

Hamanaka W, Motoi N, Ishikawa S, Ushijima M, Inamura K, Hatano S, Uehara H, Okumura S, Nakagawa K, Nishio M, Horai T, Aburatani H, Matsuura M, Iwasaki A and Ishikawa Y	A subset of small cell lung cancer with low neuroendocrine expression and good prognosis: a comparison study of surgical cases to inoperable cases with biopsy	Hum Pathol	In press		2014
Asada K, Ando T, Niwa T, Nanjo S, Watanabe N, Okochi-Takada E, Yoshida T, Miyamoto K, Enomoto S, Ichinose M, Tsukamoto T, Ito S, Tatematsu M, Sugiyama T and Ushijima T	FHL1 on chromosome X is a single-hit gastrointestinal tumor-suppressor gene and contributes to the formation of an epigenetic field defect	Oncogene	32	2140-2149	2013
Okochi-Takada E, Hattori N, Tsukamoto T, Miyamoto K, Ando T, Ito S, Yamamura Y, Wakabayashi M, Nobeyama Y and Ushijima T	ANGPTL4 is a secreted tumor suppressor that inhibits angiogenesis	Oncogene	In press		2014
Daino K, Imaoka T, Morioka T, Tani S, Iizuka D, Nishimura M and Shimada Y	Loss of the BRCA1-interacting helicase BRIP1 results in abnormal mammary acinar morphogenesis	PLoS ONE	8	E74013	2013
Shimura T, Hamada N, Sasatani M, Kamiya K, Kunugita N	Nuclear accumulation of cyclin D1 following long-term tractionated exposures to low-dose ionizing radiation in normal human diploid cells	Cell Cycle	In press		2014
Gu Y, Wang J, Li S, Kamiya K, Chen X and Zhou P	Determination of the biochemical properties of full-length human PIF1 ATPase	Prion	7	341-347	2013

Tomida J, Itaya A, Shigechi T, Unno J, Uchida E, Ikura M, Masuda Y, Matsuda S, Adachi J, Kobayashi M, Meetei AR, Maehara Y, Yamamoto K, <u>Kamiya K</u> , Matsuura A, Matsuda T, Ikura T, Ishiai M and Takata M	A novel interplay between the Fanconi anemia core complex and ATR-ATRIP kinase during DNA cross-link repair	Nucleic Acids Res	41	6930-6941	2013
Gent SC, Fujii Y, Maeda J, Genet MD, <u>Miyagawa K</u> and Kato TA	Hyperthermia inhibits homologous recombination and sensitizes cells to ionizing radiation in a time- and temperature-dependent manner	J Cell Physiol	228	1473-1481	2013
Enomoto A, Fukasawa T, Takamatsu N, Ito M, Morita A, Hosoi Y and <u>Miyagawa K</u>	The HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin modulates radiosensitivity by downregulating serine/threonine kinase 38 via Sp1 inhibition	Eur J Cancer	49	3547-3558	2013
Fujii Y, Genet MD, Roybal EJ, Kubota N, Okayasu R, <u>Miyagawa K</u> , Fujimori A and Kato TA	Comparison of the bromodeoxyuridine-mediated sensitization effects between low-LET and high-LET ionizing radiation on DNA double-strand breaks	Oncol Rep	29	2133-2139	2013
Ohishi W, Cologne JB, Fujiwara S, Suzuki G, <u>Hayashi T</u> , Niwa Y, Akahoshi M, Ueda K, Tsuge M and Chayama K	Serum Interleukin-6 Associated with Hepatocellular Carcinoma Risk: A Nested Case-Control Study	Int J Cancer	134	154-163	2013
<u>Hayashi T</u> , Ito R, Cologne J, Maki M, Morishita Y, Nagamura H, Sasaki K, Hayashi I, Imai K, Yoshida K, Kajimura J, Kyoizumi S, <u>Kusunoki Y</u> , Ohishi W, Fujiwara S, Akahoshi M and Nakachi K	Effects of <i>IL-10</i> haplotype and atomic-bomb radiation exposure on gastric cancer risk	Radiat Res	180	60-69	2013

Yoshida K, Kusunoki Y, Cologne JB, Kyoizumi S, Maki M, Imai K, Nakachi K and Hayashi T	Radiation-dose response of glycoprotein A somatic mutation in erythrocytes associated with gene polymorphisms of p53 binding protein 1	Mutat Res	755	49-54	2013
Kyoizumi S, Kubo Y, Kajimura J, Yoshida K, Imai K, Hayashi T, Nakachi K, Young LF, Moore MA, van den Brink MRM and Kusunoki Y	Age-associated changes in the differentiation potentials of human circulating hematopoietic progenitors to T- or NK- lineage cells	J Immunol	190	6164-6172	2013

Stroma-directed imatinib therapy impairs the tumor-promoting effect of bone marrow-derived mesenchymal stem cells in an orthotopic transplantation model of colon cancer

Kei Shinagawa¹, Yasuhiko Kitadai¹, Miwako Tanaka¹, Tomonori Sumida¹, Mieko Onoyama¹, Mayu Ohnishi¹, Eiji Ohara¹, Yukihito Higashi², Shinji Tanaka³, Wataru Yasui⁴ and Kazuaki Chayama¹

¹ Department of Gastroenterology and Metabolism, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

² Department of Cardiovascular Physiology and Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

³ Department of Endoscopy, Hiroshima University Hospital, Hiroshima, Japan

⁴ Department of Molecular Pathology, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Bone marrow-derived mesenchymal stem cells (MSCs) are reported to contribute to formation of tumor-promoting stromal cells. We reported recently that, in an orthotopic nude mice model of colon cancer, MSCs traveled to tumor stroma, where they differentiated into carcinoma-associated fibroblast (CAF)-like cells. We also found that CAFs express platelet-derived growth factor receptor (PDGFR) at a high level and that imatinib therapy targeting PDGFR in CAFs inhibits growth and metastasis of human colon cancer. These findings led us to examine whether the tumor-promoting effect of MSCs is impaired by blockade of PDGFR signaling achieved with imatinib. Orthotopic transplantation and splenic injection of human MSCs along with KM12SM human colon cancer cells, in comparison with transplantation of KM12SM cells alone, resulted in significantly greater promotion of tumor growth and liver metastasis. The KM12SM + MSC xenograft enhanced cell proliferation and angiogenesis and inhibited tumor cell apoptosis. When tumor-bearing animals were treated with imatinib, there was no significant increase in primary tumor volume or total volume of liver metastases, despite the KM12SM+MSC xenograft, and survival in the mixed-cell group was prolonged by imatinib treatment. Moreover, the ability of MSCs to migrate to tumor stroma was impaired, and the number of MSCs surviving in the tumor microenvironment was significantly decreased. In *in vitro* experiments, treatment with imatinib inhibited migration of MSCs. Our data suggest that blockade of PDGF signaling pathways influences the interaction between bone marrow-derived MSCs and tumor cells in the tumor microenvironment and, hence, inhibits the progressive growth of colon cancer.

Key words: mesenchymal stem cells, carcinoma-associated fibroblasts, orthotopic colon cancer model, platelet-derived growth factor receptor, imatinib

Abbreviations: CAF: carcinoma-associated fibroblast; DAPI: 4',6-diamidino-2-phenylindole; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; HBSS: Hanks' balanced salt solution; MSC: mesenchymal stem cell; MVA: microvessel area; PCNA: proliferating cell nuclear antigen; PCNA-LI: PCNA labeling index; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor

Grant sponsors: Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan, Ministry of Health, Labor and Welfare of Japan

DOI: 10.1002/ijc.27735

History: Received 12 Oct 2011; Accepted 28 Jun 2012; Online 23 Jul 2012

Correspondence to: Yasuhiko Kitadai, Department of Gastroenterology and Metabolism, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-Ku, Hiroshima 734-8551, Japan, Tel.: +81-82-257-5191, Fax: +81-82-257-5194, E-mail: kitadai@hiroshima-u.ac.jp

Many recent reports have referred to tumor-stromal interactions as essential events for tumor progression.¹⁻³ Of the constituents of tumor stroma, it has become clear that activated fibroblasts, called carcinoma-associated fibroblasts (CAFs), promote tumor growth and metastasis.⁴⁻⁸ We previously reported that the platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) signaling pathway plays a critical role in the growth and metastasis of gastric and colon cancers in orthotopic transplantation models.^{4,8} Tumor cells express PDGFs, and PDGFRs are expressed by CAFs. The origin of CAFs is not fully understood, but they may arise from fibroblasts residing in local tissues,⁷ periaortic cells including pericytes and vascular smooth muscle cells,⁹ endothelial cells,¹⁰ and bone marrow-derived cells including various stem cells.¹¹

Mesenchymal stem cells (MSCs) can be recruited from bone marrow to inflamed or damaged tissues by local endocrine signals, resulting in tissue regeneration.^{12,13} Although MSCs are known to migrate to a variety of tumors, such as melanomas,¹⁴ gliomas,^{15,16} and colon,^{17,18} pancreatic,^{19,20} and breast cancers,²¹⁻²³ the mechanisms of the migratory effect are very complex, and the key factors have not been identified. Experimental co-injection of tumor cells and MSCs has revealed

What's new?

Platelet-derived growth factor (PDGF) signaling pathways are crucial for the migration of metastasis-promoting mesenchymal stem cells (MSCs) to the tumor microenvironment in human colon cancer. In this study employing orthotopic transplantation of human MSCs and colon cancer cells in mice, inhibition of PDGF signaling with the drug imatinib was found to influence interactions between bone marrow-derived MSCs and tumor cells, suggesting that stroma-directed imatinib therapy could be effective against colon cancer progression.

that MSCs promote tumor growth and metastasis^{22,24–33} and are implicated in tumor invasion and angiogenesis,^{17,24,30,31} immunosuppression,^{25,26} inhibition of apoptosis,²⁹ and formation of a cancer stem cell niche.³² Our recent study showed that MSCs accumulating in the tumor microenvironment differentiate into CAFs and promote growth and metastasis of colon cancer.³⁴ Very recently, it was shown in a mouse model of gastric cancer that at least 20% of CAFs originate from bone marrow-derived MSCs.³⁵ PDGF signaling pathways are known to be crucial to migration and survival of MSCs.^{15,19,36}

In an orthotopic transplantation model of human colon cancer, we examined the effect of imatinib, known to be a potent tyrosine kinase inhibitor of PDGFR,³⁷ on migration and survival of MSCs in the tumor microenvironment and on promotion of tumor growth and metastasis by MSCs. We found that treatment with imatinib inhibited tumor-MSC interactions and modified tumor-supporting stroma.

Material and Methods**Cell culture**

Human MSCs were harvested from the iliac crest and isolated and cultured according to a protocol approved by the Ethics Committee of Hiroshima University Graduate School of Medicine, as described previously.^{34,38} The capacity for chondrogenic, adipogenic, and osteogenic differentiation was confirmed with the use of a Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, MN). Cell surface antigens on the MSCs were analyzed by fluorescence-activated cell sorting, and it was confirmed that the cells were positive for CD29, CD44, CD73, CD90, CD105, CD166, and MHC-DR but negative for CD14, CD34, and Flk-1, as described previously.³⁸ MSCs were fluorescently labeled with red fluorescent linker dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma, Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions.

Human colon cancer cell line KM12SM³⁹ was kindly gifted by Dr. Isaiah J. Fidler (University of Texas). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine, and a penicillin-streptomycin solution. The cultures were maintained for no longer than 12 weeks after recovery of cells from frozen stock.

Animals and transplantation of tumor cells

Female athymic BALB/c nude mice were obtained from Charles River Japan (Tokyo, Japan). The mice were main-

tained under specific pathogen-free conditions and used at 8 weeks of age. The study was carried out after permission was granted by the Committee on Animal Experimentation of Hiroshima University.

To produce cecal tumors, KM12SM cells in 50 μ L of Hanks' balanced salt solution (HBSS) were injected into the cecal wall of nude mice under observation with a zoom stereomicroscope. To produce experimental liver metastases, the cells were injected into the spleen of nude mice as described previously.³⁹

Anticancer drug

Imatinib (imatinib mesylate or Gleevec; Novartis Pharma AG, Basel, Switzerland) was diluted in sterile water for administration by oral gavage.

Effect of imatinib on tumor tropism of MSCs in vivo

Because we have found that MSCs migrate to orthotopic and metastatic tumor sites,³⁴ we examined whether imatinib affects the tumor tropism of MSCs. We injected 0.5×10^6 KM12SM cells into the cecal wall in six mice on day 0. One week after the orthotopic implantation of tumor cells (on day 7), mice were randomized into two groups: one given water daily (control group) and the other given 50 mg/kg/day imatinib by oral gavage once daily until day 28. Three weeks after tumor cell transplantation (on day 21), each mouse was injected with 1.0×10^6 PKH26-labeled MSCs (in 200 μ L of HBSS) in the tail vein. One week after this injection (on day 28), the mice were killed and necropsied.

KM12SM cells (0.5×10^6) were transplanted into the spleen of six additional mice on day 0. One week after tumor cell transplantation (on day 7), mice were randomized into two groups: one given water daily (control group) and the other given 50 mg/kg/day imatinib by oral gavage once daily until day 28. At the same time (on day 7), each mouse was injected with 1.0×10^6 PKH26-labeled MSCs (in 200 μ L of HBSS) in the tail vein. Three weeks after this injection (on day 28), the mice were killed.

Necropsy procedures and histologic studies

Mice bearing orthotopic tumors were killed with an overdose of methophane. Necropsy was performed, after which tumors were excised and measured. For immunohistochemical and hematoxylin and eosin (H&E) staining, one part of the tumor tissue was fixed in formalin-free IHC Zinc Fixative (Phar-Mingen, San Diego, CA) and embedded in paraffin, and the

other part was embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at -80°C . Sections of PKH26-labeled tissues were analyzed by means of fluorescence confocal microscopy.

Effect of imatinib on interactions between tumor cells and MSCs in orthotopic colon tumor

To examine the effect of imatinib on the tumor-MSc interactions in orthotopic colon tumors, co-injection studies were carried out. Mice were divided into four groups according to the kind(s) of implanted cells and the treatment received: (i) KM12SM cells alone and daily administration of water by oral gavage (0.5×10^6 , $n = 19$), (ii) KM12SM cells mixed with MSCs and daily administration of water by oral gavage (KM12SM:MSC ratio, $0.5 \times 10^6:1.0 \times 10^6$ [1:2]; $n = 21$), (iii) KM12SM cells alone and daily oral gavage 50 mg/kg imatinib (0.5×10^6 , $n = 8$), or (iv) KM12SM cells mixed with MSCs and daily oral gavage 50 mg/kg imatinib [KM12SM:MSC ratio, $0.5 \times 10^6:1.0 \times 10^6$, [1:2]; $n = 12$).

Treatment with imatinib was begun 1 week after intracecal transplantation of cells and lasted 5 weeks. Surviving mice were then killed and necropsied [(i) $n = 18$, (ii) $n = 10$, (iii) $n = 8$, (iv) $n = 10$]. Tumor volume, incidence of liver metastasis, and survival were evaluated. Tumor volume (V) was calculated as $V = (1/2)ab^2$, where a is the largest diameter of the tumor and b is the smallest diameter.

Quantification of the remaining MSCs and phosphorylation of PDGFR- β in MSCs coimplanted with tumor cells in vivo

To evaluate how many MSCs coimplanted with tumor cells remained at the orthotopic tumor site, and whether PDGFR- β in MSCs was phosphorylated, KM12SM cells mixed with PKH26-labeled MSCs ($0.5 \times 10^6:1.0 \times 10^6$ [1:2]) were injected into the cecal wall in three mice. Three weeks after intracecal transplantation, tumors were excised and analyzed by means of fluorescence confocal microscopy to detect PKH26-labeled MSCs and to confirm phosphorylation of PDGFR- β in MSCs by immunofluorescence staining as described previously.³⁴ For quantification of the remaining MSCs, 10 random fields were captured with a $20\times$ objective lens, and red fluorescence areas were measured. The areas were calculated with the use of NIH ImageJ software.

Effect of imatinib on interactions between tumor cells and MSCs in a liver metastasis model

To evaluate the effect of imatinib on the tumor-MSc interactions in liver metastases, we developed a model of liver metastasis by injecting tumor cells into mice spleen as described previously.³⁹ Mice were divided into four groups according to the kind(s) of implanted cells and the treatment received ($n = 10$ in each group): (i) KM12SM cells alone and daily administration of water by oral gavage (0.5×10^6), (ii) KM12SM cells mixed with PKH26-labeled MSCs and daily administration of water by oral gavage (KM12SM:PKH26-labeled MSC ratio, $0.5 \times 10^6:1.0 \times 10^6$ [1:2]), (iii) KM12SM

cells alone and daily oral gavage 50 mg/kg imatinib (0.5×10^6), (iv) KM12SM cells mixed with PKH26-labeled MSCs and daily oral gavage 50 mg/kg imatinib (KM12SM:PKH26-labeled MSCs, $0.5 \times 10^6:1.0 \times 10^6$ [1:2]).

One week after intrasplenic transplantation of cells, mice were treated with imatinib for 3 weeks; surviving mice were then killed and necropsied ($n = 10$ in each group). Tumor nodules on the liver surface were counted macroscopically. Metastatic tumors were analyzed by means of fluorescence confocal microscopy to detect PKH26-labeled MSCs.

Antibodies

Rabbit anti-PDGFR- β and rabbit antiphosphorylated PDGFR- β (p-PDGFR- β) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse antiproliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark), and rat anti-mouse CD31 (BD Pharmingen, San Diego, CA) were used as primary antibodies. Biotinylated rabbit anti-rat IgG (Dako) and biotinylated goat anti-mouse IgG (Dako) were used as secondary antibodies. Alexa Fluor[®] 488-labeled goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) was used as fluorescent secondary antibody.

Immunohistochemical determination of PCNA, apoptotic cells, and microvessel area (MVA)

Paraffin-embedded tissues cut into $4 \mu\text{m}$ sections and frozen tissues cut into $8 \mu\text{m}$ sections were used for immunohistochemical identification of PCNA and CD31, respectively. Immunohistochemistry was performed as described previously.⁴⁰ The PCNA labeling index (PCNA-LI) was taken as the ratio of positively stained tumor cells to the total tumor cells and expressed as a percentage for each case.³⁴ Apoptotic cells in paraffin sections of KM12SM tumors were detected by terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The apoptotic index (AI) was taken as the ratio of positively stained tumor cells and apoptotic bodies to all tumor cells and expressed as a percentage for each case.³⁴ Angiogenic activity was evaluated according to the areas of microvessels stained with anti-mouse CD31 antibody. For quantification of the MVA, 10 random fields at $100\times$ magnification were captured for each tumor, and the outline of each microvessel including a lumen was manually traced. The area was then calculated with the use of NIH ImageJ software.

Confocal microscopy

Confocal fluorescence images were captured with a $20\times$ or $40\times$ objective lens on a Zeiss LSM 510 laser scanning microscopy system (Carl Zeiss Inc., Thornwood, NY) equipped with a motorized Axioplan microscope, argon laser (458/477/488/514 nm, 30 mW), HeNe laser (543 nm, 1 mW), HeNe laser (633 nm, 5 mW), LSM 510 control and image acquisition software, and appropriate filters (Chroma Technology Corp., Brattleboro, VT). Confocal images were exported to Adobe Photoshop software, and image montages were prepared.

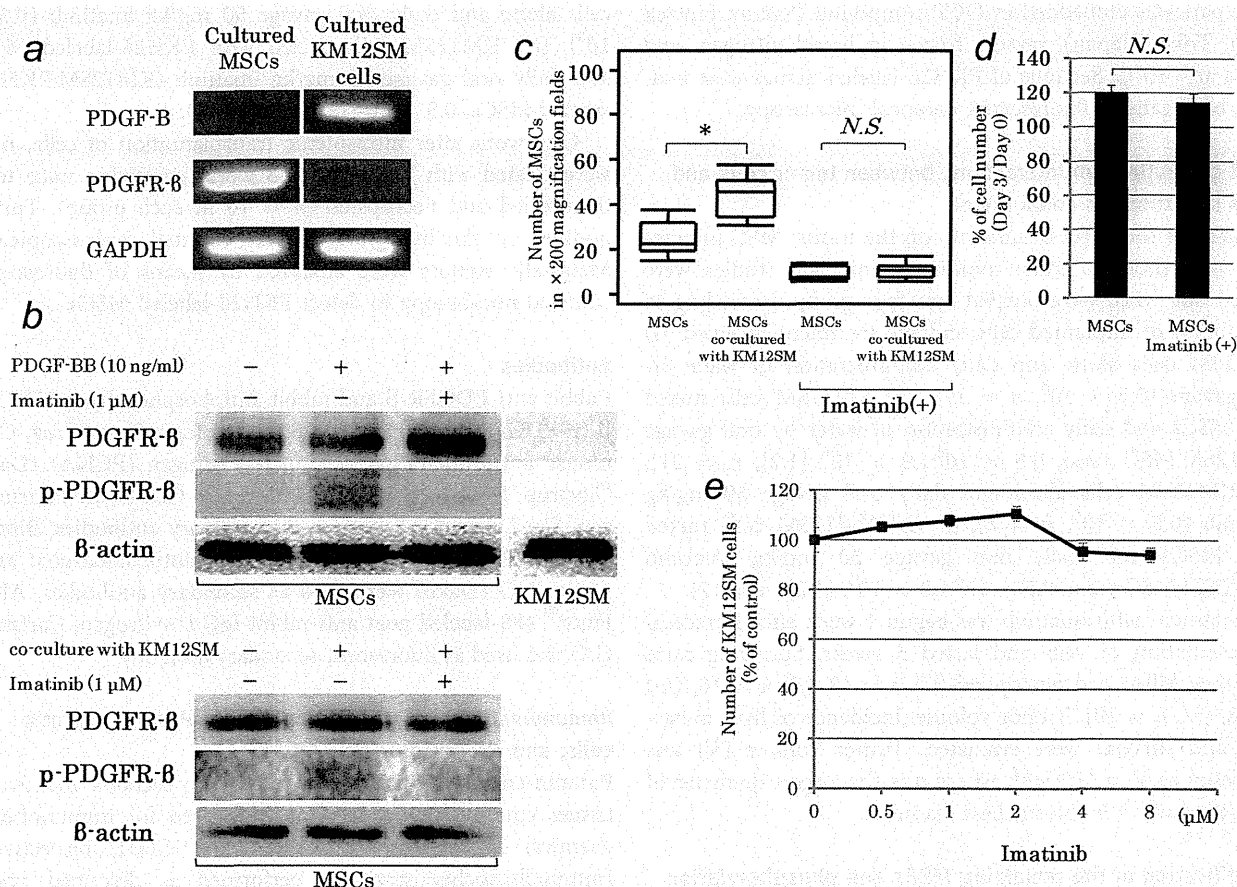


Figure 1. Effect of imatinib on KM12SM cells and MSCs *in vitro*. (a) Reverse transcription-polymerase chain reaction and (b) Western blot analyses for expression of PDGF-B and PDGFR-β in MSCs and KM12SM cells. PDGFR-β in MSCs was phosphorylated by coculture with KM12SM cells. Phosphorylation of PDGFR-β in MSCs was inhibited by 1 μM imatinib. (c) Migratory ability of MSCs evaluated by transwell assay. The tumor tropism of MSCs was impaired by imatinib treatment, but 1 μM imatinib did not affect proliferation of MSCs (d). (e) Dose-response curve for imatinib and its growth inhibiting effect in KM12SM cells. * $p < 0.05$, bars: SE.

Migration assay

The migratory ability of MSCs was assayed with the use of a 24-well microchamber plate with uncoated inserts (8 μm pore size, Corning Costar, Tokyo, Japan). Either 2.0×10^4 KM12SM cells in DMEM with 0.5% FBS or medium alone was plated into the lower chambers. After 24 hr of incubation at 37°C, upper chambers containing 2.0×10^4 MSCs in DMEM with 0.5% FBS or 0.5% FBS and 1 μM imatinib, were set into the lower chambers. Three wells were used for each experiment. After 28 hr of incubation at 37°C, inserts were fixed with 10% buffered formalin solution and stained with hematoxylin. The cells on the upper surface of the membranes were removed with cotton swabs. The number of migrating cells was counted in three random fields per filter at 200× magnification.

Proliferation assay

The effect of imatinib on the proliferation of KM12SM cells was analyzed. KM12SM (4.0×10^5) cells were seeded in a 6-well plate in DMEM with 0.5% FBS and 0, 0.5, 2.0, 4.0, or 8.0 μM imatinib. The medium was changed every day, and

the number of cells was counted on days 3 and 6 ($n = 6$ in each group).

In vitro growth of KM12SM cells was measured with a Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions. Cells were seeded in a 96-well plate at a density of 0.5×10^4 cells/well and incubated overnight in 200 μL culture medium containing 10% FBS. After incubation for 24 hr, cells were cultured in serum-free culture medium containing 10 μM BrdU with or without imatinib for 24 hr, and cell proliferation was measured in a plate reader (Microplate Manager 5.2.1, BIO-RAD, Hercules, CA) at 450 nm.

Western blot analysis

MSCs were cultured in serum-free culture medium for 1 hr and then stimulated with 10 ng/mL PDGF-BB for 10 min. Furthermore, to determine whether PDGFR-β in MSCs is phosphorylated by co-culture with KM12SM cells, 2.0×10^5 MSCs in serum-free culture medium were plated into the lower chambers, and 2.0×10^5 KM12SM cells were cultured in a 6-well microchamber plate with uncoated inserts (0.4

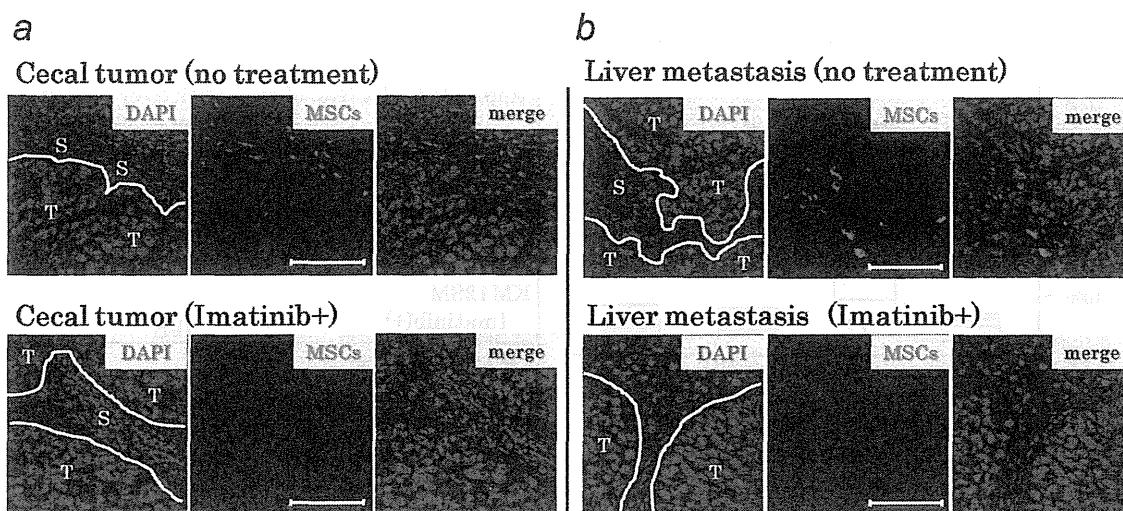


Figure 2. Migration of MSCs to orthotopic colon tumors and metastatic liver tumors. Systemically administered and PKH26-labeled MSCs (red) migrated to (a) the stroma of orthotopic colon tumors and (b) metastatic liver tumors. Imatinib treatment inhibited tumor-homing ability of MSCs *in vivo*. 4',6-diamidino-2-phenylindole (DAPI) staining for cell nuclei (blue) shows outline of the stroma within tumor tissue. T: tumor nest, S: stroma, scale bars: 100 μm .

μm pore size, Corning Costar). After 24 hr, upper chambers containing KM12SM cells were set into the lower chambers in serum-free culture medium with or without 1 μM imatinib. After 1 hr of incubation at 37°C, inserts were removed. After 3 washes with cold phosphate-buffered saline containing 1 mmol/L sodium orthovanadate, MSCs were lysed. Unstimulated KM12SM cells were also lysed. Proteins (total protein 20 μg) were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). The immune complexes were visualized by enhanced chemiluminescence with an ECL Plus Kit (GE Healthcare, Buckinghamshire, UK).

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from gastric carcinoma cell lines with an RNeasy Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was performed with the isolated RNA (1 μg). cDNA was generated from 1 μg of total RNA with a first-strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, UK). Semiquantitative RT-PCR was performed with an AmpliTag Gold Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. RT-PCR reactions without reverse transcription showed no specific bands. Respective primer sequences, annealing temperatures, and PCR cycles were as follows: PDGFR- β forward, AGC TACCCCTCAAGGAATCATAG and PDGFR- β reverse, CTC TGGTGGATGGATTAAGACTG (PDGFR- β PCR product, 376 bp; 58°C; 30 cycles); and GAPDH forward, ATCATCCCT GCCTCTACTGG and GAPDH reverse, CCCTCCGACGC CTGCTTAC (GAPDH PCR product, 188 bp; 58°C; 28 cycles); and PDGF-B forward, CGAGTTGGACCTGAAC

ATGA and PDGF-B reverse, GTCACCGTGGCCTTCTTAAA (PDGF-B PCR product, 339 bp; 58°C; 30 cycles).

Statistical analysis

Values are expressed as mean \pm standard error (SE). Survival curves were drawn by the Kaplan and Meier method, and the log rank test was used to analyze differences in survival rates. Student's- or Welch's *t*-test or the Wilcoxon test was used to analyze differences in other variables, as appropriate. Probability values of <0.05 were considered significant. All statistical analyses were performed with JMP software (SAS Institute, Cary, NC).

Results

Effect of imatinib on interaction between KM12SM cells and MSCs *in vitro*

RT-PCR and Western blotting revealed that MSCs expressed PDGFR- β and KM12SM cells expressed PDGF-B in culture conditions (Figs. 1a and 1b). PDGFR- β was expressed by MSCs but not by KM12SM cells, and phosphorylation of PDGFR- β in MSCs was completely inhibited by 1 μM imatinib (Fig. 1b). When the effects of imatinib on migration of MSCs were examined by migration assay, we found more MSCs migrated toward tumor cell culture than toward the medium without tumor cells; however, this effect was impaired with imatinib treatment (24.9 ± 2.5 vs. 43.4 ± 3.0 cells/field, $p < 0.05$; 9.3 ± 1.1 vs. 10.7 ± 1.1 cells/field) (Fig. 1c). We performed cell proliferation assay and confirmed that 1 μM imatinib did not affect cell proliferation of MSCs at least for 3 days (Fig. 1d).

To examine the effect of imatinib on the growth of KM12SM cells *in vitro*, cell proliferation assay was performed.

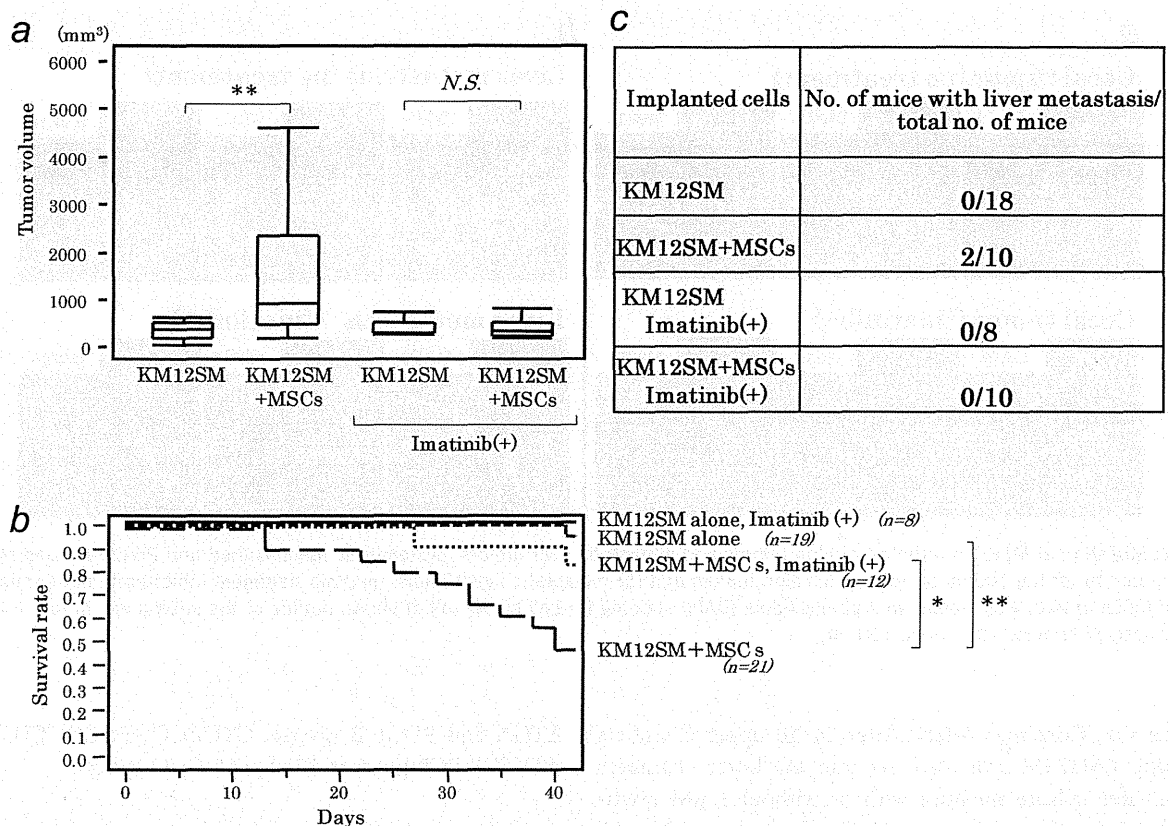


Figure 3. Comparisons of outcomes (cecal coinjection studies). Mice were divided into four groups according to the kind(s) of implanted cells and the treatment received, and mice surviving to day 42 were necropsied: (i) KM12SM cells alone without imatinib treatment ($n = 18$), (ii) KM12SM cells mixed with MSCs without imatinib treatment ($n = 10$), (iii) KM12SM cells alone with imatinib treatment ($n = 8$), or (iv) KM12SM cells mixed with MSCs with imatinib treatment ($n = 10$). (a) Transplantation of KM12SM cells mixed with MSCs ($0.5 \times 10^6:1.0 \times 10^6$, $n = 10$) resulted in significantly greater tumor volume than that resulting from transplantation of KM12SM cells alone (0.5×10^6 , $n = 18$). Imatinib treatment impairs tumor promoting effect of MSCs (KM12SM cells alone, $n = 8$ mice; KM12SM cells with MSCs, $n = 10$ mice). $**p < 0.01$, bars: SE. (b) Kaplan-Meier curves showing survival of mice bearing KM12SM cells alone (with or without imatinib treatment $n = 8$ or $n = 19$) and KM12SM cells mixed with MSCs (with or without imatinib treatment; $n = 12$ or $n = 21$). The survival rate was significantly lower in the mixed-cell group, and imatinib treatment significantly improved the survival rate in the mixed-cell group. $*p < 0.05$, $**p < 0.01$ (by log rank test). (c) Surviving mice were necropsied on day 42. The number of mice with liver metastasis is indicated. Macroscopic liver metastasis was seen only in the mixed-cell group that did not receive imatinib.

The reported clinically effective plasma concentration of imatinib is 1 to 5 μM . The proliferation of KM12SM cells was not affected by imatinib treatment when the concentration of imatinib was increased up to 8 μM (Fig. 1e).

Imatinib inhibited MSC migration to orthotopic colon tumors and metastatic liver tumors

After injection of PKH-labeled MSCs into the tail veins of tumor-bearing mice, MSCs were detected specifically in the tumor stroma both at the primary site (colon) and the metastatic site (liver). In the imatinib treatment groups, no MSCs were detected in tumor stroma (Figs. 2a and 2b).

Imatinib inhibited tumor-promoting effect of MSCs in the orthotopic colon tumor model

The volumes of tumors resulting from transplantation of mixed cells was significantly greater than the volumes of tumors

resulting from transplantation of tumor cells alone ($511 \pm 127 \text{ mm}^3$ vs. $1,478 \pm 467 \text{ mm}^3$, $p < 0.01$). In the imatinib treatment groups, the tumor-promoting effect of MSCs was impaired ($417 \pm 61 \text{ mm}^3$ vs. $408 \pm 67 \text{ mm}^3$) (Fig. 3a). The survival of mice was significantly lower in the mixed-cell group than in the group that received KM12SM cells alone ($p < 0.01$), and survival in the mixed-cell group that received imatinib was significantly improved ($p < 0.05$) (Fig. 3b). Macroscopic liver metastases were seen only in the mixed-cell group that did not receive imatinib (Fig. 3c). To clarify the mechanisms underlying the inhibitory effect of imatinib, we examined proliferation (PCNA-LI), apoptosis (AI), and angiogenesis (MVA) in primary tumors by immunohistochemistry. Between groups not given imatinib treatment, the PCNA-LI was significantly higher in the mixed-cell group than in the tumor-cell-alone group (30.6 ± 4.7 vs. $62.8 \pm 4.6\%$, $p < 0.01$) (Fig. 4a), the AI was significantly lower in the mixed-cell group ($7.2 \pm$

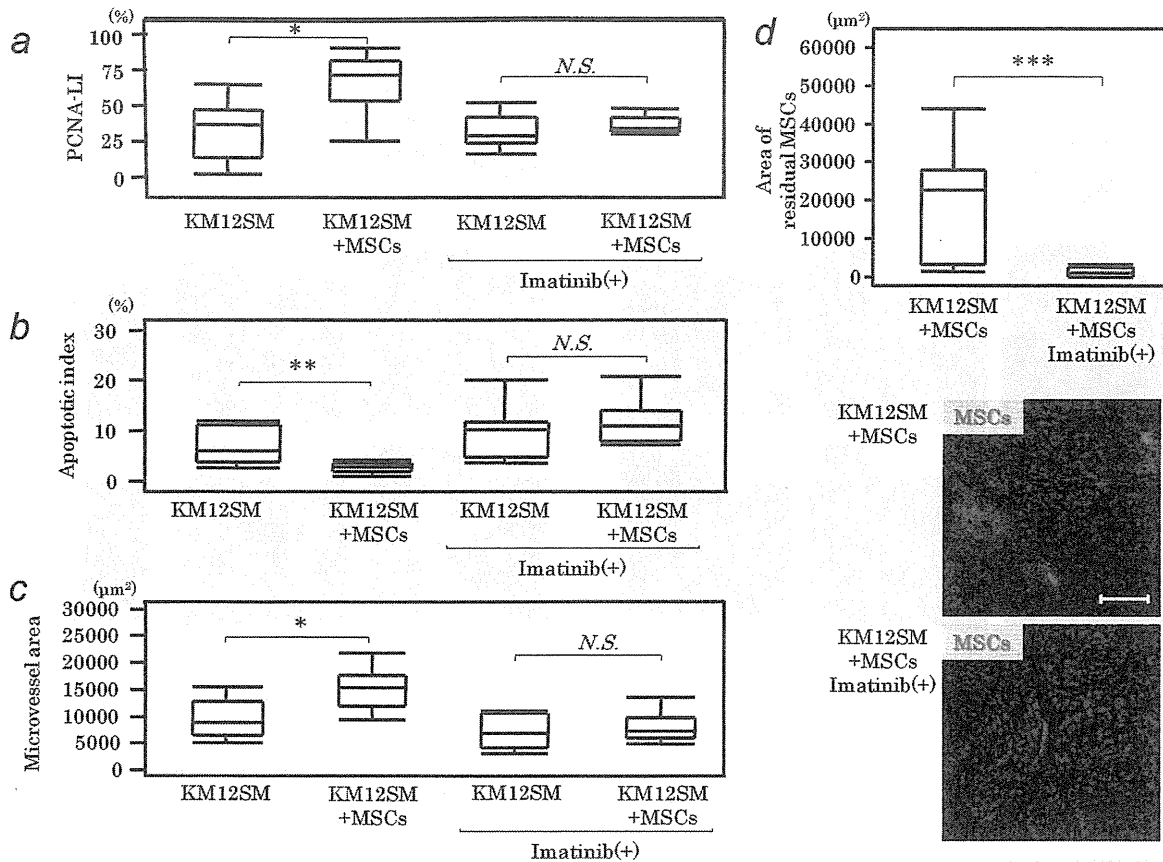


Figure 4. Proliferation, apoptosis, and angiogenesis in the four groups classified according to the kind(s) of implanted cells and the treatment received. (a) Proliferating cell nuclear antigen labeling index (PCNA-LI), (b) apoptotic index, and (c) microvessel area were analyzed by immunohistochemistry of the orthotopic colon tumors. Tumors from the mixed-cell group had a significantly higher PCNA-LI, a significantly lower AI, and significantly greater MVA than tumors from the group that received KM12SM cells alone. Imatinib treatment impaired the effects of MSCs. * $p < 0.05$, bars: SE; ** $p < 0.01$, bars: SE. (d) Surviving MSCs in the tumor microenvironment *in vivo*. Three weeks after cecal transplantation of KM12SM cells mixed with PKH26-labeled MSCs, surviving MSCs were significantly fewer in the imatinib treatment group than in the control group. Lower panels are representative photomicrographs of stained sections. *** $p < 0.001$, bars: SE. Scale bars: 100 μm .

1.1 vs. $2.8 \pm 0.3\%$, $p < 0.01$) (Fig. 4b), and the MVA was significantly greater in the mixed-cell group ($9,715 \pm 1,156$ vs. $15,180 \pm 1,225 \mu\text{m}^2$, $p < 0.05$) (Fig. 4c). With imatinib treatment, there was no significant difference between mice injected with tumor cells alone and those injected with mixed cells in PCNA-LI (33.4 ± 2.8 vs. $39.4 \pm 2.2\%$), in AI (9.5 ± 1.6 vs. $11.8 \pm 1.3\%$), or in MVA ($7,227 \pm 967$ vs. $7,919 \pm 835 \mu\text{m}^2$) (Figs. 4a–4c).

Survival of MSCs decreased with imatinib treatment *in vivo*

Three weeks after transplantation of KM12SM cells mixed with PKH26-labeled MSCs, the PKH26-positive areas were significantly smaller in the imatinib treatment group than in the group not treated with imatinib ($19,830 \pm 4,485$ vs. $2,005 \pm 868 \mu\text{m}^2$, $p < 0.001$) (Fig. 4d).

PDGFR- β in MSCs was phosphorylated, which was inhibited by imatinib treatment

We found that PDGFR- β in MSCs was phosphorylated 3 weeks after transplantation of KM12SM cells mixed with

PKH26-labeled MSCs, and it was inhibited by imatinib treatment (Fig. 5).

Imatinib inhibited the tumor-promoting effect of MSCs in the liver metastasis model

Transplantation of KM12SM cells alone into the spleen of nude mice resulted in formation of liver metastases in 70% of the animals, and the rate increased to 100% by co-implantation with MSCs. However, the rate of liver metastasis decreased to 50% and 60%, respectively, with imatinib administration (Fig. 6a). The total volume of liver metastases at 4 weeks after transplantation of KM12SM cells mixed with MSCs was significantly greater than that after transplantation of KM12SM cells alone ($66.7 \pm 39.6 \text{ mm}^3$ vs. $345.9 \pm 172 \text{ mm}^3$, $p < 0.05$) (Fig. 6b), but with imatinib administration, the total volume did not differ significantly between metastases resulting from transplantation of tumor cells alone or transplantation of mixed cells ($4.7 \pm 2.3 \text{ mm}^3$ vs. $28.8 \pm 20.7 \text{ mm}^3$).

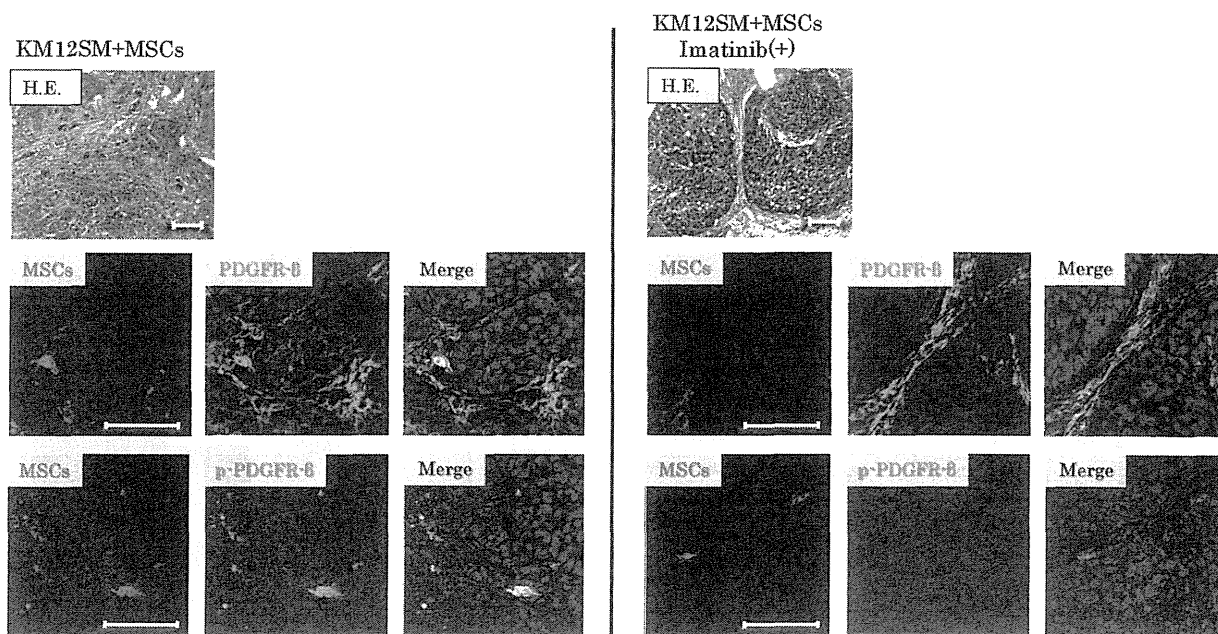


Figure 5. Phosphorylation of PDGFR- β in MSCs with or without imatinib treatment *in vivo*. PDGFR- β in MSCs was phosphorylated 3 weeks after transplantation of KM12SM cells mixed with PKH26-labeled MSCs, and it was inhibited by imatinib treatment.

Discussion

In this study, we found that tumor tropism of MSCs was inhibited by treatment with imatinib *in vivo* and *in vitro*. Oral administration of imatinib significantly inhibited the tumor growth- and metastasis-promoting effects of MSCs in our orthotopic colon cancer and liver metastasis models, and it prolonged survival of the mice. The treatment with imatinib also decreased the number of MSCs in the tumor stroma and impaired the cell proliferation- and angiogenesis-promoting effects of MSCs as well as the apoptosis-inhibiting effect of MSCs.

We previously showed in surgical specimens of human colon cancer that expression of PDGFR- β in tumor stroma is associated with vascularity and tumor stage.⁵ Most cancer cells secrete PDGFs, but do not express PDGFRs, suggesting that PDGF may act as a paracrine growth factor.^{41–43} In orthotopic mice models of colon and gastric cancers, PDGF-B was expressed by cancer cells, whereas PDGFR- β was expressed mainly by CAFs, and blockade of PDGFR signaling by imatinib in combination with irinotecan significantly inhibited tumor growth and metastasis.^{4,8} The antitumor effects were obvious in PDGF-B high-expressing tumors in comparison with effects in PDGF-B low-expressing tumors, in which the stromal reaction is minimal. These findings indicate that PDGFR signaling in CAFs plays an important role in the growth and progression of colon carcinoma.

Paracrine activation of PDGFRs is reported to act as a potent signal for tumor stroma recruitment of fibroblasts in experimental models.^{41,44} Ishii *et al.* showed that CAFs derived from human lung cancer tissue display significantly higher migratory activity in response to PDGF-B than that of

fibroblasts from corresponding noncancerous tissue.⁴⁵ We recently reported functional incorporation of MSCs into the stroma of orthotopic colon tumors, where they differentiate into CAFs with high PDGFR- β expression.³⁴ In addition, Goldstein *et al.*³³ recently showed that endogenous MSCs can migrate from the bone to primary tumors. Molecules such as CXCL12 (SDF-1)/CXCR4,¹⁸ CCL2 (MCP-1)/CCR2,²¹ TGF- β ,³³ and PDGF/PDGFR,¹⁹ are reportedly involved in the tumor-homing ability of MSCs. The mechanisms of MSC migration seem to be complex, but among involved factors, PDGF-B is reported to be the strongest.^{15,19} PDGF-B is also known to be important to the survival of MSCs transplanted *in vivo*.³⁶ Thus, we hypothesized that PDGFR tyrosine kinase inhibitor can impair migration of MSCs toward primary tumor as well as the tumor promoting effect of MSCs.

Although recent studies have revealed that MSCs promote tumor growth and metastasis in co-transplantation models,^{22,24–31} the molecular mechanisms underlying these effects remain unclear. Our present study showed that treatment with imatinib inhibits migration and survival of MSCs in tumor stroma, suggesting the importance of the PDGF/PDGFR system in the progression of colon cancer. Treatment with imatinib impaired the tumor-promoting effect of MSCs. However, imatinib had no effect on tumor growth when cancer cells were transplanted alone, indicating that the contribution of host-derived MSCs to tumor progression was at best minimal in this model. This may be, at least in part, because these MSCs and tumor cells were derived from different species.

The PDGF/PDGFR signaling pathway is known to play an important role in increasing interstitial fluid pressure (IFP) in

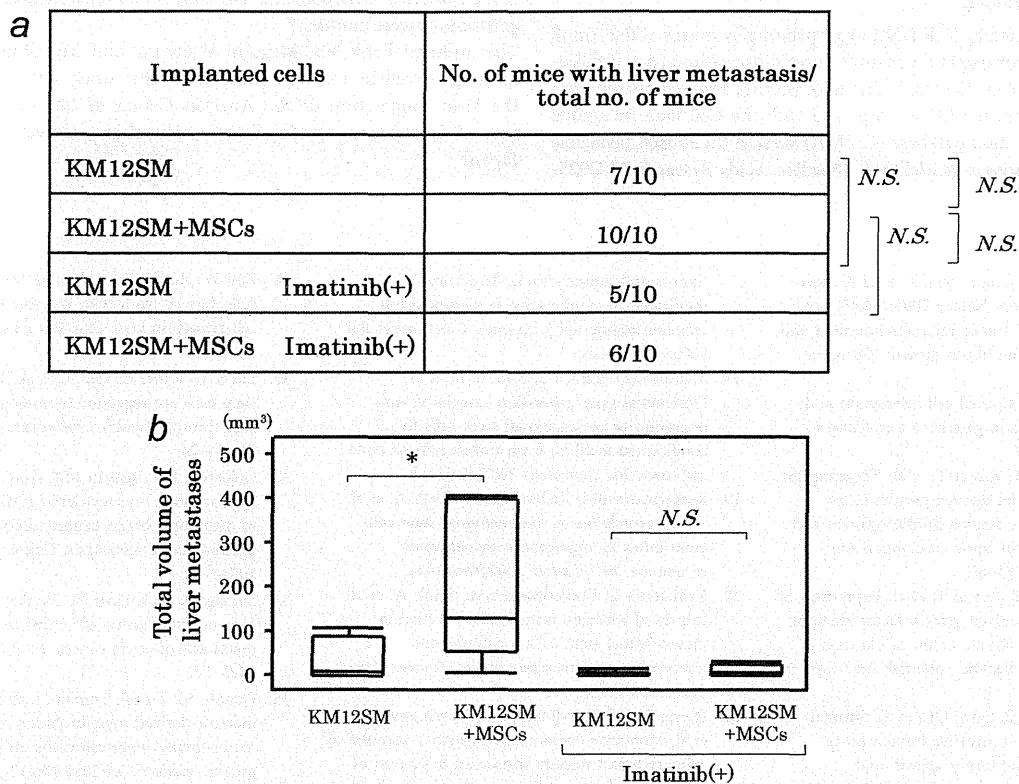


Figure 6. Formation and total volume of liver metastases in the four groups of mice. (a) Transplantation of KM12SM cells plus MSCs into the spleen resulted in 100% formation of liver metastases, but transplantation of KM12SM cells alone, or transplantation of KM12SM cells with or without imatinib treatment decreased the rate to 70%, 50% and 60%, respectively. (b) Total volume of liver metastases was significantly greater in the mixed-cell group without imatinib treatment than in other groups (each group, $n = 10$). * $p < 0.05$, bars: SE.

tumor stroma.^{40,46} Increased IFP can prevent effective distribution of antitumor drugs in the tumor microenvironment. Inhibition of the PDGF/PDGFR signaling pathway can decrease this pressure and, hence, enhance the effects of chemotherapeutic agents.⁴⁷ Our findings suggest that the tumor-inhibiting effect of imatinib may involve a reduction in CAFs derived from MSCs in the tumor microenvironment. However, imatinib is not a PDGFR-specific inhibitor; it can also inhibit the protein-tyrosine kinase activity of c-KIT, the receptor for KIT ligand, discoidin domain receptors (DDR-1 and -2), receptors for collagen, CSFR-1, the receptor for colony stimulating factors, and of ABL together with the chronic myeloid leukemia oncoprotein, BCR-ABL.⁴⁸ It has been reported that some solid tumor cell lines such as small cell lung⁴⁹ and pancreatic cancer cell lines,⁵⁰ coexpress c-Kit and Kit ligand. We recently found that cultured MSCs coexpress mRNA for c-kit and kit ligand, but there were few mononuclear cells that express c-kit and kit ligand in the xenograft resulting from transplantation of KM12SM cells mixed with MSCs (data not shown). In addition, imatinib might affect other tyrosine kinases expressed in tumor cells. Further studies are needed to clarify whether c-Kit in MSCs or other tyrosine kinases in tumor cells are involved in tumor progression *via* tumor-MSC interaction.

In addition, though we showed that MSCs promote tumor growth and metastasis in cotransplantation models, we think it would be more convincing if we could show that intravenous administration of MSCs also enhances tumor progression. In the natural setting, MSCs exist at low frequencies in human adult bone marrow, accounting for 0.00001% to 0.001% of mononuclear cells in bone marrow. However, MSCs may travel from bone marrow to tumor stroma continuously. We administered 1.0×10^6 MSCs by one-shot intravenous injection. In this model, MSCs did not promote tumor growth and metastasis (data not shown). We speculate that this is because the number of MSCs was not adequate for enhancement of tumor growth. It would be very interesting to know whether MSCs affect tumor growth if administered continuously, but if we do this in mice, they will die of pulmonary embolism. Thus, it is experimentally difficult to inject MSCs continuously as the same as a natural setting. Thus, it is experimentally difficult to mimic continuous MSC release.

Our study findings point to a possibility that migration of MSCs and the tumor-promoting effects of MSCs can be controlled by molecularly targeted antitumor drugs aimed at bone marrow-derived cells.

Acknowledgements

Cell lines were validated by STR DNA fingerprinting by means of the AmpF STR Identifier kit according to the manufacturer's instructions (Applied Biosystems catalog number 4322288). The STR profiles were compared with known ATCC fingerprints (ATCC.org) and with the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (available at: <http://bioinformatics.istge.it/clima/>) (Nucleic Acids Research 37:D925-

D932 PMID: PMC2686526). The STR profiles matched known DNA fingerprints or were unique.

The authors thank Ms. Megumi Wakisaka and Mr. Shinichi Norimura for their excellent technical assistance. This work was carried out with the kind cooperation of the Analysis Center of Life Science and Institute of Laboratory Animal Science, Hiroshima University (Hiroshima, Japan).

References

- Mantovani A, Allavena P, Sica A, et al. Cancer-related inflammation. *Nature* 2008;454:436–44.
- Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 2008;27:5904–12.
- Kitadai Y. Cancer-stromal cell interaction and tumor angiogenesis in gastric cancer. *Cancer Microenviron* 2009.
- Kitadai Y, Sasaki T, Kuwai T, et al. Targeting the expression of platelet-derived growth factor receptor by reactive stroma inhibits growth and metastasis of human colon carcinoma. *Am J Pathol* 2006;169:2054–65.
- Kitadai Y, Sasaki T, Kuwai T, et al. Expression of activated platelet-derived growth factor receptor in stromal cells of human colon carcinomas is associated with metastatic potential. *Int J Cancer* 2006;119:2567–74.
- Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121:335–48.
- De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. *J Pathol* 2003;200:429–47.
- Sumida T, Kitadai Y, Shinagawa K, et al. Anti-stromal therapy with imatinib inhibits growth and metastasis of gastric carcinoma in an orthotopic nude mouse model. *Int J Cancer* 2010;128:2050–62.
- Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006;6:392–401.
- Zeisberg EM, Potenta S, Xie L, et al. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res* 2007;67:10123–8.
- Direkze NC, Hodivala-Dilke K, Jeffery R, et al. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res* 2004;64:8492–5.
- Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.
- Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: molecular control of expansion and differentiation. *Exp Cell Res* 2005;306:330–5.
- Studeny M, Marini FC, Champlin RE, et al. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002;62:3603–8.
- Nakamizo A, Marini F, Amano T, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;65:3307–18.
- Nakamura K, Ito Y, Kawano Y, et al. Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004;11:1155–64.
- Hung SC, Deng WP, Yang WK, et al. Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive in vivo positron emission tomography imaging. *Clin Cancer Res* 2005;11:7749–56.
- Menon LG, Picinich S, Koneru R, et al. Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. *Stem Cells* 2007;25:520–8.
- Beckermann BM, Kallifatidis G, Groth A, et al. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer* 2008;99:622–31.
- Kallifatidis G, Beckermann BM, Groth A, et al. Improved lentiviral transduction of human mesenchymal stem cells for therapeutic intervention in pancreatic cancer. *Cancer Gene Ther* 2008;15:231–40.
- Dwyer RM, Potter-Berirne SM, Harrington KA, et al. Monocyte chemoattractant protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clin Cancer Res* 2007;13:5020–7.
- Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;449:557–63.
- Klopp AH, Spaeth EL, Dembinski JL, et al. Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment. *Cancer Res* 2007;67:11687–95.
- Annabi B, Naud E, Lee YT, et al. Vascular progenitors derived from murine bone marrow stromal cells are regulated by fibroblast growth factor and are avidly recruited by vascularizing tumors. *J Cell Biochem* 2004;91:1146–58.
- Djouad F, Bony C, Apparailly F, et al. Earlier onset of syngeneic tumors in the presence of mesenchymal stem cells. *Transplantation* 2006;82:1060–6.
- Djouad F, Plence P, Bony C, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;102:3837–44.
- Mishra PJ, Mishra PJ, Glod JW, et al. Mesenchymal stem cells: flip side of the coin. *Cancer Res* 2009;69:1255–8.
- Mishra PJ, Mishra PJ, Humeniuk R, et al. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res* 2008;68:4331–9.
- Ramasamy R, Lam EW, Soeiro I, et al. Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia* 2007;21:304–10.
- Sun B, Zhang S, Ni C, et al. Correlation between melanoma angiogenesis and the mesenchymal stem cells and endothelial progenitor cells derived from bone marrow. *Stem Cells Dev* 2005;14:292–8.
- Zhu W, Xu W, Jiang R, et al. Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. *Exp Mol Pathol* 2006;80:267–74.
- Liu S, Ginestier C, Ou SJ, et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* 2011;71:614–24.
- Goldstein RH, Reagan MR, Anderson K, et al. Human bone marrow-derived MSCs can home to orthotopic breast cancer tumors and promote bone metastasis. *Cancer Res* 2010;70:10044–50.
- Shinagawa K, Kitadai Y, Tanaka M, et al. Mesenchymal stem cells enhance growth and metastasis of colon cancer. *Int J Cancer* 2010;127:2323–33.
- Quante M, Tu SP, Tomita H, et al. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* 2011;19:257–72.
- Krausgrill B, Vantler M, Burst V, et al. Influence of cell treatment with PDGF-BB and reperfusion on cardiac persistence of mononuclear and mesenchymal bone marrow cells after transplantation into acute myocardial infarction in rats. *Cell Transplant* 2009;18:847–53.
- Druker BJ, Lydon NB. Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest* 2000;105:3–7.
- Ishii M, Koike C, Igarashi A, et al. Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem Biophys Res Commun* 2005;332:297–303.
- Morikawa K, Walker SM, Nakajima M, et al. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res* 1988;48:6863–71.
- Hwang RF, Yokoi K, Bucana CD, et al. Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. *Clin Cancer Res* 2003;9:6534–44.
- Forsberg K, Valyi-Nagy I, Helden CH, et al. Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proc Natl Acad Sci USA* 1993;90:393–7.
- Micke P, Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 2004;45(Suppl 2):S163–75.
- Paulsson J, Sjoblom T, Micke P, et al. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. *Am J Pathol* 2009;175:334–41.

44. Skobe M, Fusenig NE. Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. *Proc Natl Acad Sci USA* 1998;95: 1050-5.
45. Ishii G, Hashimoto H, Asada K, et al. Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: a novel character of tumor educated fibroblasts. *Int J Oncol* 2010; 37:317-25.
46. Pietras K, Ostman A, Sjoquist M, et al. Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. *Cancer Res* 2001;61:2929-34.
47. Pietras K. Increasing tumor uptake of anticancer drugs with imatinib. *Semin Oncol* 2004;31:18-23.
48. Manley PW, Stiefl N, Cowan-Jacob SW, et al. Structural resemblances and comparisons of the relative pharmacological properties of imatinib and nilotinib. *Bioorg Med Chem* 2010;18:6977-86.
49. Hibi K, Takahashi T, Sekido Y, et al. Coexpression of the stem cell factor and the c-kit genes in small-cell lung cancer. *Oncogene* 1991;6: 2291-6.
50. Yasuda A, Sawai H, Takahashi H, et al. The stem cell factor/c-kit receptor pathway enhances proliferation and invasion of pancreatic cancer cells. *Mol Cancer* 2006;5:46.

Expression of *miR-486* is a potential prognostic factor after nephrectomy in advanced renal cell carcinoma

KEISUKE GOTO^{1,2}, NAOHIDE OUE¹, SHUNSUKE SHINMEI^{1,2}, KAZUHIRO SENTANI¹, NAOYA SAKAMOTO¹, YUTAKA NAITO¹, TETSUTARO HAYASHI², JUN TEISHIMA², AKIO MATSUBARA² and WATARU YASUI¹

Departments of ¹Molecular Pathology and ²Urology, Hiroshima University Institute of Biomedical and Health Sciences, Hiroshima 734-8551, Japan

Received October 3, 2012; Accepted November 6, 2012

DOI: 10.3892/mco.2012.46

Abstract. Renal cell carcinoma is the most frequently occurring neoplasm in the adult kidney, leading to poor prognosis. Therefore novel biomarkers are required for the prediction of early metastasis following nephrectomy. The aim of the present study was to investigate whether or not expression levels of *miR-486*, detected in RNA isolated from formalin-fixed paraffin-embedded tissue sections, can predict prognosis for patients with renal cell carcinoma (RCC). Expression levels of *miR-486* were measured by quantitative reverse transcriptase-polymerase chain reaction in 150 RCC cases. Expression of *miR-486* in RCC samples was ~2.7-fold higher than in corresponding non-neoplastic kidney samples ($P < 0.0001$). In stage III and IV RCC cases ($n=46$), a high *miR-486* expression in tumors was associated with worse cancer-specific mortality, independent of clinical covariates, including TNM staging ($P=0.0064$). In addition, *miR-486* expression tended to be associated with cancer-specific mortality in stage III and IV RCC patients who were not treated with interferon- α (Kaplan-Meier analysis, $n=14$, $P=0.0574$). These results suggest that *miR-486* is a promising biomarker to identify poor prognosis in RCC patients. As expression of *miR-486* was measured from formalin-fixed paraffin-embedded (FFPE) samples, this study demonstrated that measurement of *miR-486* may be readily translated into clinical applications.

Introduction

Renal cell carcinoma (RCC) is the most common neoplasm in the adult kidney and accounts for 2-3% of malignant diseases in adults. Locally extensive or metastatic RCC, even following complete resection, have worse prognoses compared to

organ-confined diseases. Therefore, there is a need to identify novel biomarkers that enable prediction of early metastasis after nephrectomy, and to develop novel targeted therapies.

Cancer develops as a result of multiple genetic and epigenetic alterations. Better knowledge of changes in gene expression that occur during carcinogenesis may lead to improvements in diagnosis, treatment and prevention. Potential biomarkers identifying high-risk patients have been reported, however, assessment methods for these biomarkers often involve RNA-based techniques and require fresh frozen tissues. By contrast, formalin-fixed paraffin-embedded (FFPE) tissue samples have been collected during decades of routine histopathological examination and those are the most widely available materials in clinical use. However, formaldehyde-containing fixatives cause cross-linkage between nucleic acids and proteins, rendering subsequent extraction and quantification of RNA challenging (1). A major obstacle to RNA expression analysis of FFPE tissues has been the uncertainty as to whether or not gene expression analyses from routinely archived tissues accurately reflect the expression levels prior to fixation, since the fixation process is likely to cause a high degree of RNA fragmentation (2). Given that naturally occurring small RNAs are not affected by fragmentation and therefore do not experience loss of quality, targeting miRNAs is more suitable for analysis of RNA extracted from FFPE samples.

miRNAs are 18- to 25-nucleotide, non-coding RNA molecules that regulate the translation of several genes (3). miRNA expression levels are altered in most types of human cancers (4-6). Several RCC studies have examined miRNAs by microarray analysis using a relatively small amount of frozen tissue samples. Various miRNAs that are dysregulated in RCC have been identified (7,8). Several lines of evidence indicate that *miR-486* has oncogenic properties. Overexpression of *miR-486* has been reported in cutaneous T-cell lymphoma (9). High serum or plasma expression levels of *miR-486* have been reported in non-small cell lung (10) and gastric cancers (11). *miR-486* has also been reported to target *PTEN* and *FOXO1*, which negatively affect PI3K/Akt signaling (12). The Akt-signaling pathway functions as a potent activator of growth and survival signaling (13). However, expression of *miR-486* has not been investigated in RCC.

In the present study, we investigated the association of *miR-486* expression with RCC patient prognosis ($n=150$) using

Correspondence to: Professor Wataru Yasui, Department of Molecular Pathology, Hiroshima University Institute of Biomedical and Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

E-mail: wyasui@hiroshima-u.ac.jp

Key words: miRNA, *miR-486*, renal cell carcinoma, prognosis

quantitative reverse transcription polymerase chain reaction (qRT-PCR) of FFPE samples. In addition, since it has been reported that *olfactomedin 4* (*OLFM4*), which encodes the olfactomedin 4 protein, is one of the direct targets of *miR-486* (14), immunohistochemical analysis of olfactomedin 4 was carried out in RCC tissues.

Materials and methods

Tissue samples. In a retrospective study design, 150 primary tumors were collected consecutively from patients diagnosed with RCC who underwent surgery between 2001 and 2008 at the Hiroshima University Hospital (Hiroshima, Japan). Only patients without preoperative radiotherapy or chemotherapy were enrolled in the study. Of 150 patients, 129 were at stages I, II and III, while 21 were at stage IV. The 129 patients with stage I, II and III RCC underwent curative resection. Of the 129 patients with stage I, II and III RCC, 20 received adjuvant therapy (interferon- α) (15). Of the 21 patients with stage IV RCC, 17 received postoperative therapy using interferon- α (15). Postoperative follow-up was scheduled every 1, 2 or 3 months during the first 2 years after surgery, and every 6 months thereafter unless more frequent follow-up was deemed necessary. Chest-abdominal computed tomography scan and serum chemistries were performed during each 6 month visit at least. Patients were monitored by their physician until they succumbed to the disease or the date of the last documented contact. The median follow-up period was 64 months (range, 2-120). Operative mortality was defined as death within 30 days of the patient leaving the hospital, and these patients were omitted from the analysis.

For qRT-PCR analysis, archival FFPE tissues were used. Histological classification was based on the World Health Organization system. RCC cases were classified into clear cell RCC (ccRCC) and non-ccRCC. Tumor staging was performed according to the TNM grouping system. Since written informed consent was not obtained, for reasons of strict privacy protection, identifying information for the samples was removed prior to analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

RNA extraction and qRT-PCR. FFPE samples were sectioned (10 μ m), deparaffinized and stained with hematoxylin and eosin (H&E) to ensure that the sectioned block contained tumor cells. The tumor areas in the adjacent sections were marked under a light microscope without hematoxylin staining. Tumor areas were macrodissected with sterile disposable scalpels and subjected to RNA isolation using the Recover All™ Total Nucleic Acid Isolation kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Expression levels of *miR-486* and *RNU6B* were measured using TaqMan® assays for miRNA (Applied Biosystems, Austin, TX, USA) in such a manner that the identity and clinical outcomes of samples were blinded. Complementary DNA (cDNA) was synthesized using miRNA-specific primers and the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 40 ng of RNA was reverse transcribed in a 20 μ l reaction with gene-specific RT probes. qRT-PCR was performed using the ABI 7900 Version

Table I. Association between *miR-486* expression and clinicopathological characteristics.

Characteristics	<i>miR-486</i> expression		
	High (%)	Low	P-value
Age (years)			
<66	48 (69)	22	0.1084
≥66	64 (80)	16	
Gender			
Male	80 (73)	29	0.5552
Female	32 (78)	9	
T classification			
1	71 (77)	21	0.5009
2	12 (71)	5	
3	27 (69)	12	
4	2 (100)	0	
N classification			
0	107 (76)	34	0.4049
1	2 (50)	2	
2	3 (60)	2	
M classification			
0	98 (75)	32	0.6119
1	14 (70)	6	
Tumor stage			
I	71 (79)	19	0.1792
II	10 (71)	4	
III	16 (67)	8	
IV	15 (68)	7	
Histological classification			
ccRCC	101 (74)	36	0.3649
Non-ccRCC	11 (85)	2	
Venous invasion			
Positive	35 (74)	12	0.9699
Negative	77 (75)	26	
Interferon- α treatment			
Received	23 (62)	14	0.0519
Not received	89 (79)	24	

ccRCC, clear cell renal cell carcinoma.

2.3 Sequence Detection System (Applied Biosystems). *RNU6B* was used as an endogenous normalization control for *miR-486*. The assays were performed in triplicate. Quantification of *miR-486* relative expression was calculated using the RQ manager 1.2 (Applied Biosystems).

Immunohistochemistry. FFPE samples were sectioned, deparaffinized and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically with the Dako EnVision+ Mouse Peroxidase Detection System (DakoCytomation, Carpinteria, CA, USA). Antigen retrieval

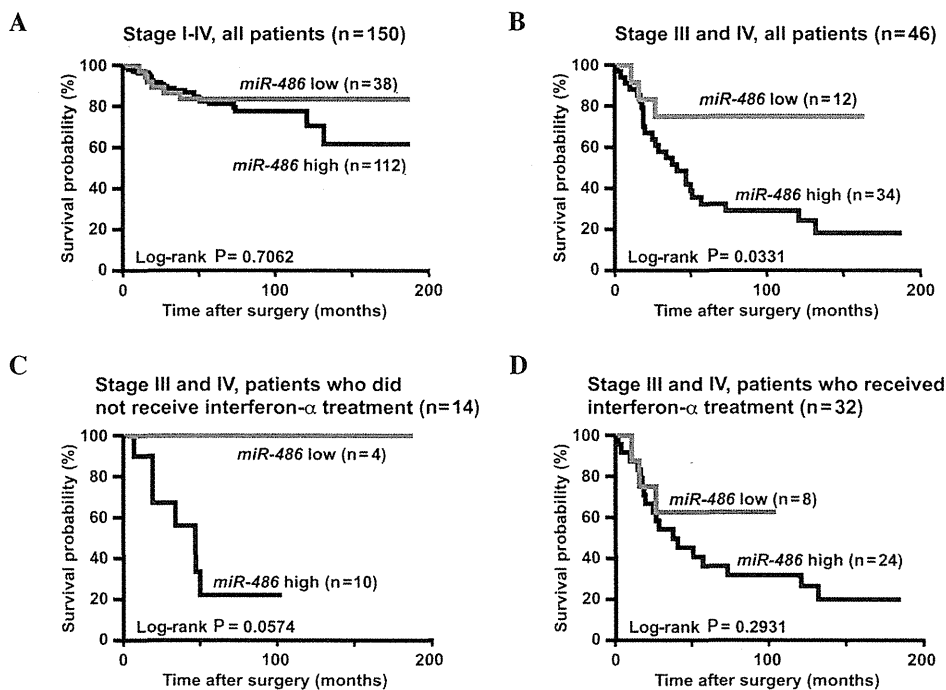


Figure 1. (A) Kaplan-Meier curves of *miR-486*-high or -low renal cell carcinoma (RCC) in stage I-IV patients. Cancer-specific mortality did not statistically vary in high- and low-*miR-486* cases. (B) Kaplan-Meier curves of *miR-486*-high or -low RCC in stage III and IV patients. High-*miR-486* expression had significantly worse cancer-specific mortality compared to those with low-*miR-486* expression. (C) Kaplan-Meier curves of *miR-486*-high or -low RCC in stage III and IV patients who did not receive interferon- α treatment. Cancer-specific mortality of patients with high-*miR-486* expression tended to be worse compared to that of patients with low-*miR-486* expression. (D) Kaplan-Meier curves of *miR-486*-high or -low RCC in stage III and IV patients who received interferon- α treatment. Expression of *miR-486* was not significantly associated with therapeutic outcome.

was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. After peroxidase activity was blocked with 3% H₂O₂ methanol for 10 min, sections were incubated with normal goat serum (DakoCytomation) for 20 min to block non-specific antibody binding sites. Sections were incubated with primary antibodies against olfactomedin 4 (1:50 dilution, using an anti-olfactomedin 4 antibody raised in our laboratory) (16) for 1 h at room temperature, followed by incubations with EnVision+ anti-mouse peroxidase for 1 h. Staining was completed with 10-min incubation with the substrate-chromogen solution. Sections were counterstained using 0.1% hematoxylin.

Statistical analysis. Associations between clinicopathological characteristics and *miR-486* expression were analyzed using the Fisher's exact test. To evaluate the associations between clinical covariates and cancer-specific mortality univariate and multivariate Cox regression analysis was used and conducted using SPSS software (SPSS Inc., Chicago, IL, USA). Hazard ratio (HR) and 95% confidence interval (CI) were estimated from the Cox proportional hazard models. For the analyses, age was treated as a categorical variable (65 plus >65 vs. <65 years old). For the final multivariate Cox regression models, the variables that were moderately associated ($P < 0.10$) with cancer-specific mortality were included. Differences in *miR-486* expression levels between the two groups were determined by the Mann-Whitney U test using Graphpad Prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA). Kaplan-Meier survival curves were constructed for high- and low-*miR-486* patients using the Graphpad Prism 5.0 software. Differences between survival curves were tested for statistical

significance using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of *miR-486* in RCC and non-neoplastic kidney tissue. The expression of *miR-486* and the corresponding non-neoplastic kidney tissue samples were assessed in 86 RCC cases using qRT-PCR. The expression of *miR-486* in RCC samples was ~2.7-fold higher compared to that in corresponding non-neoplastic kidney samples ($P < 0.0001$, Mann-Whitney U-test).

Association between *miR-486* expression levels and clinicopathological characteristics. Expression of *miR-486* was examined in 64 additional RCC tissue samples using qRT-PCR. We investigated the association between the clinicopathological characteristics and *miR-486* expression levels in 150 RCC cases (Table I). These 150 RCC cases were divided into high- and low-*miR-486* cases. When low-*miR-486* expression was classified according to the lowest quartile, the number of high- and low-*miR-486* cases was 112 and 38, respectively. Expression of *miR-486* was not associated with clinicopathological characteristics such as gender, T, N and M classifications, tumor stage or venous invasion.

Association between *miR-486* expression and survival. The association between *miR-486* expression levels and cancer-specific mortality was evaluated. The Kaplan-Meier analysis for the 150 RCC cases (stages I-IV) was performed.

Table II. Univariate and multivariate Cox regression analysis of *miR-486* expression and cancer-specific mortality in stage III and IV RCC.

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)				
<66	1 (Ref.)	0.5719		
≥66	1.24 (0.58-2.66)			
Gender				
Male	1 (Ref.)	0.5919		
Female	0.73 (0.17-2.08)			
Histological classification				
Non-ccRCC	1 (Ref.)	0.5714		
ccRCC	0.64 (0.18-4.00)			
Venous invasion				
Negative	1 (Ref.)	0.9318		
Positive	1.04 (0.42-3.10)			
T classification				
1/2	1 (Ref.)	0.8861		
3/4	1.09 (0.37-4.60)			
N classification				
0	1 (Ref.)	0.0315	1 (Ref.)	0.9832
1/2	2.73 (1.10- 6.24)		1.01 (0.32-2.89)	
M classification				
0	1 (Ref.)	0.0035	1 (Ref.)	0.2398
1	3.16 (1.46-7.08)		0.31 (0.05-2.47)	
Tumor stage				
III	1 (Ref.)	0.0005	1 (Ref.)	0.0386
IV	4.03 (1.82-9.54)		9.72 (1.14-59.82)	
Interferon- α treatment				
Not received	1 (Ref.)	0.5558		
Received	1.28 (0.57-3.28)			
<i>miR-486</i> expression				
Low	1 (Ref.)	0.0202	1 (Ref.)	0.0064
High	3.38 (1.18-14.26)		4.33 (1.45-18.71)	

RCC, renal cell carcinoma; ccRCC, clear cell RCC; HR, hazard ratio; CI, confidence interval.

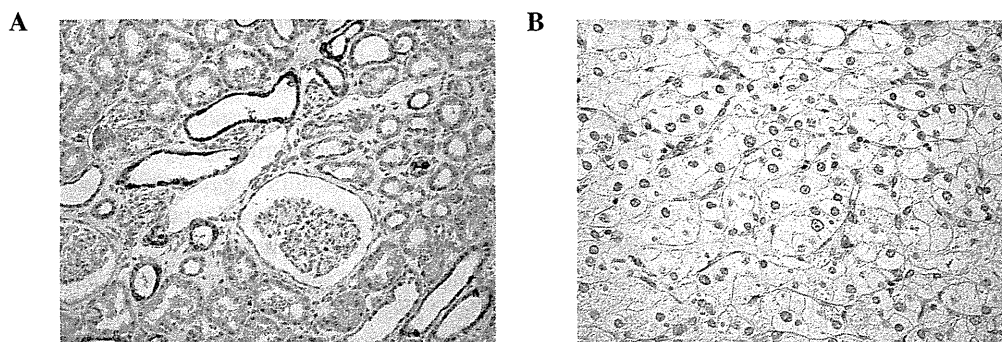


Figure 2. Immunohistochemical analysis of olfactomedin 4. (A) In non-neoplastic kidney, olfactomedin 4 staining was observed in uriniferous tubule but not glomerulus (original magnification, x200). (B) In renal cell carcinoma, olfactomedin 4 staining was not present (original magnification, x400).

As shown in Fig. 1A, cancer-specific mortality did not statistically vary in high- and low-*miR-486* cases.

Patients with RCC at stage I and II have a good rate of survival, whereas it is difficult to predict patient survival with stage III and IV RCC. These patients would benefit greatly from prognostic biomarkers. Therefore, we analyzed the prognostic value of *miR-486* expression in patients with stage III and IV RCC (n=46). Using the Kaplan-Meier analysis, we found that RCC cases with high-*miR-486* expression had significantly worse cancer-specific mortality compared to those with low-*miR-486* expression (P=0.0331, log-rank test, Fig. 1B). Since treatment with interferon- α for patients with stage III and IV RCC may affect patient survival, individuals who did not receive interferon- α treatment (n=14) were assessed. Although cancer-specific mortality among patients with high- and low-*miR-486* expression exhibited no statistically significant difference (P=0.0574, log-Rank test, Fig. 1C), cancer-specific mortality of patients with high-*miR-486* expression tended to be worse compared to that of patients with low-*miR-486* expression.

To evaluate the potential for *miR-486* expression as a prognostic predictor in patients with stage III and IV RCC, univariate and multivariate Cox proportional hazards analyses were used to evaluate the association of *miR-486* expression with cancer-specific mortality (Table II). Findings of the univariate analysis showed that N (HR, 2.73; 95% CI, 1.10-6.24; P=0.0315) and M classifications (HR, 3.16; 95% CI, 1.46-7.08; P=0.0035), tumor stage (HR, 4.03; 95% CI, 1.82-9.54; P=0.0005) and *miR-486* expression (HR, 3.38; 95% CI, 1.18-14.26; P=0.0202) were significantly associated with cancer-specific mortality. According to the multivariate model, which included N and M classifications, tumor stage and *miR-486* expression, expression of *miR-486* was an independent prognostic classifier of cancer-specific mortality (HR, 4.33; 95% CI, 1.45-18.71; P=0.0064).

Survival analysis was performed using an alternatively defined cut-off point. When low-*miR-486* expression was defined on the basis of lower than median levels of expression of *miR-486*, the number of high- as well as low-*miR-486* *miR-486* cases was 75. Kaplan-Meier analysis revealed that cancer-specific mortality did not exhibit statistically significant differences in high- and low-*miR-486* cases (data not shown). When low-*miR-486* expression was classified according to the lowest tertile, the number of high- and low-*miR-486* cases was 100 and 50, respectively. Kaplan-Meier analysis revealed that cancer-specific mortality did not exhibit statistically significant differences in high- and low-*miR-486* cases (data not shown).

Expression of *miR-486* and therapeutic outcomes. Biomarkers that predict therapeutic outcomes may provide tools to allow physicians to better stratify patients to more effective treatments. Therefore, we analyzed the association between *miR-486* expression and therapeutic outcomes in stage III and IV RCC patients treated with interferon- α (n=32). However, expression of *miR-486* was not significantly associated with therapeutic outcome (P=0.2931, log-rank test, Fig. 1D).

Expression of *OLFM4* in RCC. *OLFM4*, which encodes the *OLFM4* protein, has been reported as one of the direct targets of *miR-486* (14). Although alteration of *OLFM4* expression

has been reported in several types of human cancer (16-18), expression of *OLFM4* in RCC has not been investigated. Therefore, the expression of *OLFM4* was analyzed using immunohistochemistry in 86 RCC cases. In non-neoplastic kidney, *OLFM4* staining was observed in uriniferous tubules but not in glomeruli (Fig. 2A). By contrast, *OLFM4* staining was not expressed in RCC cells (Fig. 2B). No *OLFM4* staining was detectable in the 86 RCC cases.

Discussion

Several lines of evidence have suggested that miRNAs are useful as biomarkers as well as therapeutic targets for cancer. Prediction of the survival of patients with stage III and IV RCC remains difficult, and these groups would benefit from the detection of prognostic markers that identify individuals for whom adjuvant or post-operative treatment would be advantageous. In the present study, RNA from 150 FFPE RCC tissues was prepared, since biomarkers developed from FFPE samples may be more readily translated into clinical application. Expression of *miR-486* in patients with stage III and IV RCC was significantly associated with cancer-specific mortality according to a Kaplan-Meier analysis. The univariate and multivariate Cox proportional hazards analyses demonstrated that expression of *miR-486* was an independent prognostic classifier. Furthermore, since treatment with interferon- α for patients with stage III and IV RCC affects patient survival, individuals who did not receive interferon- α treatment were assessed. Although cancer-specific mortality among patients with high- and low-*miR-486* expression showed no statistically significant difference, cancer-specific mortality of patients with high-*miR-486* expression tended to be worse compared to that of patients with low-*miR-486* expression. This finding suggests that *miR-486* expression is associated with a more aggressive RCC histology. Taken together, these results indicate that *miR-486* is a promising biomarker to identify patients with poor prognosis in stage III and IV RCC.

In the present study, we demonstrated that *miR-486* expression was associated with cancer-specific mortality in patients with stage III and stage IV RCC. In addition, *miR-486* expression was associated with cancer-specific mortality in stage III and IV RCC patients who were not treated with interferon- α . Therefore, measurement of *miR-486* expression may help identify patients with a high risk of disease recurrence, while treatment with interferon- α may be indicative for patients with *miR-486*-positive RCC. However, it is unclear whether such patients may benefit from interferon- α treatment. To address this issue, we examined whether or not *miR-486* expression was able to identify patients for whom interferon- α treatment is beneficial in stage III and IV RCC. However, expression of *miR-486* was not significantly associated with therapeutic outcome. These results suggest that even when patients with a high risk of disease recurrence are identified by *miR-486* measurement, those patients may not benefit from interferon- α treatment. Novel therapeutic methods may be more effective for these patients.

miR-486 has been reported to have oncogenic properties. Overexpression of *miR-486* has been reported in several human malignancies. In the present study, although expression of *miR-486* in RCC samples was significantly higher compared

to that in corresponding non-neoplastic kidney samples, expression of *miR-486* was not associated with tumor stage. Therefore, high-*miR-486* expression is likely to be involved in carcinogenesis, but not in tumor progression. Since targets of *miR-486* are *PTEN* and *FOXO1* (12), expression of *PTEN* and *FOXO1* may be low in high-*miR-486* RCC cases. In addition to *PTEN* and *FOXO1*, one of the targets of *miR-486* is *OLFM4* (14). In the present study, although *OLFM4* staining was observed in uriniferous tubules, staining of *OLFM4* was not detected in the 86 RCC cases. Previously, we showed that patients with *OLFM4*-positive gastric cancer had a better survival rate compared to patients with *OLFM4*-negative gastric cancer (16). Forced expression of *OLFM4* has also been reported to decrease cell adhesion and migration (17,18). Taken together, *OLFM4* is likely to have tumor suppressive properties in RCC. Given that staining of *OLFM4* was not detected in the 86 RCC cases, overexpression of *miR-486* is unlikely to have a major role in the loss of *OLFM4*.

In summary, we showed that a high-*miR-486* expression is an independent prognostic classifier in stage III and IV RCC. Therefore, measurement of *miR-486* helps identify high-risk patients. In this study, expression of *miR-486* from FFPE samples was assessed. Therefore, measurement of *miR-486* can be readily translated into clinical applications.

Acknowledgements

This study was supported in part by grants-in-aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan and in part by a grant-in-aid for the Third Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan. The authors would like to thank Mr. Shinichi Norimura for his excellent technical assistance and advice. This study was conducted in collaboration with the Research Center for Molecular Medicine of the Faculty of Medicine of Hiroshima University. We also thank the Analysis Center of Life Science of Hiroshima University for the use of their facilities.

References

1. Srinivasan M, Sedmak D and Jewell S: Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 161: 1961-1971, 2002.
2. Specht K, Richter T, Muller U, Walch A, Werner M and Hofler H: Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* 158: 419-429, 2001.
3. Huntzinger E and Izaurralde E: Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet* 12: 99-110, 2011.
4. Lu J, Getz G, Miska EA, *et al.*: MicroRNA expression profiles classify human cancers. *Nature* 435: 834-838, 2005.
5. Schetter AJ, Heegaard NH and Harris CC: Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* 31: 37-49, 2010.
6. Ueda T, Volinia S, Okumura H, *et al.*: Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* 11: 136-146, 2010.
7. Gottardo F, Liu CG, Ferracin M, *et al.*: Micro-RNA profiling in kidney and bladder cancers. *Urol Oncol* 25: 387-392, 2007.
8. White NM, Bao TT, Grigull J, *et al.*: miRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. *J Urol* 186: 1077-1083, 2011.
9. Narducci MG, Arcelli D, Picchio MC, *et al.*: MicroRNA profiling reveals that miR-21, miR486 and miR-214 are upregulated and involved in cell survival in Sézary syndrome. *Cell Death Dis* 2: e151, 2011.
10. Hu Z, Chen X, Zhao Y, *et al.*: Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol* 28: 1721-1726, 2010.
11. Konishi H, Ichikawa D, Komatsu S, *et al.*: Detection of gastric cancer-associated microRNAs on microRNA microarray comparing pre- and post-operative plasma. *Br J Cancer* 106: 740-747, 2012.
12. Small EM, O'Rourke JR, Moresi V, *et al.*: Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. *Proc Natl Acad Sci USA* 107: 4218-4223, 2010.
13. Crackower MA, Oudit GY, Kozieradzki I, *et al.*: Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell* 110: 737-749, 2002.
14. Oh HK, Tan AL, Das K, *et al.*: Genomic loss of miR-486 regulates tumor progression and the *OLFM4* antiapoptotic factor in gastric cancer. *Clin Cancer Res* 17: 2657-2667, 2011.
15. Medical Research Council Renal Cancer Collaborators: Interferon-alpha and survival in metastatic renal carcinoma: early results of a randomised controlled trial. *Medical Research Council Renal Cancer Collaborators. Lancet* 353: 14-17, 1999.
16. Oue N, Sentani K, Noguchi T, *et al.*: Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensitive biomarker for gastric cancer patients. *Int J Cancer* 125: 2383-2392, 2009.
17. Koshida S, Kobayashi D, Moriai R, Tsuji N and Watanabe N: Specific overexpression of *OLFM4*(GW112/hGC-1) mRNA in colon, breast and lung cancer tissues detected using quantitative analysis. *Cancer Sci* 98: 315-320, 2007.
18. Liu W, Liu Y, Zhu J, Wright E, Ding I and Rodgers GP: Reduced hGC-1 protein expression is associated with malignant progression of colon carcinoma. *Clin Cancer Res* 14: 1041-1049, 2008.