Integrin alphavbeta6 mediates the potential for colon cancer cells to colonize in and metastasize to the liver

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Integrin alphavbeta6 (αvβ6) is correlated with colon cancer progression. To detect the effects of αvβ6 on liver metastasis, the specificity of αvβ6 against the monoclonal antibody (mAb) 2G2 was examined by immunoprecipitation. Integrin αvβ6-immunoreactivity (IR) in liver metastasis tissues (63 cases) and colon carcinoma (358 cases) were examined. These results showed that αvβ6 was specifically recognized by the mAb 2G2, and that rates of αvβ6 positivity in liver metastatic tissues (71.4%, 45/63) were higher than that for primary colon cancer (34.0%, 122/358) ($P < 0.01$). Patients who were αvβ6-positive had higher liver metastasis rates (17%, 21/122) than those who were αvβ6-negative (only 3%, 7/236) ($P < 0.01$). To examine the underlying mechanisms associated with αvβ6 regulating colonic metastasis in the liver, experimental liver metastasis (intrasplenic injection of HT29 transfectants) and liver colonization assays (direct injection of WiDr transfectants into the liver) in nude mice were performed; these demonstrated that αvβ6 contributed to the promotion of the metastatic potential and the survival of cancer cells in the liver. Matrix metalloproteinase-9 (MMP-9) levels in the cultures of both HT29 and WiDr cells were detected by the Biotrak MMP-9 activity assay system and gelatin zymography assay, and showed that suppression of αvβ6-IR inhibited MMP-9 activity and secretion. Transwell migration assay *in vitro* also showed that αvβ6 promoted migration on fibronectin for HT29/WiDr mock compared with HT29/WiDr antisense β6 transfects ($P < 0.01$). We concluded that αvβ6 may mediate the potential for colon cancer cells to colonize in and metastasize to the liver. The mechanisms that αvβ6 may be involved in include the promotion of MMP-9 secretion, the enhancement of migration on fibronectin, and the survival of cancer cells in the liver. (*Cancer Sci* 2008; 99: 879–887)

Despite welcome declines in the mortality rate over the past decade, the prevention of colonic metastasis to the liver in patients with colon carcinoma remains one of the most challenging issues in cancer research. Surgical resection can provide a potentially curative outcome, but nearly a quarter of patients with colon carcinoma will develop a recurrence in the first 5 years, with about 40–50% accounting for liver metastasis.[1] The conventional paradigm of liver metastasis in colon cancer is based on a multistep tumor genesis model defined by a series of progressive somatic genetic alterations, which give malignant cells the ability to proceed through the many phases of metastasis. When a colon carcinoma *in situ* develops into a metastatic tumor in the liver, the degradation of extracellular matrix (ECM) barriers by matrix metalloproteinase-9 (MMP-9), cell migratory capability, and survival in a new environment, play important roles in facilitating tumor cellular dissemination and metastasis.[2] They are the common prerequisite conditions involved in liver metastasis in colon cancer. It is well known that the expression of MMPs is involved in liver metastasis in human colon cancer.[3,4] MMP-9 is also named gelatinase B or Mr 92 000 type IV collagenase, partly because of its ability to degrade type IV collagen, the major structural component of basement membranes, and because of the important role it is believed to play in cellular invasion.[5] A change in the binding properties of metastatic cells promotes their release from the stem tissue, cell migration, and/or transport, and the homing of the target tissue. Integrins are transmembrane glycoprotein receptors comprising an alpha (α) and a beta (β) subunit in non-covalent association that mediate dynamic linkages between the actin cytoskeleton and the ECM as well as transducing signals to and from the cell interior.[6,7] Within the αv subfamily, the αvβ6 subunit form pivotal molecules involved in this progression.[8] Integrin αvβ6 is a fibronectin receptor expressed predominantly by epithelial cells,[9] which is not expressed on normal epithelial cells; however, it becomes highly expressed during tumorigenesis[10,11] and de novo expression of αvβ6 has been observed in colon cancer.[12] Integrin-mediated cell attachment, spreading, cell migration, and tumor invasion were well known.[13,14] Bates *et al*.[15] reported that elevated αvβ6 expression facilitates the invasion and dissemination of colon carcinoma cells, and suggested that integrin αvβ6 created a tumor microenvironment more amenable to progression. We have demonstrated that the expression of αvβ6 results in MMP-9 secretion, demonstrating a delicate balance between αvβ6 expression and MMP-9 levels.[16] Kawashima *et al*.[17] also reported that increased αvβ6 in gastric carcinoma is associated with lymph node metastasis. However, the links between αvβ6 expressed in primary colon cancer and both colonic liver colonization and colonic metastasis to the liver remain unknown, and the mechanisms by which integrin αvβ6 may mediate liver colonization and liver metastasis are also unclear.

In this study, integrin-αvβ6 expression in hepatic metastatic tissues and malignant colon carcinoma was examined by immunohistochemical (IHC) staining. A three-year follow-up for liver metastasis of the patients with malignant colon carcinoma was conducted. In addition, experimental liver metastatic assays (intrasplenic injection of human colon cancer cell line, HT-29) and liver colonization assays (direct injection of human colon cancer cell line, WiDr, into the liver) on nude mice were performed. MMP-9 levels in the cultures of both HT-29 and WiDr transfectants were detected by the Biotrak MMP-9 activity assay system and gelatin zymography. The migratory capabilites on fibronectin of both HT-29 and WiDr transfectants *in vitro* were detected by Transwell migration assay.

Materials and Methods

Cell lines and transfections. The human colon cancer cell lines (SW480, HT29, and WiDr) and ras-transformed NIH 3T3 cells were obtained from ATCC (Rockville, MD, USA) and maintained as a monolayer in standard medium comprising Dulbecco's

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Modified Eagle's Medium (DMEM) (4.5 g/L of glucose). SW480 β6 transfectants were obtained from SW480 wild cells stably transfected with the expression vector pcDNA1Neo β6 which contains the full-length β6 gene under the control of the human cytomegalovirus immediate early enhancer.[18] A selected metastatic HT29 variant was kindly supplied by Rong Chen (School of Medicine, Shandong University, China), and it was isolated from the HT29 cell line by selection techniques as Chen *et al*. previously described.[19] HT29 variant transfected with the expression plasmid only was designated as HT29 mock transfectant. Antisense β6 transfectants of HT29 and WiDr were obtained from HT29 variant and WiDr cell lines transfected with the β6 gene construct in antisense orientation, and stable transfectants were selected continuously in puromycin (HT29, 2.5 μg/mL; WiDr, 1 μg/mL) as previously described.[20] WiDr cells transfected with the expression plasmid only were established as WiDr mock transfectants.

Antibodies and reagents. The anti-αvβ6 monoclonal antibody (mAb) 2G2 was obtained from Biogen Idec (Cambridge, MA, USA). The specificity of the antibodies has recently been reported by Bates[15] and Weinreb.[21] The anti-β6 mAb E7P6, function-blocking mAbs 10D5, and phycoerythrin conjugated goat-antimouse IgG were obtained from Chemicon (Temecula, CA, USA). Reagents for SDS-PAGE, SDS-gelatin gels, and purified MMP-9 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Tumor-conditioned medium (TCM) for MMP-9 estimation was prepared as previously described by Gu *et al*.[22] The Biotrak MMP-9 activity assay system was obtained from Amersham (Aylesbury, UK).

Immunoprecipitation. To demonstrate the specificity of the IHC results for the against-β6 mAb 2G2 immunoprecipitation assay was performed as Niu and Bates have previously described.[15,23] Briefly, equal cell numbers from WiDr wild, SW480 wild, and SW480 β6 transfectants were harvested using trypsin/EDTA and labeled at the cell surface by biotinylation (biotin ester from Sigma Aldrich, Milwaukee, WI, US), before lysis in the immunoprecipitation buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM CaCl2, 1% Triton X-100, 0.1% SDS, 0.1% NP-40, 1 mM vanadate, 1 μg/mL pepstatin, 1 mM PMSF, 5 μg/mL aprotinin, and 1 μg/mL of leupeptin) for 1 h. Lysates stood at 4°C for 30 min and were clarified at 10 000*g* for 10 min at 4°C to remove detergent-insoluble material. For immunoprecipitations, the lysates were precleared twice with antimouse IgG-agarose (Sigma-Aldrich). Immunoprecipitations were carried out directly using the against-β6 mAb 2G2 or isotype-matched control IgG1, and captured with antimouse IgG-agarose. Precipitates were analyzed by 7.5% SDS-PAGE under non-reducing conditions.

IHC and tissue microarray. Sixty-three cases of hepatic metastatic specimens obtained from January 1995 to January 2005, and 358 cases of colon carcinoma specimens obtained from January 2001 to December 2002, were selected randomly from Qilu Hospital (Shandong University, China; tissue procurement was approved by the institutional review board of Shandong University). Hepatic metastatic tissues and colon carcinoma specimens were formalin-fixed and paraffin-embedded. Tissue microarray (TMA) analysis as a screening method was performed in a large sample of colon carcinoma specimens. Since the cores of TMA include only small parts of tumors, and tissue heterogeneity is considered to ensure the cores including target antigen (i.e. tumor tissues or tumor fronts) and quality control in IHC, in the process of constructing TMA, the protocols shown in Fig. 1 were followed. First, all of the colon carcinoma specimens were diagnosed by whole tumor tissues in the department of pathology of Qilu Hospital (Shandong University, China). Second, cores were selected from the formalin-fixed, paraffin-embedded tissue blocks by screening and labeling with a marker on hematoxylin–eosin-stained (HE) standard serial tissue section slides for optimal tumor content, and were placed into a recipient master block using a Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, US). The greatest dimension of each core measured 1.0 mm, including the target antigen (tumor tissue), and was selected and spaced 0.8 mm apart. TMAs (200 points/each) constructed by Chaoying Biochip Company (Xi'an, China) as previously described by Rimm,[24] were sliced into 5-μm sections and adhered to slides by an adhesive tape-transfer system (Instrumedics, St. Louis, MO, US) and UV cross-linking. Third, the IHC staining against αvβ6 was performed on the TMAs. In the preliminary studies, 30 cases of standard tissue sections and 30 cores (the greatest dimension of each core measuring 1.0 mm) were subjected to HE and IHC assay. The schematic diagram for HE and IHC in serial sections is shown in Fig. 1a. The results indicated that there were the same diagnosed outcomes for HE staining between cores of TMA and standard tissue sections, and that the diagnosed outcomes of 96.7% (29/30) cores for IHC on TMA were consistent with those on standard tissue sections ($P < 0.05$, cores *versus* standard tissue sections). The results also suggested that the TMA technique was an effective screening method.

Integrin-αvβ6 immunoreactivity (IR) was examined according to the avidin-biotin complex protocol[25] on both the paraffin-embedded hepatic metastatic tissues and the TMA slides. Antigen retrieval for αvβ6 was performed by incubation of the slides in a pepsin solution (0.4%; Zymed Laboratories, San Diego, CA, US) at 37°C for 15 mins. The slides were incubated by anti-αvβ6 mAbs 2G2 primary antibody (1.67 μg/mL; Biogen Idec). The antibody is specific for αvβ6 and does not recognize the av or other av integrins.) The slides were stored overnight at 4°C and then biotinylated antimouse IgG (1:200; DakoCytomation, Glostrup, Denmark) was added for 30 mins at 37°C. The slides were subsequently treated using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and stained with HE. Negative control samples were treated identically but with the primary antibody omitted.

Scoring of integrin-αvβ6 IR. Integrin-αvβ6 IR was evaluated independently by three pathologists (G-YY, J-SW, and RC) who were blinded to patients' outcomes. The percentage of positive tumor cells and staining intensity were determined by three observers with 100% agreement. Differences of opinions were resolved by reaching a consensus during a meeting. Five microscopical fields (200 ×; 100 cells/field) in each core were assessed for positive DAB staining according to the modified methods described previously.[26–28] The proportion score represented the estimated fraction of positive staining cells (0, 0%; 1, <20%; 2, 20–50%; 3, 51–75%; 4, > 75%). The intensity score represented the estimated average staining intensity of positive cells (0, none, no staining; 1, weak, pale brown; 2, moderate, brown; 3, strong, dark brown). We took into account both the intensity and the proportion of positive cells to give a semiquantitative estimate of the expression levels of antigen in the tissue core. A combined score was derived by adding the intensity and proportion scores. The combined score for DAB staining in < 2 of tumor cells was designated as negative, 2–4 as low (weak) expression, and = 5 as high (strong) expression. Both low and high expressions were graded as αvβ6-IR positive.

Patients and follow-up. From January 2001 to December 2002, 358 patients who underwent curative resection by the same surgical team for pathologically confirmed colon cancer at the Department of Pathology of Qilu Hospital (Shandong University, China) were divided into two groups (αvβ6-IR positive and αvβ6-IR negative) to investigate postoperative liver metastases. In our protocol for the follow-up, all patients were followed every 3 months in the first year and at least every 6 months afterwards, with regular monitoring of metastasis by liver ultrasonography or computed tomography (CT) studies of the liver. New lesions diagnosed in the liver based on typical imaging findings from CT scans was diagnosed as liver metastasis.

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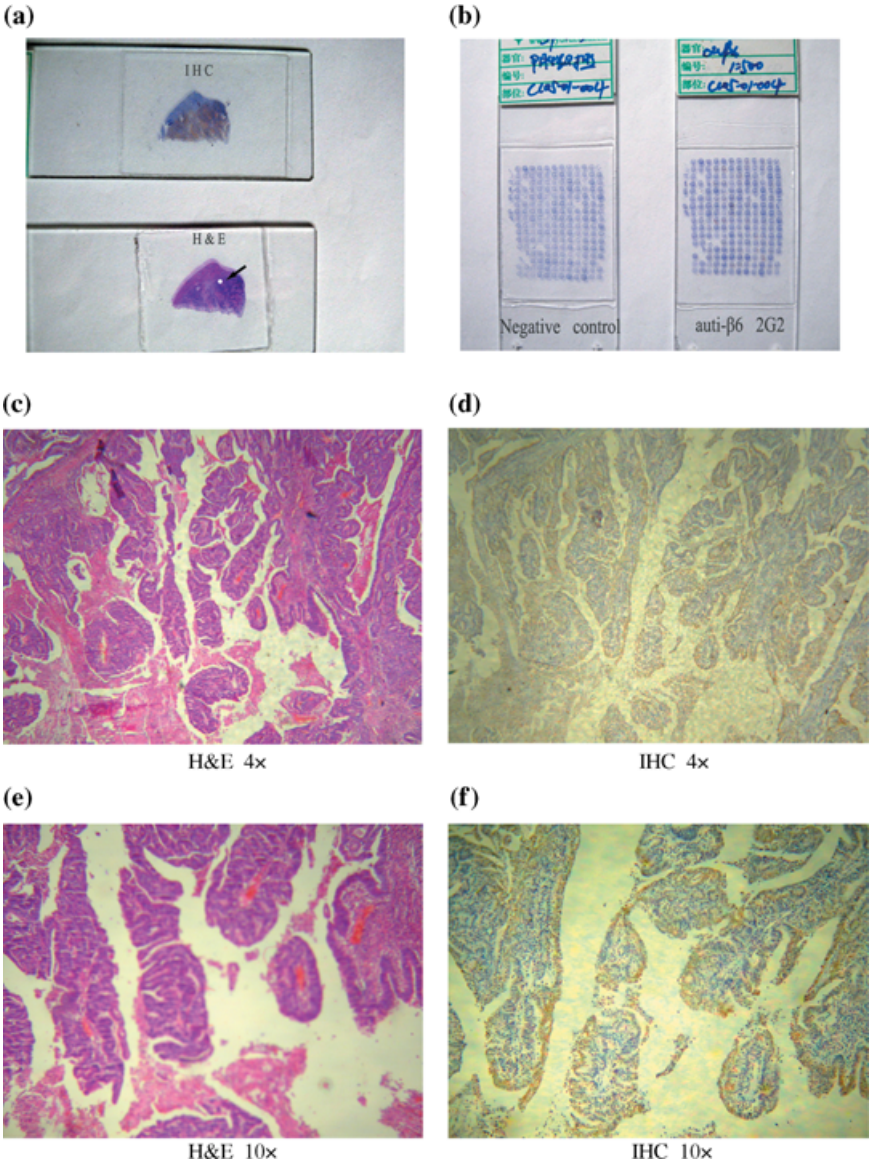

Fig. 1. The schematic diagram of the experimental process of tissue microarray (TMA) analysis, showing immunohistochemical (IHC) and hematoxylin–eosin (HE) staining for serial standard tissue section slides (the point shown with arrow in the HE section is the localization site for the optimal tumor core in paraffin-embedded tissue blocks). (b) TMAs constructed with cores for both the IHC staining against integrin αvβ6 monoclonal antibody (mAb) 2G2 and negative controls. (TMAs, 200 points each, including 358 cases of tumors, 20 cases of normal colon tissue, six cases of smooth muscle tissue, two cases of cancer adjacent colon tissue, 14 dots which maybe missing or histologically unidentifiable). (c,e) Standard tissue section slides with HE staining. (d,f) From one core (obtained from the same paraffin-embedded tissue block as the HE section) of the TMA by IHC staining against αvβ6 mAb 2G2.

Preparation and treatment for tumor cells, and animals. To investigate whether reduced αvβ6-IR in colon cancer cells altered the role of colonic metastasis to the liver, experimental liver metastases in nude mice were generated by intrasplenic injection of the human colon cancer cell line, HT29 transfectants, which expresses αvβ6 and possesses the potential of metastasis.[29–31] Forty BALB/C female nude mice at 6 weeks of age were randomly divided into two groups of 20 each and all mice within each group were inoculated with either HT29 mock cells or HT29 antisense β6 transfectants.

For survival in liver environment assays, the liver colonization model was generated by direct injection of WiDr colon cancer cell transfectants into the liver, which express avβ6 but are non-metastatic cell lines. Another 40 BALB/C female nude mice at 6 weeks of age were also divided into two groups of 20 each, and all mice within each group were inoculated with either WiDr mock-expressing αvβ6 or WiDr antisense β6 transfectants.

We harvested HT29 mock, WiDr mock, HT29 antisense β6, and WiDr antisense β6 transfectants using trypsin-EDTA (Life Technologies, Rockville, MD, US), followed by centrifugation at 300*g* for 15 min at room temperature. We then resuspended cells in serum-free DMEM (Life Technologies), and the cell number was adjusted to a final concentration of 1×10^7 cells/mL. A 1-cm incision was made in the mice under i.p. anesthesia with pentobarbital (Abbott Laboratories, North Chicago, IL, USA) at 70 mg/kg body weight. Using a 27-gauge needle and a 1-mL syringe, we injected 100 μL of tumor cell suspension (1×10^6) into the spleen (liver metastasis model) or into the liver (liver colonization model) of mice. All of the animal studies were conducted using a protocol approved by the institutional animal Care and use committee at the School of Medicine, Shandong University. Four weeks (HT29 groups) or six weeks (WiDr groups) later, the mice were sacrificed under i.p. anesthesia with pentobarbital. The presence of one or more nodules was defined as positive for metastasis or new tumor formation. Then the visible liver tumors were excised, fixed in formalin and embedded in paraffin. Sections were used for HE and IHC staining to inspect routinely for the presence of both metastases and αvβ6-IR.

Flow cytometry analysis. Monolayer cultures of HT29 and WiDr transfectants were harvested with 20 mmol/L EDTA and then blocked with goat serum at 4°C for 10 min. Cells were incubated with either the anti-β6 mAb E7P6 (10 μg/mL) or an isotype-matched control IgG (10 μg/mL in phosphate-buffered saline, PBS) for 30 mins. After washing, cells were stained with

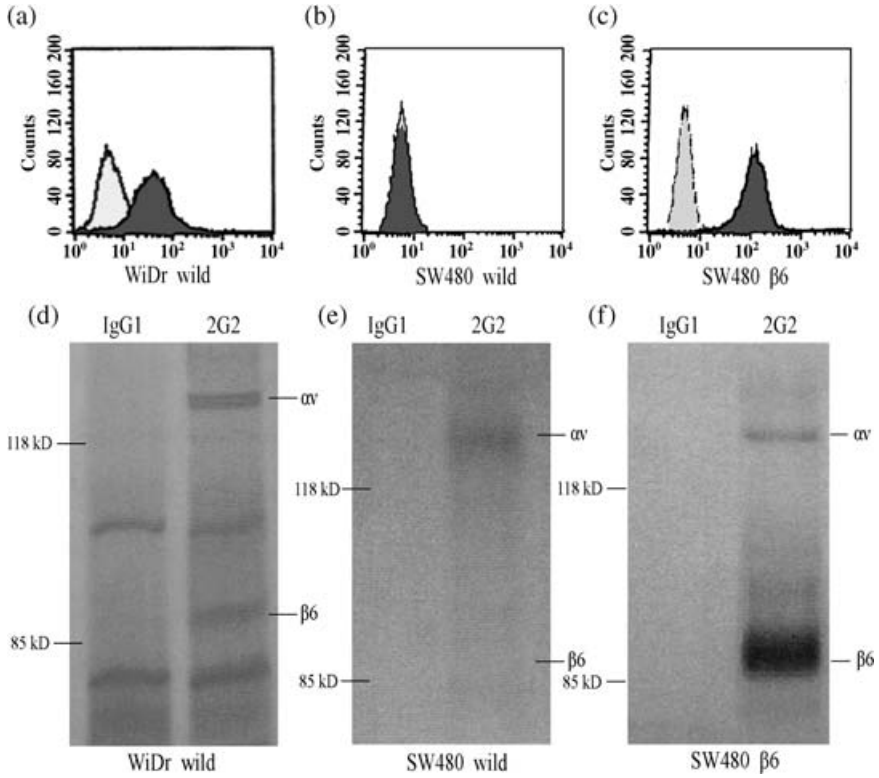

Fig. 2. Integrin avβ6 was specifically recognized by the against β6 monoclonal antibody (mAb) 2G2. (a) Expression of αvβ6 in WiDr wild, (b) SW480 wild, and (c) SW480 β6 were examined by flow cytometry. Pale and black histograms represent analyses in the absence/presence of primary-antibody recognizing β6 (mAb, E7P6). The human colon caner lines (d) WiDr wild, (e) SW480 wild, and (f) sw480 β6 transfectants were surface-labeled with biotin followed by immunoprecipitation with against β6 mAb 2G2 or isotype-matched control antibody mAb IgG1, and analyzed by SDS-PAGE under non-reducing conditions. Relative molecular masses are shown to the left in kDa. The positions of αv and its associated chains β6 are indicated to the right.

secondary antibody (goat antimouse IgG) conjugated with phycoerythrin for 30 mins in the dark, prior to flow cytometry analysis.

MMP-9 activity assay. MMP-9 levels in TCM obtained from HT29 mock, WiDr mock, HT29 antisense β6, and WiDr antisense β6 transfectants were assayed using the Biotrak MMP-9 activity assay system. This assay measures total MMP-9 levels (inactive pro-enzyme activated artificially plus endogenous active enzyme forms), and MMP-9 secretion is calculated on a per-cell basis.

Gelatin zymography assay. The levels of MMP-9 (gelatinase B) in TCM obtained from HT29 and WiDr transfectants were analyzed using 10% SDS-gelatin (1 mg/mL final concentration) gels under non-reducing conditions. TCM collected under serum-free conditions was mixed with a substrate gel sample buffer (0.5 M Tris [pH 6.8], 5% SDS, 20% glycerol, and 1% bromophenol blue) at 1:1 ratio. Following electrophoresis, gels were washed twice, then incubated at 37°C overnight in the substrate buffer. Gels were stained with 0.15% Coomassie blue R250 (Biorad, Hercules, CA, USA) in 50% methanol, 10% glacial acetic acid for 20 min at room temperature, and de-stained in the same solution without Coomassie blue. MMP-9 was identified as clear bands against the blue background of the stained gel.

Transwell migration assay. Migration assays were performed by assessment of the ability of cells on fibronectin using 6.5-mm Costar Transwell chambers (8-μm pore size; Corning, Corning, NY, US). Transwell membranes were coated on both surfaces with 20 μg/mL laminin (Roche Diagnostics, Mannheim, Germany) or 50 μg/mL human cellular fibronectin (Sigma-Aldrich) by immersion overnight at 4°C, before aspiration and three washes in PBS. Five × 10^4 HT29 mock, WiDr mock, HT29 antisense β6, and WiDr antisense β6 transfectants/0.5 mL were added to the upper chambers, and fibroblast conditioned medium prepared by ras-transformed NIH 3T3 cells was placed in the lower chamber as a chemoattractant to induce chemotaxis.[32] For antibody-inhibition studies, cells were incubated in a medium containing either an anti-β6 function-blocking mAb (10D5) or an isotype-matched control IgG antibody (100 μg/mL). Allowed to migrate for 24 h at 37°C, cells were removed from the upper face of the filters using cotton swabs. The cells that had migrated to the lower surface were fixed in methanol, stained with crystal violet, and counted. Each experiment was repeated in triplicate wells, and within each well, counting was done in four randomly selected microscopic fields (400 × magnification).

Statistical analysis. Continuous variables were expressed as mean ± SD and were compared between groups by using the Student's *t*-test or one-way ANOVA. Categorical variables were compared by using the χ^2-test. All statistical analyses were completed by using statistical software (SPSS 11.5; SPSS, Chicago, IL, USA). Statistical significance was defined as a *P*-value < 0.05.

Results

Integrin αvβ6 was specifically recognized by the against-β6 mAb 2G2. The human colon cancer cell line, WiDr wild cells, has previously been shown to express the integrin avβ6 (examined by flow cytometry shown in Fig. 2a), avβ1, avβ3, and avβ5 subunits.[12] The SW480 colon cancer cell line lacks constitutive avβ6 expression (Fig. 2b) and, moreover, does not express avβ1.[10,12] To confirm the specificity of the IHC results for the against-β6 mAb 2G2, equal numbers of WiDr wild, SW480 wild, and SW480 β6 transfectants expressing full length β6 (Fig. 2c), harvested with trypsin/EDTA, were surface-labeled with biotin (Sigma Aldrich), followed by immunoprecipitation with against-β6 mAb 2G2, or isotype-matched control mAbs (IgG1). Immunoprecipitates were analyzed by non-reducing SDS-PAGE as shown in Figure 2d–f. The results indicated that integrin avβ6 was specifically recognized by the against-β6 mAb 2G2 and that there is no β6 band on SW480 wild column.

Integrin αvβ6-IR in primary colon carcinoma and its association with the potential of colonic metastasis to the liver. To examine αvβ6-

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Table 1. The background for 63 patients with colonic metastasis to the liver

Characteristics	Number of patients	Percentages
Sex		
Male	41	65.1
Female	22	34.9
Age (years)		
Less than 30	11	17.5
30–60	29	46.0
60 or more	23	36.5
Tumor differentiation		
Well	6	9.5
Moderate	26	41.3
Poor	28	44.4
Unknown	3	4.8
Lymph node metastasis		
0	4	6.3
1–4	15	23.8
5 or more	44	69.9
Venous vessel invasion		
Absent	9	14.3
Present	54	85.7
αvβ6 expression		
Negative	18	28.6
Positive (Low expression)	14	22.2
Positive (High expression)	31	49.2

Table 2. The clinicopathologic characteristics for 358 patients with colon cancer

Characteristics	Number of patients	Percentages
Sex		
Male	203	56.7
Female	155	43.3
Age (years)		
Less than 30	38	10.6
30–60	184	51.4
60 or more	136	38.0
Tumor differentiation		
Well	64	17.9
Moderate	213	59.5
Poor	50	14.0
Unknown	31	8.6
TNM Stage		
I and II	168	46.9
III	131	36.6
IV	59	16.5
Lymph node metastasis		
0	159	44.4
1–4	150	41.9
5 or more	49	13.7
Venous vessel invasion		
Absent	274	76.5
Present	84	23.5
αvβ6 expression		
Negative	236	66.0
Positive (Low expression)	66	18.4
Positive (High expression)	56	15.6

IR in hepatic metastatic tissues from patients with colon cancer, αvβ6 IHC staining was assessed on 63 randomly selected liver specimens. The baseline clinicopathological characteristics are shown in Table 1. The integrin αvβ6 is a transmembrane glycoprotein receptor and its IHC staining located in both its membrane and cytoplasm.[27] Aggressive colon carcinoma cells were easily discernible by HE staining of a section of liver specimens (Fig. 3a). Integrin-αvβ6 staining of the same metatatic tumor tissues showed strong IR in the tumor cells and the liver was negative (Fig. 3b). Positive rates for αvβ6 were 71.4% (low 14/63, high 31/63) in liver slides. In normal colon tissues, however, there was no αvβ6 expression. The results suggested that there was a high association between αvβ6-IR and liver metastatic tumors because a higher αvβ6 positive rate (71.4%) found in hepatic metastasis specimens.

To examine the relationship between αvβ6-IR in primary colon cancer and liver metastasis rate, αvβ6-IR on tissue microarrays (200 points/each) of 358 cases of malignant colon tumors were detected by IHC. Table 2 summarizes the patients' clinical and pathological information. The effect for αvβ6-IR was that 34% (low 66/358, high 56/358) of cases overall were αvβ6-positive. At the stage of tumor progression, tumor cells migrate into and invade the surrounding tissue either as single cells or in collective clusters, which defined as tumor budding[33] if there are fewer than five tumor cells, thereby forming an invasive front.[34] Numerous tumor buddings observed in the stroma of the actively invasive region are shown in Figure 3c. The boundary between invasive tumor cells and those adjacent to the stroma is unclear; however, αvβ6-IR was positive in the margin of budding. An intense up-regulation and, particularly, preferential localization at the edges of both aggressive tumor islands and tumor budding were frequently observed (Fig. 3d). The results of the three-year follow-up showed that the three-year liver metastasis rate was 7.8% (28/358) overall, and surprisingly, 17% (21/122) for the αvβ6-positive group and only 3% (7/236) for the αvβ6-negative group. Integrin αvβ6-IR in primary colon cancer resulted in a much more significant impact on the liver metastases, highlighted by a greater-than-14% elevation, compared with that of patients with no αvβ6 expression ($P < 0.01$).

Integrin αvβ6 contributes to the survival of colon cancer cells in the liver and promotes their metastatic potential in vivo. To test the effect of integrin αvβ6 on enhanced liver colonization and metastatic potential of colon cancer cells in tumor progression, we performed *in vivo* studies using the liver colonization model (direct injection of WiDr cells into the liver, since they have non-metastatic potential), and the liver metastatic model (intrasplenic injection of HT29 cell lines, which have metastatic potential). The levels of αvβ6 expression for WiDr and HT29 transfectants are shown in Figure 4a–d. The results showed that 55% (11/20) of the mice injected with WiDr mock transfectants expressing αvβ6 developed liver metastatic tumors (Fig. 4e), and that 90% (18/20) of the mice injected intrasplenically with HT29 mock transfectants developed visible liver metastatic tumors. However, none of visible metastatic nodules in liver were observed in the mice injected with either WiDr antisense β6 (Fig. 4f) or HT29 antisense β6 transfectants. Furthermore, we observed that the tumor xenografts were besieged by neoformative fibrous tissue (more encapsulation Fig. 4g,h), but there was no invasion of the blood vessels. The liver metastatic lesions showed strong αvβ6-IR in the tumor cells, especially in the margin of invasive tumor buddings[33] (Fig. 4i). Positive αvβ6 expression was also observed on hepatic metastatic tumors (Fig. 4j) receiving an intrasplenic injection of HT29 mock transfectants. These results indicated that down-regulation of αvβ6-IR attenuates the capability of liver colonization and metastatic potential for colon cancer cells in nude mice. It also suggested that αvβ6 contributes to the survival of cancer cells in the liver, and promotes colonic metastasis to the liver.

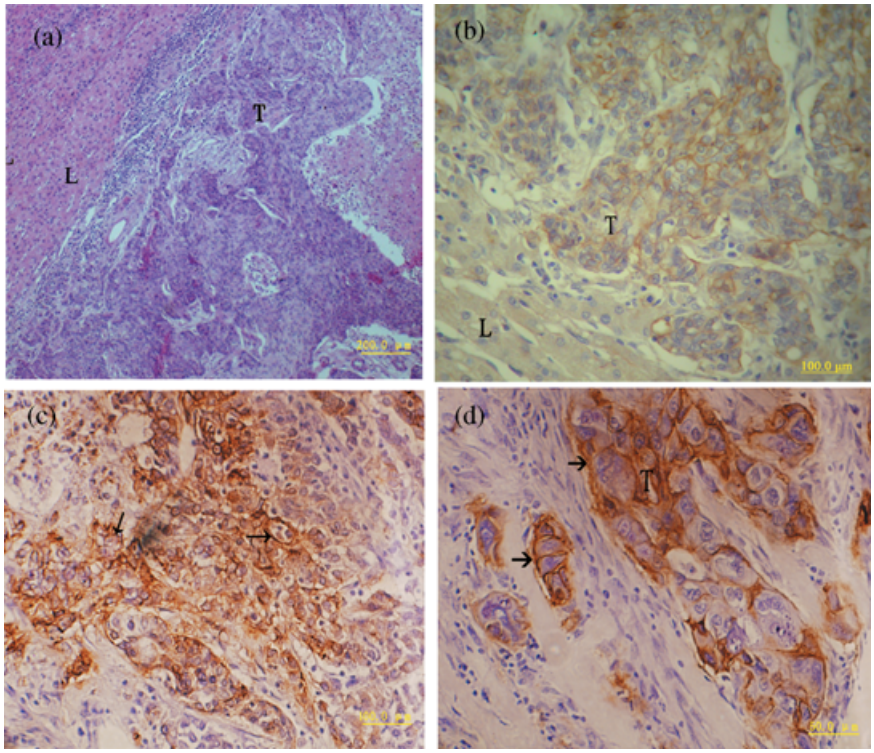

Fig. 3. (a–d) Integrin αvβ6 expression on both liver metastasis tissues and colon carcinoma tissues. (a) Colonic metastases to liver (L) shown by hemotoxylin–eosin staining (T, tumor). (b) Positive αvβ6 expression in the metastatic tumor cells (T) of liver and negative in the normal stroma and liver (L). In the isolated tumor cell and collective metastatic clusters, cell membranes exhibited strong αvβ6 expression. (c) A tumor sample with both tumor cells infiltrating the stroma and the margin of tumor buddings (arrows) showing αvβ6 high expression. (d) Positive αvβ6 expression at the edge (arrows) of both tumor cell islands (T) and tumor budding. Scale bar shown in the lower-right corner of each figure.

Suppression of αvβ6-IR inhibits MMP-9 levels in the cultures of human colon cancer cell lines. We detected the effect of avβ6 on levels of MMP-9, which facilitates cell invasion and migration by degrading ECM. The Biotrak MMP-9 activity assay showed that the amount of MMP-9 secreted per cell into serum-free TCM was approximately three-fold higher for HT29/WiDr mock compared with HT29/WiDr antisense-β6 transfectants ($P < 0.01$) (Fig. 5a). Gelatin zymography assay indicated that WiDr mock transfectants not only secreted markedly more MMP-9 into TCM than WiDr antisense β6, but that this was inhibited by the function-blocking β6-antibody 10D5; The antibody reduced MMP-9 secretion to levels seen for WiDr antisense β6 (Fig. 5b). Gelatin zymography assay also indicated that the levels of MMP-9 for HT29 mock transfectants was similar to WiDr mock as shown in Figure 5c.

Integrin αvβ6 mediates migratory capacity of colon cancer cell lines on fibronectin. To ascribe a functional role for αvβ6 associated with the capability of colonic metastasis to the liver, we focused initially on the classical role of integrin αvβ6 as a fibronectin receptor.[35] Using Transwells coated with specific matrix proteins, we observed that HT29/WiDr mock transfectants expressing the normal level of αvβ6, compared with HT29/WiDr antisense β6, were significantly more chemotactic when the Transwells were coated with fibronectin ($P < 0.01$) (Fig. 6a). The enhanced migration for either HT29 mock or WiDr mock transfectants on fibronectin was mediated by αvβ6 since it was inhibited by the function-blocking β6 antibody 10D5 ($P < 0.01$) (Fig. 6b).

Discussion

The development of liver metastasis is an ominous event in the natural history and progression of colon cancer. The fact that not all disseminated colon cancer cells develop into macrometastases indicates that subpopulations of malignant cells evolve a genetic advantage to adhere, migrate, and invade through ECM, and survive in new liver environment. That adhesion molecules are implicated in the progression of colorectal cancer is well known.[36] Integrin αvβ6, one member of the adhesion molecules, is involved in cell–cell adhesion and tumor growth in colon cancer progression.[13,14] In our study, preliminary studies also showed that the TMA technique was an effective screening method, and that the specificity of the against-β6 mAb 2G2 was well demonstrated using immunoprecipitation assay. The enhanced αvβ6-IR in hepatic metastatic sections (71.4%) compared with that in colon cancer (34%) was observed. The results of a three-year follow-up indicated that patients who were αvβ6-positive had higher liver metastasis rate (17%, 21/122) compared with those who were αvβ6-negative (only 3%, 7/236). Surprisingly, we found that higher expression of αvβ6 in primary colon carcinoma was coincident with the higher liver metastasis rate of patients with colon carcinoma. According to the results, it is likely that integrin αvβ6-IR in primary colon cancer is associated with the properties functionally linked to the progression of colonic metastasis to the liver.

The experimental liver metastasis model where tumor cells are injected into the portal system through the spleen is well established and validated.[37] In our study, 90% (18/20) of mice injected intrasplenically with HT29 mock transfectants developed visible liver metastatic tumors, and only 10% (2/20) of mice failed to manifest a high metastatic capacity. However, none of the visible metastatic nodules in the liver were observed in the mice injected with HT29 antisense β6 transfectants. The reason may be that the sinusoidal network of the spleen provides ready access to the portal venous system. Furthermore, it is also possible that the continued growth of the injected tumor in the spleen allowed the seeding of very large numbers of tumor cells into the circulation and that greater numbers of cells traversed the initial capillary bed of the liver. Although intrasplenic injection appears to allow maximum expression of metastatic capacity, it apparently does not induce metastasis in HT29 antisense β6 transfectants. The intrinsic characteristics of the tumor line transfectants from the HT29 variant resulted in different levels of

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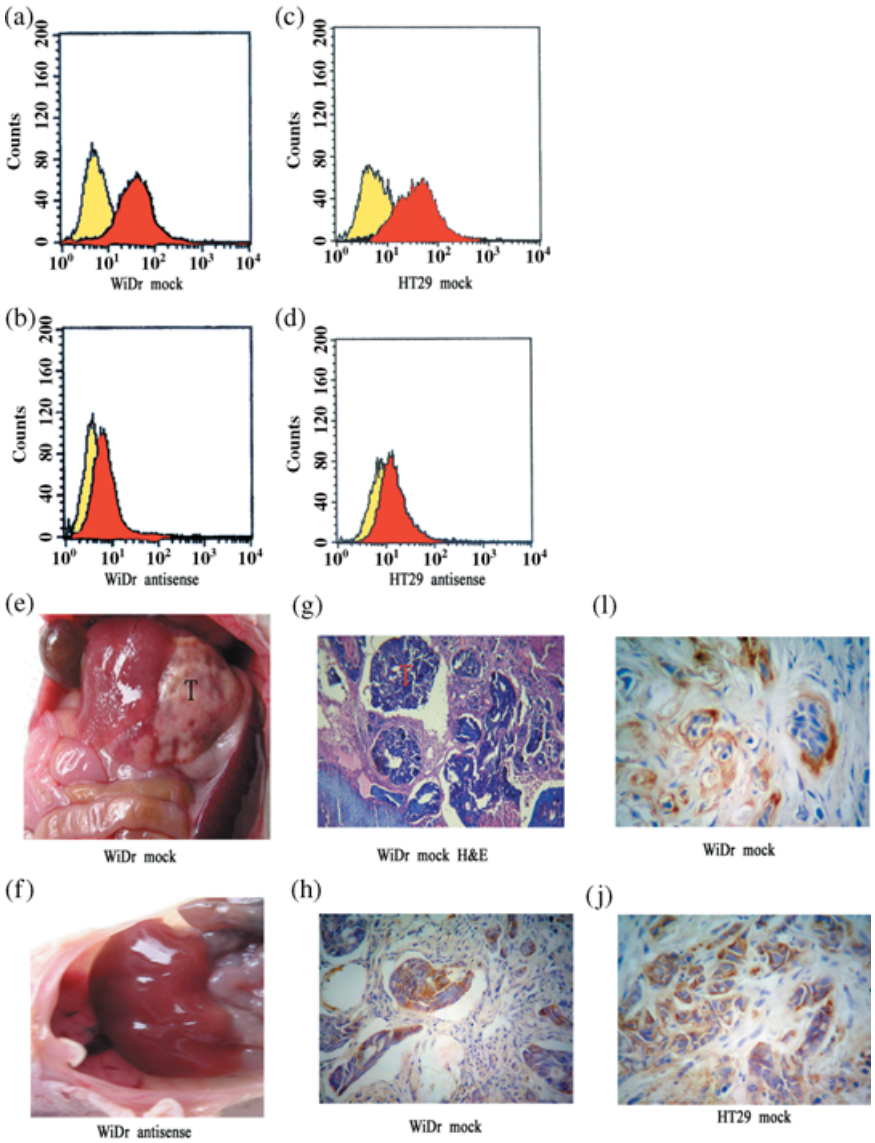

Fig. 4. Down-regulation of αvβ6-IR attenuates liver colonization and metastatic potential for human colon cancer (WiDr and HT29) cell lines in nude mice. (a) Expression of αvβ6 in WiDr mock, (b) WiDr antisense, (c) HT29 mock, (d), and TH29 antisense β6 transfectants were examined by flow cytometry. Yellow and red histograms represent analyses in the absence/presence of primary-antibody recognizing β6 (mAb, E7P6). Hepatic tumor growth after 6 weeks following direct liver injection of 10^6 viable cells of (e) WiDr mock, and (f) WiDr antisense β6 transfectants. (g) Hepatic xenograft tumor (T) infected with WiDr mock transfectants shown by hematoxylin–eosin staining (100 × magnification). (h, 200 ×; i, 400 ×) Positive αvβ6 expression on xenograft tumors injected with WiDr mock and preferential localization at the edge of tumor islands and tumor muddings. (j 400 ×) Positive αvβ6 expression on hepatic metastatic tumors with intrasplenic injection with HT mock transfectants.

αvβ6-IR between HT29 mock and HT29 antisense β6 transfectants, which may be the major determinants in regulating metastatic spread.

A tumor cell's ability to survive in a new local environment is an important step in the development of metastasis. Normal cells lack adhesion capabilities, away from their primary site, which results in apoptosis; or cells lack the ability to evade the immune system resulting in their destruction. To determine the link between αvβ6-IR in colon cancer and the ability of cancer cells to survive in the liver, experimental liver colonization assays (direct liver injection) in nude mice were performed since WiDr cells expressing αvβ6 are non-metastatic colon cell lines. The results showed that 55% (11 of 20) of the nude mice injected with WiDr mock transfectants expressing αvβ6 developed liver metastases. None of the mice injected with WiDr antisense β6 transfectants developed visible tumor nodules in the liver. These results suggest that the down-regulation of αvβ6 expression attenuates the capability of liver colonization in mice. It is also suggested that integrin αvβ6 contributes the survival of colon cancer cells in the liver. This model indirectly confirmed that αvβ6 promoted the liver colonization potential of colon carcinoma cells in aggressive cancer. One possible mechanism for the survival of cancer cells in the liver may be via the recognizable relationship between αvβ6 and its ligands. This has been supported by staining experiments[38] in which luminal epithelial cells in the portal and hepatic veins in the normal liver strongly expressed fibronectin and tenascin, both ligands for αvβ6.[39]

To examine the underlying mechanisms associated with integrin αvβ6 that regulate the complex process of colonic metastasis to the liver, we defined two key functions of αvβ6 that can be linked to the biology of aggressive carcinoma: elevation of MMP-9 levels and promotion of migration on fibronectin matrices. First, MMP-9 plays a critical role in tumor cell invasion because of its ability to hydrolyze type IV–collagen present in basement membranes which separates the epithelial and stromal compartments. A role for ECM degradation resulting from cancer cell–mediated proteolytic activity in the invasion and metastasis of cancer cells has been investigated by Liotta and Stetler-Stevenson.[40] They showed that tumor tissue has the ability to dissolve plasma clots; normal tissue lacks this ability. MMP-9 is one of the vital proteolytic enzymes. MMP-9 expression in primary tumors is associated with tumor-infiltrating growth and metastasis in pancreatic and colon carcinomas.[41] The induction of MMP-9 mRNA in endothelial cells has also been

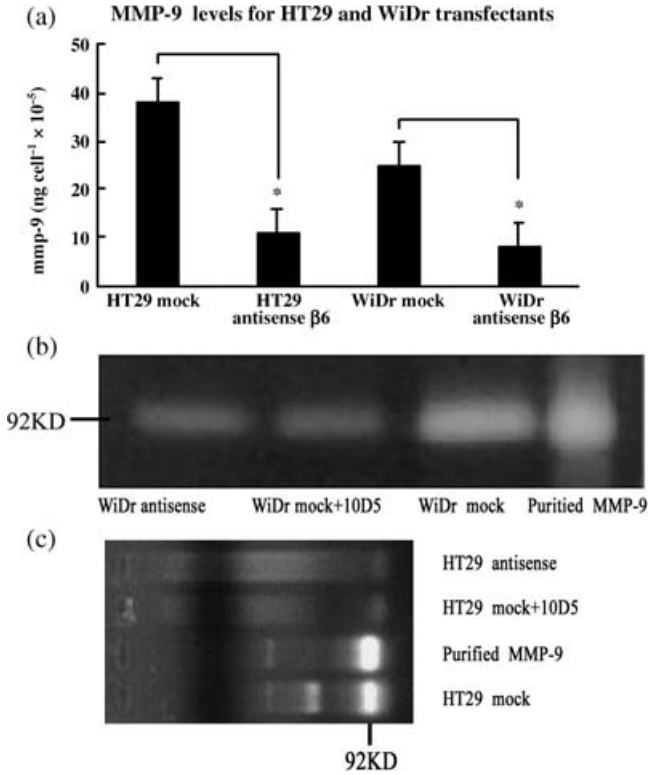

Fig. 5. Down-regulation of αvβ6 immunoreactivity inhibits matrix metalloproteinase-9 (MMP-9) secretion. (a) The Biotrak MMP-9 activity assay showing MMP-9 levels in tumor-conditioned medium (TCM) from HT29 and WiDr mock/antisense β6 transfectants. The data represent mean ± SD levels of MMP-9 (ng/cell) for three independent experiments. Asterisks denote statistically significant differences in MMP-9 secretion levels between mock and antisense β6 transfectants (*$P < 0.01$). (b) Gelatin zymography showing MMP-9 levels in TCM (concentrated 20-fold) from WiDr mock/antisense β6 transfectants. The position of purified MMP-9 is shown on the right. Results are representative of three experiments. (c) Gelatin zymography showing MMP-9 levels in TCM (concentrated 20-fold) from HT29 mock/antisense β6 transfectants. The purified MMP-9 is the positive control. Results are representative of three experiments.

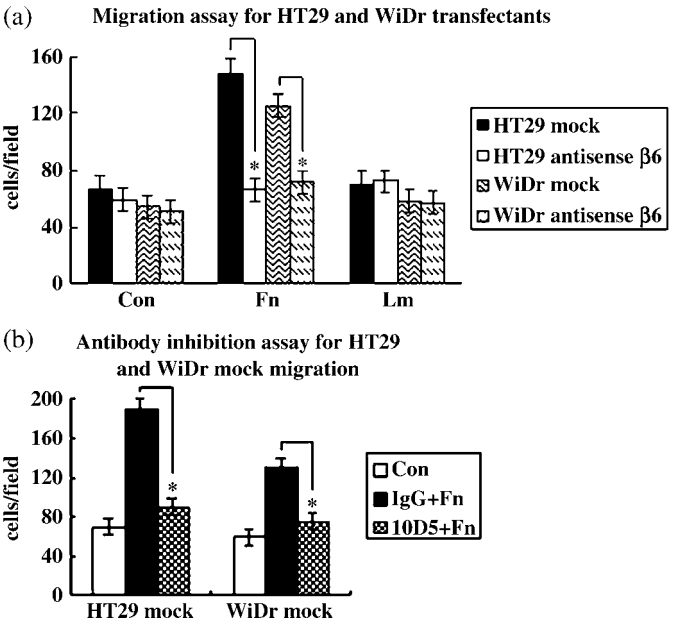

Fig. 6. Integrin αvβ6 promotes migration on fibronectin. (a) Chemotactic migration assay of HT29 and WiDr transfectants for 24 h on untreated control Transwells (Con), or Transwells coated with laminin (Lm) or fibronectin (Fn). Data are expressed as mean ± SD of four individual fields randomly selected from each well, with each experiment performed in triplicate. *$P < 0.01$. (b) Chemotactic migration for HT29 mock and WiDr mock transfectants on fibronectin (Fn) was performed in the presence of function-blocking anti-β6 mAb (10D5; 10 μg/mL) or isotype-matched antibodies (IgG; 100 μg/mL). The anti-β6 antibody reduced chemotaxis back to the levels of the uncoated (Con) migration for these cells. *$P < 0.01$.

shown to be dependent on direct cell adhesion to cancer cells.[42] Maximal expression of MMPs has also been noted at the invading margin of colonic tumor cell islands.[43] This is consistent with Ahmed *et al.*'s[27] observation of αvβ6 preferential localization in the leading edge of epithelial ovarian cancer with malignant potential for invasion and metastasis. Heterologous expression of αvβ6 in colon cancer SW480 cells lacking αvβ6 expression has been shown by us to enhance tumor growth *in vitro* and *in vivo*, which is thought to be due in part to αvβ6-mediated MMP-9 secretion.[16,44] Moreover, tumor cell proliferation within a three-dimensional collagen matrix in β6-expressing SW480 cells is associated with the conversion of tumor cell colonies from compact to spreading colonies, and the exposure of the cells to a specific MMP inhibitor abolishes β6-mediated tumor cell proliferation and colony-spreading within a collagen matrix.[44] In the present study, both the gelatin zymography assay (detecting inactive pro-enzymes) and Biotrak MMP-9 activity assay system (detecting inactive pro-enzymes activated artificially plus endogenous active-enzyme forms) show that down-regulation of constitutive αvβ6 expression dramatically reduced MMP-9 levels in the cultures of the human colon cancer cell lines, HT29 and WiDr. The results indirectly indicate that the degradation of ECM by enhanced secretion of MMP-9 in the colon cells is mediated in part in an αvβ6-dependent manner, since the reduction of mmp-9 levels was inhibited by the function-blocking β6 antibody 10D5.

Second, having a specific fibronectin receptor has selective advantages for tumor cells. The colonic epithelium resides upon a basement membrane, composed primarily of laminins, collagen IV, and proteoglycans, which provide an interface with the lamina propria beneath. The structural composition of this zone differs in its high fibrillar collagen content, and also in its enrichment with fibronectin and tenascin, both ligands for αvβ6.[39] Thus, an enhanced migratory capacity on fibronectin would facilitate the escape and dissemination of invading colon carcinoma cells into the lamina propria. Furthermore, the degradation of ECM by enhanced secretion of MMP-9 also contributes to this process. Our results suggest not only that both HT29 mock and WiDr mock transfectants were more chemotactic when they were on fibronectin, but also that the function-blocking β6 antibody 10D5 markedly reduced the migratory capacity of colon cells on fibronectin. The propensity of enhanced migratory capacity on fibronectin, which is abundant on the surface of hepatocytes,[45] maybe of central importance for the promotion of colon carcinoma metastasis to liver the by aiding extravasation. These findings suggest that αvβ6-IR in colon cancer provides a preferential binding to the portal and hepatic vessels mediating the development of hepatic metastasis.

In conclusion, liver metastasis of human tumor cell lines is not an uncommon event, and the incidence of metastasis is ultimately dependent on the nature of the tumor cells. Our results suggest that αvβ6-IR in human colon cancer mediates the potential of both liver colonization and liver metastasis, in part because of its high frequency of expression (71.4%) in liver metastatic tissues, and high liver metastasis rates (17%, during

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the three-year follow up) in patients who were αvβ6-positive. Furthermore, both the liver metastatic model and the liver colonization model in nude mice demonstrated that αvβ6 contributes to the promotion of the potential of liver metastasis and the survival of cancer cells in the liver. Our studies *in vitro* also suggested that integrin αvβ6 may play a critical role in mediating properties associated with metastasis, such as cell motility and MMP-9 production, which constitute the basis for colonic metastasis to the liver. Therefore, strategies that target the inhibition of αvβ6-IR in colon cancer cells could, in part, prevent the potential of colon cancer cells to colonize and metastasize in the liver.

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