Role of integrin α1 subunits in gastric cancer patients with peritoneal dissemination

KAZUMASA FUKUDA, YOSHIRO SAIKAWA, HIROSHI YAGI, NORIHITO WADA, TSUNEHIRO TAKAHASHI and YUKO KITAGAWA

Department of Surgery, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan

Received July 7, 2011; Accepted October 7, 2011

DOI: 10.3892/mmr.2011.642

Abstract. The interaction between gastric cancer (GC) cells and the peritoneum is a critical event in peritoneal dissemination. The molecular mechanisms of this dissemination, however, remain unclear. Integrins are heterodimeric cell adhesion molecules consisting of α and β subunits that serve as adhesion receptors for extracellular matrix proteins and cellular ligands, and may participate in GC peritoneal dissemination. In this study, we isolated fresh GC cells from a patient with peritoneal metastasis and examined them for integrin expression and investigated the role of integrin α1 subunit molecules in GC. Five clones (KGC1C2, KGC1F3, KGC1H3, KGC1E8, KGC1G10) were established from the clinical GC sample and used in an *in vitro* adhesion model using a single cell culture method. Each clone was transplanted into the peritoneal cavity of SCID mice, where each clone formed tumors and caused conglutination of organs in the abdominal cavity. We analyzed the expression of integrin subunits for each clone by flow cytometry and found that the expression ratio of α1 subunits paired with β1 subunits was detected at higher levels than other subunits. To verify that anti-integrin α1 subunit (CD49a) antibody inhibits cell adhesion in an *in vitro* adhesion assay, each clone was treated with anti-CD49a antibody, which significantly inhibited cell adhesion compared to the untreated group. Characterization of α1 subunit expression in GC may be useful in optimizing treatments for different individuals. Having high metastatic abilities, these 5 new GC clones may be beneficial for analyzing integrin function in tumor metastasis.

Introduction

Gastric cancer (GC) is the leading cause of gastrointestinal carcinoma and is the second most common cause of cancer-related mortality worldwide (1). Previous studies have demonstrated that peritoneal dissemination is a unique characteristic of recurrent GC and, more importantly, is related to GC prognosis, since peritoneal dissemination can cause serious clinical complications such as ileus, hydronephrosis and ascites. Thus, controlling peritoneal dissemination in GC patients, particularly in advanced cancer, is critical. Although many studies have attempted to identify the best options for controlling peritoneal dissemination, none have been able to attenuate poor patient prognosis (2,3).

In general, metastatic dissemination is mediated through exfoliation of cancer cells into the peritoneal cavity, with a key step in the process regulated by cancer cell adhesion to the peritoneum that is mediated by various cell adhesion molecules expressed on cancer cells. However, the mechanisms responsible for this disseminated metastasis remain unclear.

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix (ECM) proteins of the basement membrane or to ligands on other cells and play a decisive role in metastatic dissemination. Integrins contain large (α) and small (β) subunits of sizes 120-170 kDa and 90-100 kDa, respectively, as well as binding sites for $Mg^{2+}$and Ca^{2+}, which are necessary for their adhesive function. Mammalian integrins form several subfamilies, which share common β subunits that associate with different α subunits.

Numerous reports have shown that integrin receptors are highly expressed on cancer cells and help stimulate cell growth and motility through intracellular signaling (4,5). There have also been several studies concerning the role of integrin βl subunit in peritoneal implantation, although the relationship between integrin αl-α6βl subunits and peritoneal implantation has not yet been reported. Since the profiles of integrin expression in cancer cells are frequently associated with their malignant phenotypes, as well as invasive and metastatic potentials (6-9), integrins may be important therapeutic targets of peritoneal dissemination.

To study the role of integrins in the progression of peritoneal dissemination, we first isolated fresh GC cells from a patient with type 4 carcinoma (pT4N1H0CY1M0, stage IV) and established clones for the specific model of GC with metastatic dissemination. Five clones were created from the primary GC cells and they possessed tumorigenic properties in the peritoneal cavity of combined immunodeficient (SCID) mice. Moreover, we analyzed the expression of integrin subunits as well as other adhesion molecules of these clones

Correspondence to: Dr Yoshiro Saikawa, Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
E-mail: saiky@z8.keio.jp

Key words: gastric cancer, peritoneum dissemination, integrin

and found that the expression ratio of α1 subunits paired with β1 subunits was higher than that for other subunits. These 5 new GC clones with high metastatic ability may be beneficial for analyzing the mechanisms of progression and metastasis.

Materials and methods

Clinical sample. Clinical samples were obtained from the patient with informed consent and agreement (approved by the Ethics Committee of Keio University; no. 17-47). The patient was clinically diagnosed with GC with a pathological diagnosis of type 4 carcinoma (pT4N1H0CY1M0, stage IV). Ascites were collected into 50-ml centrifuge tubes, which were then centrifuged at 800 x g for 5 min. The GC cells were suspended in RPMI-1640 medium (Nacalai Tesque Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), penicillin-streptomycin mixed solution (penicillin 10,000 units/ml, streptomycin 10,000 μg/ml; Nacalai Tesque Inc.). In all experiments, cells were cultured at 37˚C in a humidified 5% CO_2/95% air atmosphere.

Cloning and proliferation assay. Fresh GC cells were detected as CD45- and glycopholin-A-negative cells from the peritoneum sample. The GC cells were then collected and sorted using a FACSVantage™ SE (Becton Dickinson) with a single cell in each well of a 96-well cell culture plate coated with Matrigel (Becton Dickinson, San Jose, CA, USA) as a basement membrane matrix (Becton Dickinson). Single cells were cultured in the appropriate cell culture medium as described above. Colony formation was observed daily under a microscope. Clones were seeded and cultured in 10% serum-supplemented RPMI-1640. Cells were cultured for 0, 35, 40 and 45 days and then harvested using 0.25% trypsin-1 mM EDTA, whereupon the cells were counted by the trypan blue dye exclusion method. Trypan blue is a vital stain used to selectively color dead cells blue, while live cells with intact cell membranes remain uncolored. The growth curve was drawn from cell counts and the population growth rate was calculated.

Transplantation assay. SCID (C.B-17/lcr-scid/scidJcl) mice aged 8-10 weeks (Japan Clea Laboratories, Tokyo, Japan) were used in the experiment. For transplantation, $1x10^5$ cloned cells derived from the GC clinical sample were subcutaneously inoculated intraperitoneally in the mice, which were then sacrificed using diethyl ether 7.4 weeks later and laparotomized. Intraperitoneal dissemination of the tumor was observed and the tumors weighed.

Flow cytometry. To analyze integrin expression, the cells were removed from the culture dish using 0.25% trypsin and 1 mM EDTA, pelleted by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended at 37˚C in Hank's balanced salt solution (HBSS) containing 2% FBS and 10 mM HEPES (Invitrogen). The cells were then stained with FITC- or PE-labeled monoclonal antibodies against human CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1) subunits (Pharmingen, San Jose, CA, USA). The cells were then incubated with human monoclonal antibodies for 30 min at 4˚C, and then suspended in HBSS containing 2% FBS, penicillin-streptomycin mixed solution and 10 mM HEPES. The cells were then passed through a 40-μm mesh filter and maintained at 4˚C until flow cytometric analysis. The cells were subsequently counterstained with 1 μg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) to label dead cells. Subsequently, $1x10^6$ viable cells were analyzed and sorted using a FACSVantage™ SE. The distribution of cells was analyzed using FlowJo software (Tomy Digital Biology, Tokyo, Japan).

Adhesion assay. The adhesion ability of each cell clone was examined by an adhesion assay. Briefly, a 96-well microtiter plate was coated with Matrigel (50 μl/well) (Becton Dickinson). Cells ($1x10^4$) were incubated with anti-CD49a (integrin α1) antibody for 30 min at 37˚C, and then seeded into a 96-well microtiter plate (Corning Costar, Franklin Lakes, NJ, USA). The cells were allowed to adhere to each well for 12 h at 37˚C and were then washed gently twice in PBS. Cancer cells with adhesion capacity were quantified by the WST-8 assay. The value of optical density (OD) was detected using Rainbow Sunrise (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The rate of inhibition was calculated as follows: % inhibition = (OD of treated group - blank)/(OD of control group) x100.

Statistical analysis. All data were expressed as the mean ± SD. Statistically significant differences were determined using the Student's t-test. P values <0.05 were considered to denote statistical significance.

Results

In vitro adhesion assay. To select and clone GC cells having adhesion and proliferation capacity, GC cells were evaluated using single cell sorting methods. After 14 days in culture, 5 clones (KGC1C2, KGC1F3, KGC1H3, KGC1E8, KGC1G10) having sphere or colony-like shape appeared in Matrigel-coated 96-well plates (Fig. 1). At day 45, the growth rate (number of cells) of clone KGC1C2 ($4.75x10^6$) and KGC1E8 ($5.0x10^6$) was ~1.8-fold higher compared to the other three clones (Fig. 2). These results indicate that only 5 of the 96 clones isolated could suvive and proliferate, suggesting that 5.2% of GC cells in ascites may possess adhesion and proliferation capabilities.

Evaluation of tumorigenesis in vivo. The tumorigenic ability of the clones in peritoneal tissue was examined using SCID (C.B-17/lcr-scid/scidJcl) mice. Each clone ($1x10^5$ cells) was injected into the peritoneal cavity of SCID mice. After 7.4 weeks, each clone was able to generate tumors. Moreover, these metastatic GC cells caused conglutination of surrounding organs in the abdominal cavity, including the stomach, liver and intestine. The weight of tumors derived from the KGC1H2 clone was 1.03±0.21 g (mean ± SD). The KGC1H2 clone formed more tumors than the other clones tested ($P<0.05$) (Fig. 3). These results indicate that the clones derived from the clinical sample possessed tumorigenic ability *in vivo*.

Integrin expression profiles determined by flow cytometry. Integrin expression was measured by flow cytometry with

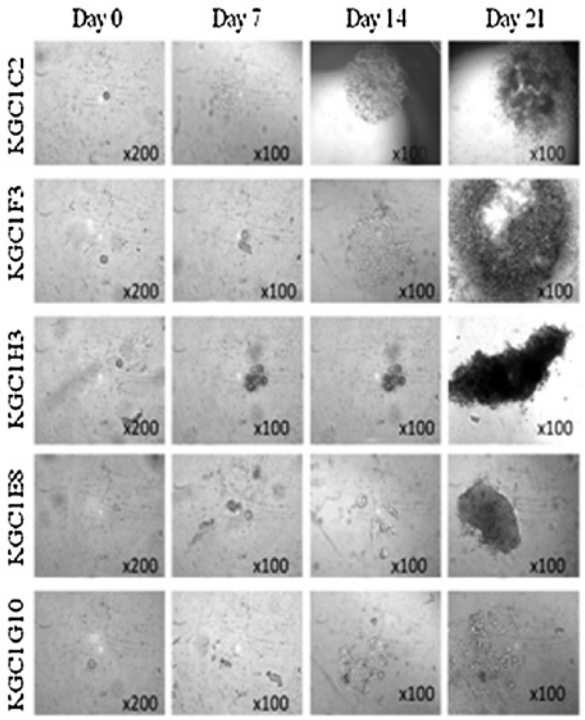

Figure 1. *In vitro* single cell culture and cell growth assay. A clinical sample was obtained from a patient with peritoneum dissemination. GC cells were isolated and sorted into Matrigel-coated 96-well cell culture clusters by flow cytometry. Microscopic colonies began to appear after day 7 of culture (magnification, x100).

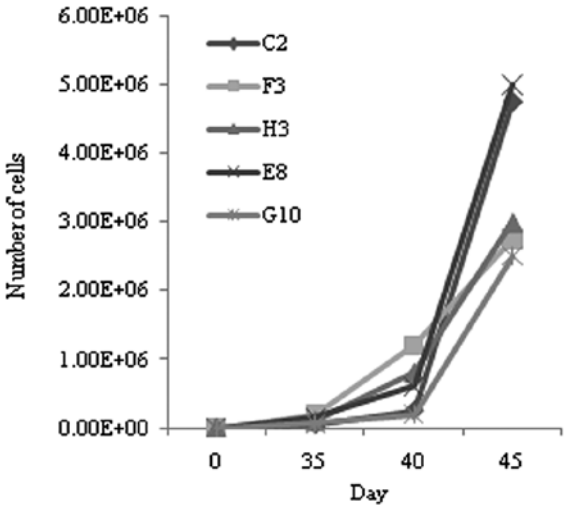

Figure 2. Cell proliferation activity. After culture for 35, 40, or 45 days, cells were removed from the culture dish using trypsin and EDTA, pelleted by centrifugation and resuspended with HBSS. The cells were stained with 0.5% trypan blue and counted.

monoclonal antibodies against human CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1) subunits. Notably, the expression levels of 1α integrin subunits were much higher compared to the other integrins evaluated (Table I). These results suggest that the

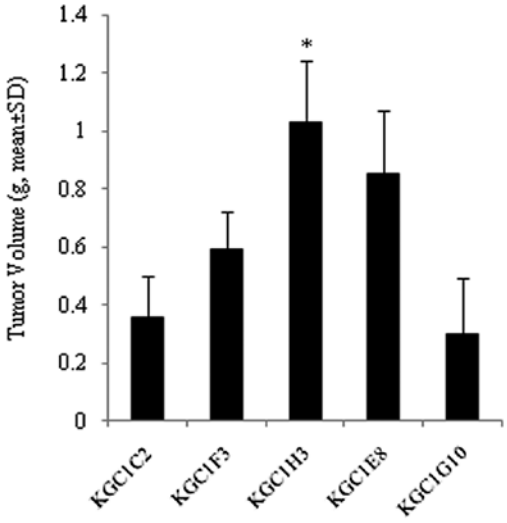

Figure 3. Assay of tumorigenesis *in vivo*. Peritoneal tumorigenic capability was examined using SCID (C.B-17/lcr-scid/scidJcl) mice, into which $1.0x10^5$ cells were injected, representative. Values are expressed as the means ± SD. $^*p<0.05$.

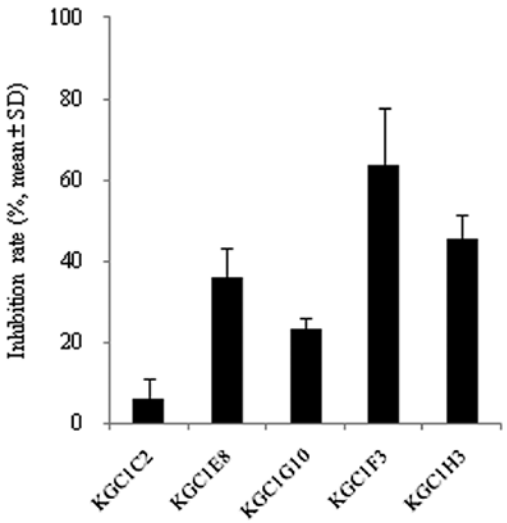

Figure 4. Effects of antibody against integrin α1 on the adhesion of GC clones. Each clone was treated with either anti-CD49a (integrin α1) antibody or PBS (control) for 30 min at 37˚C. Cells were allowed to adhere to each well for 12 h at 37˚C and were then gently washed twice in PBS. Cells that had adhered were quantified using the WST-8 assay. Inhibition rates were determined relative to the OD value of the controls.

expression of integrin α1 subunit is correlated with adhesion in the peritoneum.

Suppression of adhesion by anti-integrin antibody. To test the ability of anti-CD49a (integrin α1) antibodies to interfere with cell adhesion *in vitro*, we examined the effect of anti-CD49a on the adhesion ability of the cloned cells. The inhibition ratio of adhesion was 63.8% in the KGCF3 clone, which appeared to be effectively inhibited by antibodies against integrin α1 ($P<0.05$) (Fig. 4). This result showed that the adhesive ability of GC cells may be inhibited or controlled by the anti-CD49a (integrin α1) antibody.

Table I. Flow cytometric analysis of integrin subunit expression in each clone derived from a GC patient with peritoneal dissemination.

Integrin subunit[a]	KGC1C2	KGC1F3	KGC1H3	KGC1E8	KGC1G10
α1	18.6±1.23	9.49±0.07	83.5±6.36	36.4±5.35	28.2±5.44
2	6.07±0.37	4.00±0.36	81.7±5.39	26.6±4.01	18.7±1.08
α3	0.03±0.04	0.00	0.14±0.01	0.67±0.07	0.32±0.03
α4	1.66±0.14	5.81±0.17	45.7±5.72	44.2±4.68	12.2±0.61
α5	0.08±0.01	0.27±0.03	8.87±0.39	8.72±0.25	0.97±0.15
α6	0.06±0.01	0.53±0.07	0.39±0.04	0.77±0.08	0.98±0.18
β1	10.2±0.36	3.18±0.24	81.5±9.61	26.5±3.73	19.5±0.82

Cells were treated with monoclonal antibodies against CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1). Expression levels were estimated using the fluorescence intensity of FITC or PE in the samples. [a]Expressed as percentage of expression (mean ± SD).

Discussion

We isolated GC primary cells with adhesion capacity from a patient with peritoneal dissemination using single cell sorting methods, and established 5 clones that were found to adhere to the peritoneum *in vivo* as well as to the extracellular matrix (ECM) *in vitro*. The attachment of GC cells to the peritoneum is an important step in the initial phases of metastatic dissemination (10-12). The results of the present study showed that only a small subset of GC cells have potential metastatic ability in the peritoneum.

To elucidate the mechanism of peritoneal dissemination, we analyzed expression of integrins (α1-6 and β1), which act as adhesion molecules. Flow cytometric analysis demonstrated that the level of expression of the α1 subunit paired with β1 subunits was higher compared to other integrins in all of the clones. In addition, the results of the transplantation assay indicated that the integrin expression level was inversely correlated with adhesion.

Integrin α1 (CD49a) is also known as very late antigen 1α subunit (VLA-1α). Integrin α1 associates with integrin β1 subunits (CD29) to form α1/β1 heterodimers, and serves as a receptor for collagen and laminin-1 (13,14). Integrin α1 has been reported to play a role in cell attachment (15-17). Conversely, integrin α1 is expressed at low levels in human breast cancer cell lines (18) and is downregulated in breast and ovarian cancer (19), suggesting that its loss might be associated with malignancy. Furthermore, integrin α1 can negatively regulate EGFR signaling in prostate and cervical cancer cells (20) as well as in mesangial cells (21). In the present study, a cell adhesion blocking assay showed that anti-CD49a (integrin α1 subunit) mAb significantly reduced the adhesiveness of the clones compared to the controls. These data indicate that the α1 subunit is a molecule that is significantly involved in the adhesion of cancer cells to the peritoneum.

Peritoneal dissemination has often been detected in recurrent GC and results from a multistep phenomenon that includes detachment of malignant cells from the primary tumor, transfer to the peritoneal cavity, attachment to the peritoneum, ECM degradation, and migration of adhesive tumor cells into surrounding tissues (22). In a previous study, the expression of integrins in tumor invasion and metastasis, particularly in liver metastasis, was reported (23). Cell adhesion molecules are necessary for many different cellular functions including cell signaling, migration and apoptosis as well as maturation or formation of tight junctions (24-27). By contrast, patients known to have free GC cells in the peritoneal cavity have not always developed peritoneal implantation. It may be that only a minority of cancer cells possess metastatic capability in GC with peritoneal dissemination, so understanding the adhesion of GC cells is important to fully elucidate GC cell functions (28-30).

In conclusion, the interaction of GC cells with the ECM appears to be of considerable importance in peritoneal metastasis as cancer cells that exfoliated into the abdominal cavity were found to attach initially to the mesothelial lining, followed by their invasion through the intercellular gaps of mesothelial cells (31). This study indicated that the adhesion of integrin α1-positive GC cells to the ECM is a critical process in peritoneal dissemination. The clones derived from this patient are available for research and may be useful for defining the biological phenomena involved in GC in metastasis.

Acknowledgements

We thank Sadafumi Suzuki for his invaluable FACS expertise. This work was supported by a Grant-in-Aid from the 21st Century COE program of the Ministry of Education, Science, and Culture of Japan to Keio University and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

1. Jemal A, Siegel R, Ward E, *et al*: Cancer statistics 2008. CA Cancer J Clin 58: 71-96, 2008.
2. Jemal, A, Murray, T, Samuels A, *et al*: Cancer statistics 2003. CA Cancer J Clin 53: 535-562, 2003.
3. Yamamoto M, Matsuyama A, Kameyama T, *et al*: Prognostic re-evaluation of peritoneal lavage cytology in Japanese patients with gastric carcinoma. Hepatogastroenterology 56: 261-265, 2009.

4. Nakashio T, Narita T, Akiyama S, *et al*: Adhesion molecules and TGF-β1 are involved in the peritoneal dissemination of NUGC-4 human gastric cancer cells. Int J Cancer 70: 612-618, 1997.
5. Matsuoka T, Yashiro M, Nishimura S, *et al*: Increased expression of α2β1-integrin in the peritoneal dissemination of human gastric carcinoma. Int J Mol Med 5: 21-25, 2000.
6. Ruoslahti E: Fibronectin and its integrin receptors in cancer. Adv Cancer Res 76: 1-20, 1999.
7. Orr FW, Wang HH, Lafrenie RM, Scherbarth S and Nance DM: Interactions between cancer cells and the endothelium in metastasis. J Pathol 190: 310-329, 2000.
8. Holly SP, Larson MK and Parise LV: Multiple roles of integrins in cell motility. Exp Cell Res 261: 69-74, 2000.
9 Felding-Habermann B: Integrin adhesion receptors in tumor metastasis. Clin Ex Metastasis 20: 203-213, 2003.
10. Kawajiri H, Yashiro M, Hirakawa K, *et al*: A novel transforming growth factor β receptor kinase inhibitor, A-77, prevents the peritoneal dissemination of scirrhous gastric carcinoma. Clin Cancer Res 14: 2850-2860, 2008.
11. Sawada K, Mitra AK, Radjabi AR, *et al*: Loss of E-cadherin promotes ovarian cancer metastasis via α 5-integrin, which is a therapeutic target. Cancer Res 68: 2329-2339, 2008.
12. Kenny HA, Kaur S, Coussens LM and Lengyel E: The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. J Clin Invest 118: 1367-1379, 2008.
13. Rentala S, Yalavarthy PD and Mangamoori LN: α1 and β1 integrins enhance the homing and differentiation of cultured prostate cancer stem cells. Asian J Androl 12: 548-555, 2010.
14. Lindberg K, Ström A, Lock JG, Gustafsson JA, Haldosén LA and Helguero LA: Expression of estrogen receptor beta increases integrin α1 and integrin β1 levels and enhances adhesion of breast cancer cells. J Cell Physiol 222: 156-167, 2010.
15. Schlossman SL, Boumsell W. Gilks, *et al* (eds): Leucocyte Typing V: White Cell Differentiation Antigens. New York, NY, Oxford University Press, 1995.
16. Kishimoto T, von dem Borne AEG, Goyert SM, *et al* (eds): Leucocyte Typing VI: White Cell Differentiation Antigens. New York, NY, Garland Publishing, 1997.
17. Barclay AN, Brown MH, Law SKA, *et al*: The Leukocyte Antigen Facts Book. 2nd edition. Academic Press, San Diego, pp149-151, 1997.
18. Shaw LM: Integrin function in breast carcinoma progression. J Mammary Gland Biol Neoplasia 4: 367-376, 1999.
19. Su Al, Welsh JB, Hampton GM, *et al*: Molecular classification of human carcinomas by use of gene expression signatures. Cancer Res 61: 7388-7393, 2001.
20. Mattila E, Pellinen T, Nevo J, Vuoriluoto K, Arjonen A and Ivaska J: Negative regulation of EGFR signalling through integrin-α1β1-mediated activation of protein tyrosine phosphatase TCPTP. Nat Cell Biol 7: 78-85, 2005.
21. ChenX, Abair TD, Ibanez MR, *et al*: Integrin α1β1 controls reactive oxygen species synthesis by negatively regulating epidermal growth factor receptor-mediated Rac activation. Mol Cell Biol 27: 3313-3326, 2007.
22. Liotta LA, Rao CN and Wewer UM: Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 55: 1037-1057, 1986.
23. Yashiro M, Chung YS, Nishimura S, Inoue T and Sowa M: Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. Clin Exp Metastasis 14: 43-54, 1996.
24. Sriramarao P, Steffner P Gehlsen KR: Biochemical evidence for a hemophilic interaction of the α3β l integrin. J Biol Chem 268: 22036-22041, 1993.
25. Tonutti L, Bononi E, Paris L, Breillout F, Corvaia N, Bailly C and Bazzoni G: Effect of microtubule-targeting drugs on cell-cell and cell-matrix junctions in tumor epithelial cells. Anticancer Drugs 22: 234-244, 2011
26. Gondi CS, Gogineni VR, Chetty C, Dasari VR, Gorantla B, Gujrati M, Dinh DH and Rao JS: Induction of apoptosis in glioma cells requires cell-to-cell contact with human umbilical cord blood stem cells. Int J Oncol 36: 1165-1173, 2010.
27. Mierke CT, Frey B, Fellner M, Herrmann M and Fabry B: Integrin α5β1 facilitates cancer cell invasion through enhanced contractile forces. J Cell Sci 124: 369-383, 2011
28. Tsuji T, Kawada Y, Kai-Murozono M, *et al*: Regulation of melanoma cell migration and invasion by laminin-5 and α3β1 integrin (VLA-3). Clin Exp Metastasis 19: 127-134, 2002.
29. Izumi Y, Taniuchi Y, Tsuji T, *et al*: Characterization of human colon carcinoma variant cells selected for sialyl LeX carbohydrate antigen liver colonization and adhesion to vascular endothelial cells. Exp Cell Res 216: 215-221, 1995.
30. Galliano MF, Aberdam D, Aguzzi A, Ortonne JP and Menehguzzi G: Cloning and complete primary structure of the mouse laminin β3 chain Distinct expression pattern of the laminin α 3A and α 3B chain isoforms. J Biol Chem 270: 21820-21826, 1995.
31. Lv ZD, Na D, Liu FN, *et al*: Induction of gastric cancer cell adhesion through transforming growth factor-β1-mediated peritoneal fibrosis. J Exp Clin Cancer Res 29: 139, 2010.