Clin Oncol Cancer Res (2011) 8: 138–143
DOI 10.1007/s11805-011-0572-x

Interaction between Colon Cancer Cells and Human Liver Sinusoidal Endothelial Cells Promotes Liver Metastasis of Tumor Cells

Li-chao SUN[1]
Shu-ting LI[2]
Long YU[1]
Li-xin SUN[1]
Lu-lu HAN[1]
Tong LIU[1]
Zhi-hua YANG[1]
Yu-liang RAN[1]

[1] State Key Laboratory of Molecular Oncology, Cancer Institute (Hospital), Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, People's Republic of China.
[2] Internal Medicine, Cancer Institute (Hospital), Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, People's Republic of China.

Correspondence to: Yu-liang RAN
E-mail: ran_yuliang@126.com

Received June 13, 2011; accepted August 9, 2011

E-mail: editor@cocronline.org
Tel (Fax): 86-22-2352 2919

OBJECTIVE To investigate the effect of co-culture between colon cancer cells (SW1116) and human liver sinusoidal endothelial cells (HLSECs) on cancer cell metastasis, and to provide a novel model for studying the mechanism of colon cancer liver metastasis.
METHODS HLSECs and SW1116 were co-cultured for 21 rounds *in vitro*. Transwell migration, gelatin-zymography, CCK-8 proliferation and colony formation assays were used to examine the invasion, proliferation, and colony forming ability of cancer cells. Assays were carried out to examine tumor growth ability and liver metastasis. The associated molecular change was examined by western blotting.
RESULTS After 21 selection rounds, colon cancer cells SW1116P21 displayed a clear boundary. Compared with the SW1116 cells, SW1116P21 cells had a greater invasive ability, cell proliferation and colony formation in soft agar. A gelatin-zymography assay showed that the ability of SW1116P21 cells to secrete matrix metalloproteinase-2/9 was significantly greater than that of SW1116 cells. Additionally, the capacity for subcutaneous tumor formation of SW1116P21 was significantly increased. It was found that mice injected with SW1116P21 cells developed significantly more visually observable liver nodules than mice injected with SW1116 cells. Western blotting showed increased vimentin expression and decreased E-cadherin expression in the SW1116P21 cells, compared with the SW1116 cells.
CONCLUSION The interaction between SW1116 and HLSECs may promote tumor cell invasion, proliferation and colony formation *in vitro*, and tumor formation and liver metastasis *in vivo*. An epithelial-mesenchymal transition occurs in SW1116P21 cells, which contributes to the change in the characteristics of tumor cells.

KEY WORDS: colon cancer, human liver sinusoidal endothelial cells, co-culture, liver metastasis.

Introduction

Colorectal carcinoma is one of the major causes of cancer death worldwide. The liver is the most common target for metastasis in patients with this disease. It is estimated that approximately 50% of patients with colorectal cancer develop liver metastases[1], and about 20%–40% of these patients have synchronous liver metastasis and about 25% will develop heterochronous liver metastasis[2].

The organ-specific metastasis of tumors is closely correlated with tumor cell–endothelium interaction. First, circulating tumor cells express molecules whose ligands adhere preferentially to the endothelial cells of

the target organ. Then, once tumor cells have successfully bound to the endothelium, multiple signal transduction cascades may be triggered which affect the proliferation, migration and invasion of the tumor cells. All these processes may contribute to the specific organ metastasis of tumor cells[3,4]. It has been reported that interaction between tumor cells and endothelial cells mediated by E-selectin may promote cancer metastasis[5]. Also, Al-Mehdi et al.[6] found that the tumor cells, which adhere to the endothelium, proliferate in the blood vessels promoting metastasis of tumor cells.

In this study, we co-cultured cancer cells (SW1116) with human liver sinusoidal endothelial cells (HLSECs) and obtained subpopulations with a high potential for liver metastasis. The biological properties of these sublines were examined, providing a new model for studying the mechanism of colon cancer liver metastasis.

Materials and Methods

Materials

Reagents

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium and fetal bovine serum (FBS) were purchased from Hyclone company (Logan, UT). Matrigel was purchased from BD Bioscience (San Jose, CA, USA). Gelatin and Transwell were purchased from Corning. Calcein AM was purchased from Invitrogen. Horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG was purchased from Vector (Burlingame, CA, USA). A western blotting kit was purchased from Applygen Technologies Inc. Polyvinylidene difluoride membrane was purchased from Millipore (Bedford, MA).

Animals

BALB/c (nu/nu) mice (4–6 weeks old) were purchased from Vital River Laboratories (Beijing, PR China), and were maintained under standard specific pathogen-free (SPF) conditions.

Methods

Isolation of the subline of colorectal carcinoma cells with a high level of adhesion to HLSECs

The human colorectal carcinoma cell line SW1116 was grown in DMEM-H medium, and HLSECs were grown in DMEM-E medium, supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS. In all experiments, the cells were maintained at 37°C in a humidified 5% CO_2 incubator. To determine whether SW1116 cells had been treated correctly for the isolation of adhesive cancer cells, human liver sinusoidal endothelium was grown to 100% confluence in a T75 flask, and then the medium was changed into DMEM-H with 0.1% FBS for 24 h. The colorectal carcinoma cells were digested and resuspended with DMEM-H/0.5% bovine serum albumin, and incubated with the human liver sinusoidal endothelium for 1 h. The colorectal carcinoma cells which had not adhered to the HLSECs were washed off, and the attached cells were grown in the T75 flask. After 48 h, the attached cancer cells were isolated from the HLSECs by trypsin digestion, as HLSECs are more easily trypsinized than colon cancer cells. Finally, colorectal carcinoma cells with high adhesion to HLSECs were obtained by repeating this process 21 times; the cells obtained were named SW1116P21.

Functional analysis of SW1116

Cell adhesion assay

Adhesion of SW1116 or SW1116P21 to HLSECs was examined. The tumor cells were stained with calcein-AM (Invitrogen). LSEC cells (1×10^4) were plated in a 96-well plate then coated with 2% gelatin (w/v) for 30 min, followed by the addition of medium containing endothelial cell growth factors for 24 h. Then this medium was replaced by medium containing no endothelial cell growth factors for a further 3 days to ensure that most of the endothelial cells were in a quiescent state. The colon cancer cells were maintained for 1 h, and the non-adhesive tumor cells were washed off. The adhesive tumor cells were examined by fluorescence microscopy, and the number of cells in each visual field was counted using IPP5.1 software.

Cell proliferation assay

SW1116 and SW1116P21 cells in the exponential growth phase were plated in a 96-well plate with 2,000 cells in each well. A viable cell count was made for each well every 24 h using CCK-8.

Assay of colony-forming ability

After digestion into a single-cell suspension, the cells were counted and the cell number adjusted to 10,000 cells/mL. Agarose (0.6%) with a low melting point and 2 × cell medium were mixed 1:1 (v/v) to obtain 0.3% agar in the upper chamber. Agar (1 mL) in the upper chamber and 100 μL single-cell suspension (1,000 cells/well) were mixed in each well and solidified at room temperature. The 6-well plate was maintained for 2–3 weeks at 37°C in a humidified 5% CO_2 incubator, and then the cell colony-forming rate was calculated.

Cell invasion assay

Matrigel (10 mg/mL) was diluted to 1 mg/mL with RPMI-1640 medium on ice. Diluted gel (50 μL) was added into the Transwell upper chamber, allowed to solidify for 1 h at room temperature and washed twice with RPMI-1640 medium. RPMI-1640 medium (600 μL) containing 10% FBS was added to the Transwell lower chamber, 100 μL RPMI-1640 medium containing 0.1% FBS was added to the Transwell upper chamber to resuspend the cells, and the cells were maintained for 24 h. The invaded cells were counted under a fluorescence microscope after staining with 4′,6-diamidino-2-phenylindole.

Gelatin-zymography assay

Colon cancer cells were maintained in serum-free DMEM medium. The supernatant was harvested by centrifugation at 2,000 rpm for 10 min and then stored at

−70°C. The supernatant was subjected to electrophoresis on 100 g/L polyacrylamide gel containing 1 g/L gelatin, and the gel was rinsed 3 times (30 min each time) in 25 mL Triton X-100, followed by two rinses with 100 mL buffer (50 mmol/L Tris-HCl, pH 7.6) (20 min each time). The gel was incubated in 100 mL buffer (50 mmol/L Tris-HCl, 200 mmol NaCl, 10 mmol $CaCl_2$) for 24 h at 37°C, stained in buffer (5 g/L Coomassie brilliant blue, 300 mL/L ethanol and 100 mL/L acetic acid) for 3 h, and destained for 15, 45 and 45 min. Matrix metalloproteinases (MMPs) were detected as a transparent bands on a blue background, and the band area, width and grey level were analyzed and scored with the gel-image analysis system for statistical analysis.

Xenografted human colon cancer cells in Nude mice

SW1116 or SW1116P21 (1×10^6 cells) were injected subcutaneously into the right flanks of the mice (6 mice in each group). The major and minor diameters of the xenograft were measured every 3 days, and the volume of the xenograft was calculated with the formula: V= (JI/6) × (major diameter × minor diameter × minor diameter).

Experimental liver metastasis

All animal experiments were carried out in full compliance with institutional guidelines. SW1116 parental cells were harvested and suspended in phosphate-buffered saline at a final concentration of 2×10^7 cells/mL. Mice were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). Through a 10 mm left subcostal incision, the spleen was identified beneath the peritoneum, and exposed through an 8 mm incision. A suspension of tumor cells (100 μL) was injected into the spleen using a 27-gauge needle. After this, the spleen was returned to the abdominal cavity, the peritoneum sutured with a single stitch, and the wound closed with a clip. The number of metastases on the surface of the liver was calculated 8 weeks after the injection.

Western blot

For western blot analysis, cellular proteins were extracted into 40 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 1% (v/v) Triton X-100 and supplemented with a cocktail of protease inhibitors. An equal amount of protein was run on 10% SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane. After blocking with 5% non-fat milk, the membranes were allowed to react with primary antibody at 4°C overnight, and then with horseradish peroxidase-conjugated sheep anti-rabbit or anti-mouse IgG (Vector). The blots were washed and then developed with a super-enhanced chemiluminescence detection kit.

Statistical analysis

All data are presented as means ± SD. Statistical analysis was performed using SPSS statistical software (SPSS Inc, Chicago, IL, USA). Student's two-tailed t-test in the two groups was used to analyze the statistical significance of differences.

Results

Isolation of the colon cancer SW1116P21 cell subline with high adhesion to HLSECs in vitro

The colon cancer cell SW1116 stored in our laboratory was used for the co-culture selection. SW1116 cells were plated onto HLSECs. After 1 h the nonadherent cells were removed and the adherent cells were allowed to remain in the culture flask for 72 h. During this time, the cancer cells began to penetrate the monolayer of HLSECs, and ultimately grew into cancer cell nests. As a result of the difference in trypsin digestion of colon cancer cells and endothelium, we then removed HLSECs, taking advantage of the fact that HLSECs are much more easily trypsinized than the colon cancer cells (Fig.1A and B). The remaining tumor cells were then subjected to another round of selection. The process was repeated 21 times, and the cells finally separated were named SW1116P21. The morphology of SW1116 and SW1116P21 was observed under light microscopy. Fig. 1C shows that the morphology of SW1116P21 cells had changed dramatically: the cells had typical epithelial-like characteristics, the membrane was clear, the cellular boundary was marked. On the other hand, the

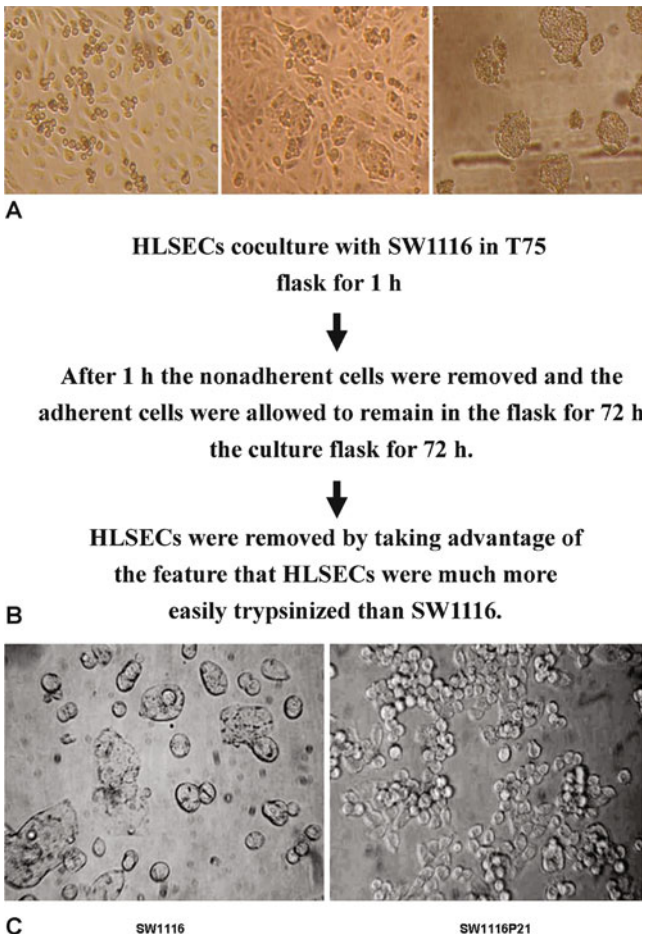

Fig.1. SW1116 cells and liver sinusoidal endothelial cells were cultured. (A) Human liver sinusoidal endothelial cells were cultured with SW1116. (B) The culture procedure. (C) Cell morphology.

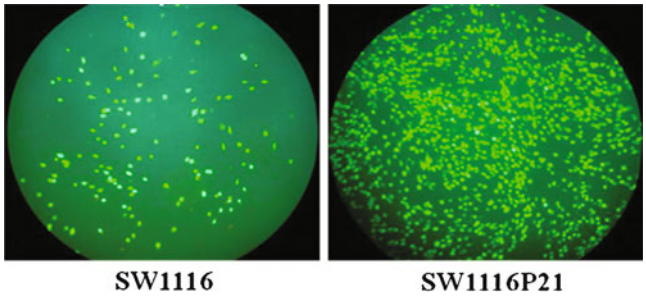

Fig.2. Adhesion of SW1116 or SW1116P21 cells to human liver sinusoidal endothelial cells.

boundary between parental cells had disappeared, a cell mass was formed and distributed as islands.

Analysis of the biological properties of SW1116P21 cells

Analysis of adhesive ability in vitro

The ability of SW1116 or SW1116P21 cells to adhere to HLSECs was examined. SW1116P21 cells had significantly greater adhesive ability than SW1116 cells (Fig.2).

Analysis of invasive ability in vitro

The invasive ability of SW1116 and SW1116P21 cells *in vitro* was investigated using a Transwell invasion chamber. It was found that the invasive ability of SW1116P21 cells was increased 2-fold compared with that of SW1116 cells (Fig.3). The numbers of SW1116 and SW1116P21 cells were 89.4 ± 8.9 and 152.7 ± 11.3, respectively.

Gelatin-zymography assay

A gelatin-zymography assay of the culture supernatant of SW1116 and SW1116P21 showed two bands at 92 and 72 KD in the SW1116P21 cell lane (Fig.4). The light level of SW1116P21 was higher than that of SW1116 cells, and the band area of SW1116P21 was greater than that of the parent cells, suggesting more MMP-2 and MMP-9 secretion and a significantly stronger invasive ability of SW1116P21 cells.

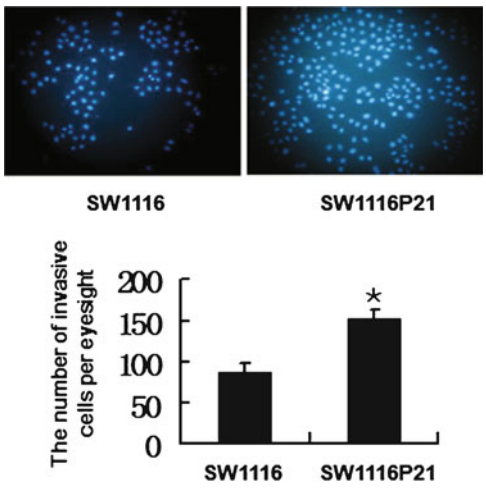

Fig.3. Invasion ability of SW1116 and SW1116P21 cells.

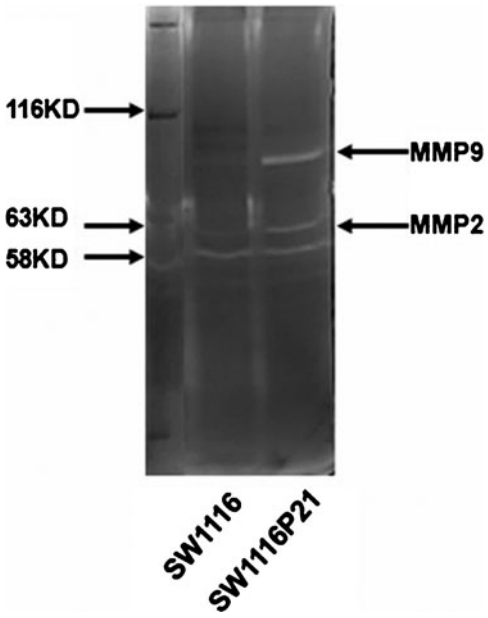

Fig.4. Gelatin zymograms of the culture supernatant of SW1116 and SW1116P21. MMP, matrix metalloproteinase.

Analysis of proliferative ability in vitro

The proliferative ability of SW1116 and SW1116P21 *in vitro* was analyzed using CCK-8 staining. Fig. 5 shows that the SW1116P21 cells grew significantly faster than the SW1116 cells after plating for 24 h, and the optical density (OD) of the SW1116P21 cells at 450 nm was increased 2-fold at 48 h compared with the parent cells.

Analysis of colony-forming capacity

The colony-forming capacity of SW1116 and SW1116P21 was investigated by a soft agar assay. Fig. 6 shows that after 3 weeks the numbers of colonies formed by SW1116P21 cells and SW1116 cells were 152.4 ± 19.6 and 89.8 ± 19.6, respectively, an almost 2-fold increase in comparison with SW1116.

Tumorigenesis of SW1116P21 in vivo

BALB/c mice were subcutaneously injected with 1×0^6 SW1116 or SW1116P21 cells, and the growth of subcutaneous xenografts was measured using calipers. Fig. 7

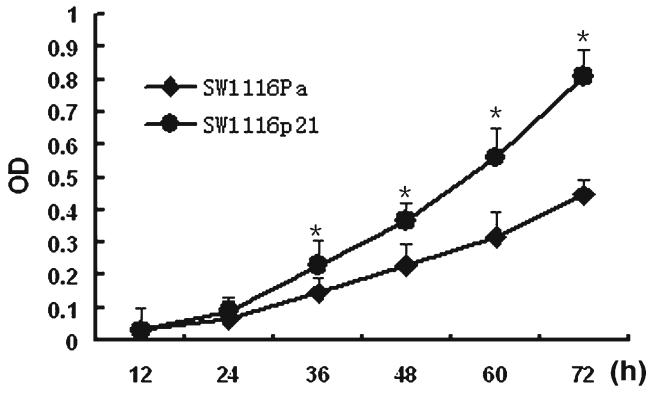

Fig.5. Proliferation of SW1116 and SW1116P21. (*, $P < 0.05$)

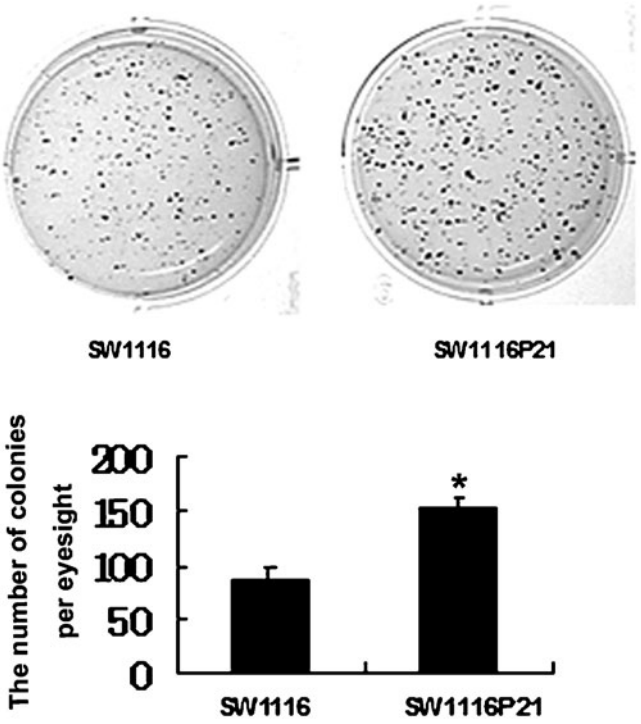

Fig.6. Colony formation of SW1116 and SW1116P21 in soft agar. (*, $P < 0.05$)

shows that the growth of SW1116P21 was significantly faster than that of SW1116 cells. The average weight of xenograft formed by SW1116P21 and SW1116 was 2.31 ± 0.89 g and 0.67 ± 0.21 g, respectively. This result indicates that the tumor-forming ability of SW1116P21 was significantly increased after its interaction with HLSECs.

Analysis of experimental liver metastasis

SW1116P21 or SW1116 cells were then injected into the spleens of 6 mice for each group. Nine weeks later, the mice were sacrificed and examined for the presence of metastases. Fig. 8 shows that SW1116P21 cells had caused significantly more visible liver metastatic foci than SW1116 cells ($P<0.05$). With the injection of SW1116P21, metastases were found on the surface of the liver in all 6 mice, with an average number of metastases of 4.67 ± 1.63. In contrast, with the injection of SW1116 cells, metastases were found in the liver of 4/6 mice, with an average number of metastases of 0.83 ± 0.79. The results suggest that interaction of SW1116P21 with HLSECs may contribute to liver metastasis.

Detection of invasion-metastasis related molecules

The changes including cell morphology, migration and invasion suggested that SW1116P21 had undergone an epithelial-mesenchymal transition (EMT). It has been reported that E-cadherin and vimentin are markers of EMT[7,8]. Therefore, we detected the protein level of E-cadherin and vimentin in SW1116P21 and SW116 by western blotting (Fig.9). The results showed that the level of E-cadherin was decreased significantly and the level of vimentin was increased significantly in SW1116P21 cells in comparison with the parental cells, suggesting that SW1116P21 cells had undergone EMT by co-culturing with HLSECs.

Discussion

Tumor metastasis occurs preferentially to certain sites: colon cancer often gives rise to hepatic metastasis[9,10], brain metastases occur frequently in patients with lung cancer[11,12], and the most common site of gastric cancer metastasis is the lymphatic and abdominal cavity[13,14]. Recent research has shown that interactions between tumor cells and the endothelium of target organs have a vital role in the specific metastasis of a tumor.

Therefore, in this study we isolated colon cancer cells attached to HLSECs *in vitro*. The highly adhesive cells were co-cultured with HLSECs for 48 h. During this time, the cancer cells began to penetrate the monolayer of the HLSECs, and ultimately grew into cancer cell nests. We then removed the HLSECs, taking advantage of the fact that they were much more easily trypsinized than the colon cancer cells. The surviving tumor cells were then subjected to another round of selection. SW1116P21 cells (the 21th cycle co-culture with HLSECs) were obtained. The different cell morphology and phenotypes in SW1116 and SW1116P21 were observed.

Then the phenotype and function of cell proliferation, invasion, colony formation in soft agar, and adhesion to HLSECs were examined. In comparison with SW1116 cells, SW1116P21 cells showed clear cellular boundaries and were characterized by a greater invasive ability, cell proliferation and colony formation in soft agar. A gelatin-zymography assay also showed that the ability of the SW1116P21 cells to secrete MMP-2/9 was significantly greater than that of the SW1116 cells. An assay showed that the ability of subcutaneous tumor formation of SW1116P21 was significantly enhanced. It was also found that mice injected with SW1116P21 cells developed significantly more visually observable liver

Fig.7. Subcutaneous tumor formation of SW1116 and SW1116P21.

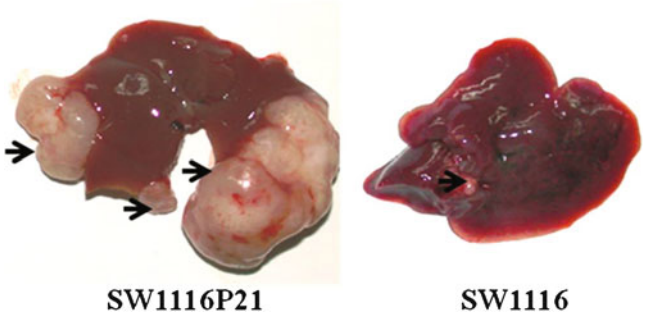

Fig.8. Experimental liver metastasis of SW1116 and SW1116P21 by intrasplenic injection.

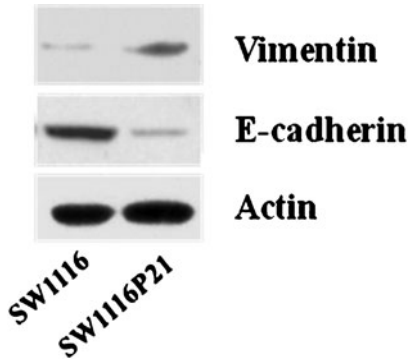

Fig.9. EMT-related molecules detected by western blotting.

nodules than the mice injected with SW1116 cells. All the results indicated that co-culture of colon cancer cells with HLSECs may promote liver metastasis.

Studies have shown that the invasion and metastasis of tumors are dependent on EMT characteristics, including loss of cell polarity, reduced contact with neighboring cells and matrix, downregulation of the cell adhesive ability and increasing cell migration and movement[15]. Based on the morphology and the results of functional assays of SW1116P21 cells, we speculated that SW1116P21 underwent EMT after interaction with HLSECs, which might contribute to the liver metastasis of colon cancer cells. It has been reported that E-cadherin and vimentin are markers of EMT[8]. Therefore, the protein level of these EMT markers was detected by western blotting. The results showed that the level of E-cadherin was downregulated and the level of vimentin was upregulated, suggesting that the metastasis of SW1116P21 cells was promoted by the EMT.

In summary, we successfully established a model of the interaction between tumor cells and the endothelium of the target organ and obtained the colon cancer cell subline SW1116P21, which showed increased invasiveness, high proliferative ability, high adhesion to the human liver sinusoidal endothelium and high metastasis *in vivo*. SW1116P21 might be useful as a basic material for the study of the molecular mechanism of colon cancer liver metastasis. In future studies we would aim to identify the different genes between SW1116 and SW1116P21, which might provide candidate molecules for therapy of colon cancer liver metastasis.

Acknowledgements

This work was supported by a grant from the Major Basic Research and Development Program (973) of China (No.2009CB521804) and by PUMC&CAMS Funds for Young Scholar.

Conflict of interest statement

No potential conflicts of interest were disclosed.

References

1 McMillan DC, McArdle CS. Epidemiology of colorectal liver metastases. Surg Oncol 2007; 16: 3–5.
2 Manfredi SC, Lepage C, Hatem C. et al. Epidemiology and management of liver metastases from colorectal cancer. Ann Surg 2006; 244: 254–259.
3 Gassmann P, Kang M-L, Mees S, et al. In vivo tumor cell adhesion in the pulmonary microvasculature is exclusively mediated by tumor cell-endothelial cell interaction. BMC Cancer 2010; 10: 177.
4 Enns A, Gassmann P, Schlüter K, et al. Integrins can directly mediate metastatic tumor cell adhesion within the liver sinusoids. J Gastrointest Surg 2004; 8: 1049–1060.
5 Brodt P, Fallavollita L, Bresalier RS, et al. Liver endothelial E-selectin mediates carcinoma cell adhesion and promotes liver metastasis. Int J Cancer 1997; 71: 612–619.
6 Al-Mehdi AB, Tozawa K, Fisher AB, et al. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med 2000; 6: 100–102.
7 Vuoriluoto K, Haugen H, Kiviluoto S, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene 2011; 30: 1436–1448.
8 Sethi S, Macoska J, Chen W, et al. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. Am J Transl Res 2010; 3: 90–99.
9 Minn AJ, Kang Y, Serganova I, et al. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J Clin Invest 2005; 115: 44–55.
10 Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003; 3: 537–549.
11 Castrucci WA, Knisely JP. An update on the treatment of CNS metastases in small cell lung cancer. Cancer J 2008; 14: 138–146.
12 Mulvenna P, Barton R, Wilson P, et al. Survival of patients with non-small cell lung cancer and brain metastases. Clin Oncol (R Coll Radiol) 2011; 23: 375–376.
13 Sheen-Chen SM, Chou CW, Chen MC, et al. Adenocarcinoma in the middle third of the stomach-an evaluation for the prognostic significance of clinicopathological features. Hepatogastroenterology 1997; 44: 1488–1494.
14 Zhu G, Zhang M, Zhang H, et al. Survival predictors of patients with gastric cancer with peritoneal metastasis. Hepatogastroenterology 2010; 57: 997–1000.
15 Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 2005; 24: 7443–7454.