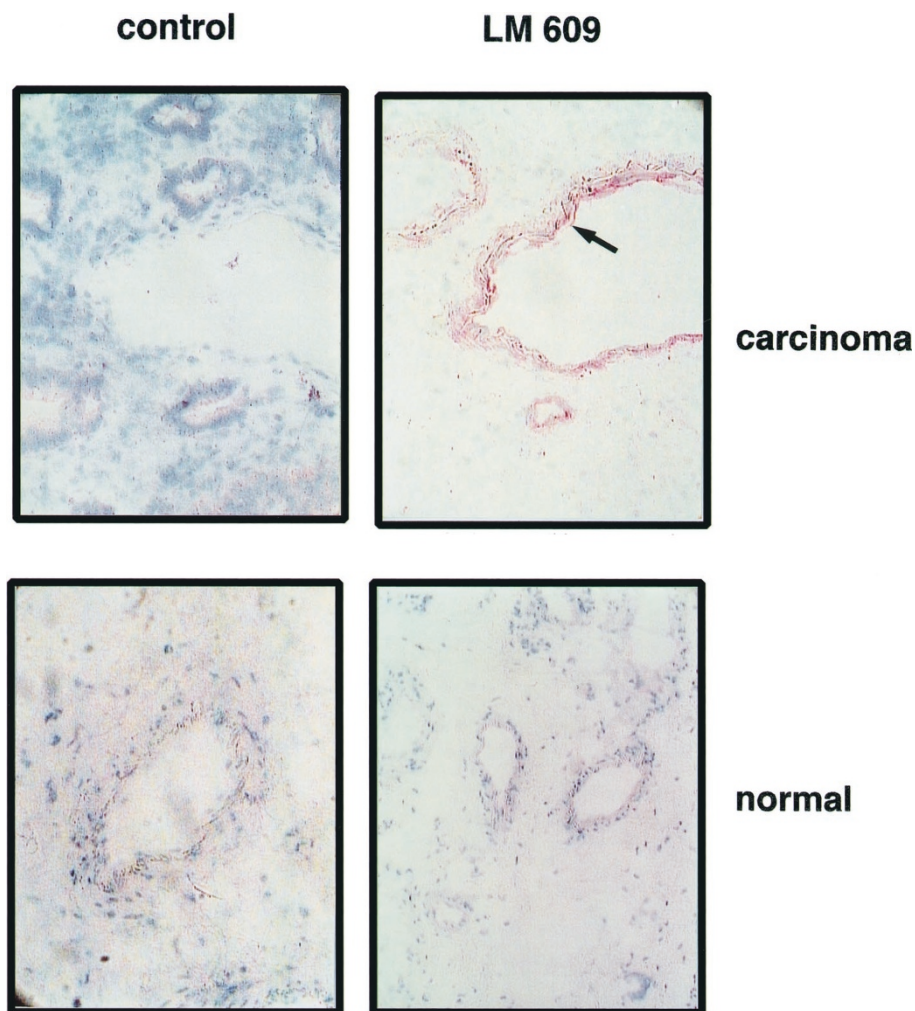
FIGURE 2. Detection of $\alpha_{v\beta3}$ integrin expression on frozen sections of colon carcinoma and normal colon tissue. $\alpha_{v\beta3}$ integrin was detected on endothelial cells of the tumor vasculature (**arrow**). In larger vessels, we observed, in addition, labeling within the vessel wall indicating expression of $\alpha_{v\beta3}$ integrin by smooth muscle cells. Control sections were not incubated with the primary antibody but were included in all following staining steps (bar = 50 μm).

F(ab)$_2$ fragments of rabbit anti-mouse immuno-globulins. After rinsing with PBS, the sections were incubated for 30 minutes with a streptavidin-alkaline phosphatase complex, washed with PBS, and reactive sites revealed by incubating the sections for 30 minutes with a compound of naphthol phosphate, fast red, and levamisole in Tris-HCl buffer 0.1 M. Counterstaining was performed by hematoxylin staining for 3 minutes. Control sections were not incubated with the mAb LM609 but included in all subsequent staining steps.

Antibodies and Other Reagents

Monoclonal mouse anti-human integrin $\alpha_{v\beta3}$ (LM609) were from Chemicon (Temecula, CA), normal rabbit serum and biotinylated F(ab')$_2$ fragment rabbit anti-mouse immunoglobulins were from DAKO A/S (Glostrup, Denmark). Streptavidin-conjugated alkaline phosphatase were from Bio-genex (San Ramon, CA) and Fast Red, from DAKO Corporation (Carpinteria, CA).

Evaluation

LM609 staining intensity was as follows: tumor sections and 6 sections of normal mucosa were assessed by three independent observers blinded to patient data. To quantify the immunohistochemical results, four categories ranging from 0 (no expression of $\alpha_{v\beta3}$ integrin) to III (high expression) were used. The staining intensity of vessels was evaluated in five distinct fields (200×) for each section, and a mean value was calculated.

Statistical Analysis

The statistical analyses have been performed using the computer package Stata (9). Survival curves have been estimated by the Kaplan-Meier method (10) and compared with the log rank test (11). The
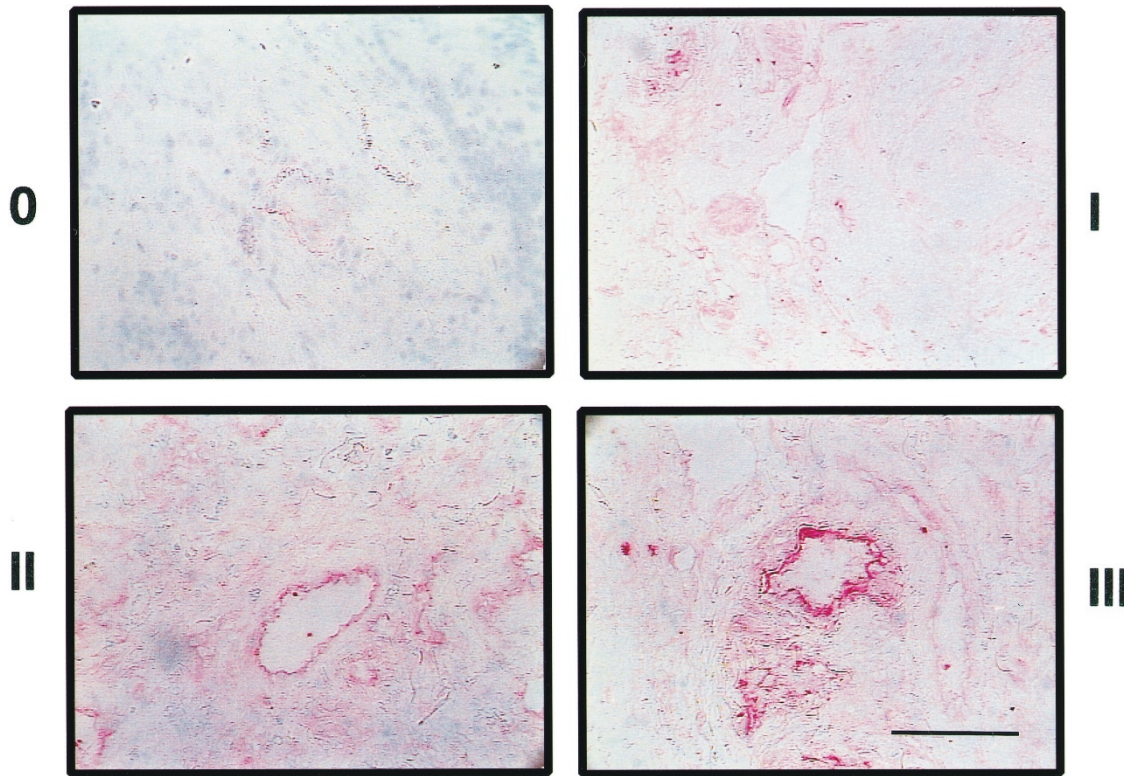
FIGURE 3. Detection and grading of $\alpha_{v\beta3}$ integrin expression on the vasculature of colon carcinoma. For statistical analysis, the tumors were classified in four groups with no expression (0), very low expression (I), intermediate expression (II), and high expression (III; bar = 50 μm).
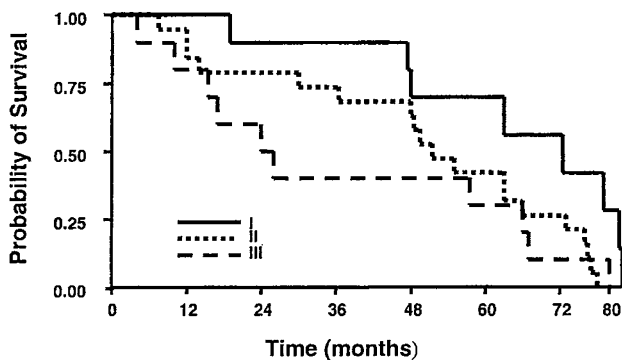

FIGURE 4. Kaplan-Meier analysis of survival of colon carcinoma patients correlated with vascular $\alpha_{v\beta3}$ integrin expression. We grouped them into categories defined in Figure 3 according to the vascular expression of $\alpha_{v\beta3}$ integrin expression. From the 40 patients, 10 are in Category I, 20 are in Category II, and 10 are in Category III.

Cox regression (12) has been used to model the effect of various factors on survival. Each continuous variable has been recoded in three categories, using as cut-offs the values corresponding to the 25th and 75th centiles of the corresponding distribution. This was done to single out observations in the tails of the distributions. For $\alpha v\beta3$ integrin, such cut-off values were 0.9 and 2 (values corresponding to the expression level as defined in Fig. 3); for VCAM-1, these values were 0.16 and 1.25; for age, they were 58 and 73 years; and for infiltration they were 36 and 78. For tumor grading, five patients

were Grade 1 and only two were Grade 3; therefore, Categories 2 and 3 were fused. All variables were then coded into binary indicators. An exploratory model including all available variables was fitted to the data. All P values reported are for two-sided tests.

RESULTS

The expression of integrin $\alpha_{v\beta3}$ was analyzed in 40 human colon carcinoma and 6 normal tissues by immunohistochemistry on frozen sections. The integrin was detected by the monoclonal anti-$\alpha_{v\beta3}$ antibody LM609. Placenta expressing high levels of $\alpha_{v\beta3}$ was used as a positive control (Fig. 1).

Angiogenesis is used as a measure of aggressive tumor growth and is accompanied by elevated $\alpha_{v\beta3}$ expression by angiogenic endothelial cells. In sections of colon carcinoma, $\alpha_{v\beta3}$ expression was restricted to the tumor vasculature (Fig. 2). Tumor cells as well as blood vessels in normal colon tissue were negative. For statistical analysis, we therefore considered only staining intensities of clearly identifiable vessels.

We next examined whether vascular $\alpha_{v\beta3}$ integrin expression on sections of colon carcinomas correlated with the aggressiveness of the tumor. Therefore, we graded the expression level of vascular $\alpha_{v\beta3}$ from 0 (no expression), I (very low expression), II

TABLE 1. Hazard Ratios of Disease Recurrence and Death for Each Variable

Categories	Hazard Ratio	Standard Error	95% Confidence Interval		P Value
Disease-free survival					
$\alpha v\beta 3$ Integrin					
<0.9	1		Reference category		
0.9–2	1.90	1.02	0.66	5.44	0.234
>2	6.12	4.05	1.68	22.39	0.006
VCAM-1					
<0.16	1		Reference category		
0.16–1.25	1.30	0.57	0.55	3.06	0.546
>1.25	1.39	0.84	0.42	4.54	0.590
Age (y)					
<58	1		Reference category		
58–73	0.79	0.39	0.30	2.06	0.631
>73	1.14	0.73	0.32	4.00	0.843
Leukocyte infiltrate					
<36	1		Reference category		
36–78	1.68	0.86	0.62	4.56	0.310
>78	2.09	1.38	0.57	7.63	0.267
T stage					
1–2	1		Reference category		
3–4	1.20	0.64	0.42	3.41	0.728
N stage					
1	1		Reference category		
2	1.15	0.58	0.43	3.08	0.775
3	8.60	5.16	2.65	27.90	<0.001
Grade					
1	1		Reference category		
2–3	0.52	0.31	0.17	1.65	0.269
Overall survival					
$\alpha v\beta 3$ Integrin					
<0.9	1		Reference category		
0.9–2	4.98	3.45	1.28	19.36	0.020
>2	10.89	8.27	2.46	48.24	0.002
VCAM-1					
<0.16	1		Reference category		
0.16–1.25	0.99	0.48	0.38	2.57	0.991
>1.25	1.81	1.25	0.47	7.00	0.388
Age					
<58	1		Reference category		
58–73	0.94	0.56	0.29	3.02	0.912
>73	1.73	1.30	0.39	7.57	0.467
Leukocyte infiltrate					
<36	1		Reference category		
36–78	2.15	1.15	0.75	6.14	0.153
>78	1.30	0.89	0.34	4.95	0.701
T stage					
1–2	1		Reference category		
3–4	1.23	0.74	0.38	3.98	0.725
N stage					
1	1		Reference category		
2	2.71	1.50	0.92	8.00	0.071
3	20.80	15.37	4.88	88.54	<0.001
Grade					
1	1		Reference category		
2–3	0.63	0.45	0.15	2.54	0.514

The number of patients were grouped into three categories using the 25th or 75th percentiles as cut off. For $\alpha v\beta 3$ integrin expression as an example, these values corresponded to <.9 (below the 25th percentile), 0.9–2 (between the 25th and the 75th percentiles), and >2 (above the 75th percentile). The values were determined according to Figure 3.

(intermediate expression), or III (high expression; Fig. 3). The photographs shown in Figure 3 were then used as a standard for the evaluation by 3 independent observers. All the six control colon tissues fall into $\alpha_{v\beta 3}$ Category 0; no tumor tissue was found with this grading. The expression level of $\alpha_{v\beta 3}$ correlated with overall survival (Fig. 4). For statistical analysis of overall and disease-free survival, Cox regression (12) has been used to model the effect of various factors. Those included as a measure of inflammation the expression of VCAM-1 by the tumor vasculature and also leukocyte infiltration. We also determined the degree of tumor invasiveness (T stage and nodal status) as well as the grade of the tumor. Each of these variables has been recoded in three categories using as cut-offs the values corresponding to the 25th and 75th percentiles of the
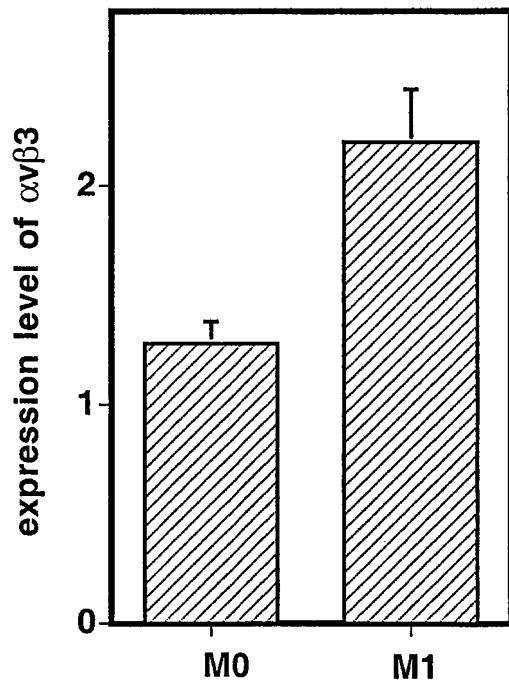
DISCUSSION

In this study, we revealed expression of $\alpha_{v\beta3}$ integrin on the vasculature of colon adenocarcinomas with the established monoclonal antibody LM609. The expression level of $\alpha_{v\beta3}$ was graded and correlated with survival data of the patients and metastatic potential of the tumors. Our data indicate that expression level of $\alpha_{v\beta3}$ integrin may thus be used as a prognostic factor in colon carcinoma.

Expression of integrin $\alpha_{v\beta3}$ was identified on tumoral vessels and was absent in vessels of normal tissue. Integrin $\alpha_{v\beta3}$ was not only found on capillaries but also in the walls of larger vessels and in tumor stroma. These findings are consistent with $\alpha_{v\beta3}$ expression on vascular and intestinal smooth muscle cells (13).

The statistical analysis showed that mean relapse-free interval proved significantly lower in patients suffering from colon carcinomas with high vascular expression of $\alpha_{v\beta3}$. These findings are in accordance with recent observations that indicate a shortened relapse-free interval in breast cancer with high vascular expression of $\alpha_{v\beta3}$ integrin (14). A more dramatic tendency was observed for the overall survival time of the patients. We found that $\alpha v\beta3$ integrin expression represents the best prognostic factor when compared with the tumor grading, node status, depth of invasion, and inflammatory response.

Colorectal cancer often gives rise to metastases in the liver. We therefore sought to correlate vascular $\alpha_{v\beta3}$ expression in colorectal cancer with liver metastases. Tumors that had spread to the liver showed an $\alpha_{v\beta3}$ integrin expression that was nearly double the level of nonmetastatic colon cancers. This finding underlines the fact that tumors with a strong angiogenic activity form metastases more easily, although it remains elusive whether this is caused by an increased dissemination of neoplastic cells through newly formed blood vessels or improved growth conditions at distant sites.

In conclusion, we show that the degree of $\alpha_{v\beta3}$ integrin expression on the vasculature of colon carcinomas is of high prognostic value.

FIGURE 5. Vascular expression of $\alpha_{v\beta3}$ integrin in colon carcinoma patients with or without liver metastasis. Expression of $\alpha_{v\beta3}$ integrin was almost twice as high in colorectal cancer with liver metastasis, compared with the case of nonmetastatic tumors.

corresponding distribution (Table 1). For $\alpha_{v\beta3}$ these values corresponded to less than 0.9 (≤25%), 0.9–2 (26–75%) and more than 2 (>75%). The median survival time for these categories were 72.5, 51.5, and 24 months, respectively ($P = .0528$) and the median disease-free survival was 60, 48, and 24 months ($P = .014$). When fitting a multivariate model, the only parameters that achieved significance for overall survival are the variables identifying the patients with $\alpha_{v\beta3}$ levels of 0.9–2, >2, and of N stage 3. All other parameters did not reach significance and were therefore of no or little prognostic value in this group of patients. Although the confidence intervals are rather wide because of the small sample size, the hazard ratios suggest a trend of increased rate of failure with increasing $\alpha_{v\beta3}$ levels. Similar results held for disease-free survival; however, the associations with $\alpha_{v\beta3}$ integrin expression appeared slightly weaker. At best, the risk of death was 10 times higher for patients expressing $\alpha_{v\beta3}$ integrin above the 75th percentile (expression level more than 2) when compared with patients falling into the 25 lower percentiles (expression level less than 0.9). The expression level of $\alpha_{v\beta3}$ integrin was also correlated with the presence of liver metastases. Tumors that had developed metastases in the liver generally showed a higher grading for $\alpha_{v\beta3}$ integrin expression than did tumors without metastases (Fig. 5).

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