เอกสารอ้างอิง (REFERENCES)

[1] S. Sriamporn, P. Pisani, V. Pipitgool, K. Suwanrungruang, S. Kamsa-ard, D.M. Parkin, Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand, Trop Med Int Health 9 (2004) 588-594.

[2] O. De Wever, P. Demetter, M. Mareel, M. Bracke, Stromal myofibroblasts are drivers of invasive cancer growth, Int J Cancer 123 (2008) 2229-2238.

[3] A.F. Olumi, G.D. Grossfeld, S.W. Hayward, P.R. Carroll, T.D. Tlsty, G.R. Cunha, Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium, Cancer Res 59 (1999) 5002-5011.

[4] A. Sadlonova, Z. Novak, M.R. Johnson, D.B. Bowe, S.R. Gault, G.P. Page, J.V. Thottassery, D.R. Welch, A.R. Frost, Breast fibroblasts modulate epithelial cell proliferation in three-dimensional in vitro co-culture, Breast Cancer Res 7 (2005) R46-59.

[5] P. Garin-Chesa, L.J. Old, W.J. Rettig, Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers, Proc Natl Acad Sci U S A 87 (1990) 7235-7239.

[6] D. Lazard, X. Sastre, M.G. Frid, M.A. Glukhova, J.P. Thiery, V.E. Koteliansky, Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue, Proc Natl Acad Sci U S A 90 (1993) 999-1003.

[7] J.L. Camps, S.M. Chang, T.C. Hsu, M.R. Freeman, S.J. Hong, H.E. Zhau, A.C. von Eschenbach, L.W. Chung, Fibroblast-mediated acceleration of human epithelial tumor growth in vivo, Proc Natl Acad Sci U S A 87 (1990) 75-79.

[8] O. Picard, Y. Rolland, M.F. Poupon, Fibroblast-dependent tumorigenicity of cells in nude mice: implication for implantation of metastases, Cancer Res 46 (1986) 3290-3294.

[9] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat Rev Cancer 6 (2006) 392-401.

[10] V. Neaud, S. Faouzi, J. Guirouilh, B. Le Bail, C. Balabaud, P. Bioulac-Sage, J. Rosenbaum, Human hepatic myofibroblasts increase invasiveness of hepatocellular carcinoma cells: evidence for a role of hepatocyte growth factor, Hepatology 26 (1997) 1458-1466.

[11] H. Nakagawa, S. Liyanarachchi, R.V. Davuluri, H. Auer, E.W. Martin, Jr., A. de la Chapelle, W.L. Frankel, Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles, Oncogene 23 (2004) 7366-7377.

[12] A.M. Scott, G. Wiseman, S. Welt, A. Adjei, F.T. Lee, W. Hopkins, C.R. Divgi, L.H. Hanson, P. Mitchell, D.N. Gansen, S.M. Larson, J.N. Ingle, E.W. Hoffman, P. Tanswell, G. Ritter, L.S. Cohen, P. Bette, L. Arvay, A. Amelsberg, D. Vlock, W.J. Rettig, L.J. Old, A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer, Clin Cancer Res 9 (2003) 1639-1647.

[13] K. Okita, I. Sakaida, K. Hino, Current strategies for chemoprevention of hepatocellular carcinoma, Oncology 62 Suppl 1 (2002) 24-28.

[14] D.B. Elkins, E. Mairiang, P. Sithithaworn, P. Mairiang, J. Chaiyakum, N. Chamadol, V. Loapaiboon, M.R. Haswell-Elkins, Cross-sectional patterns of hepatobiliary abnormalities and possible

precursor conditions of cholangiocarcinoma associated with Opisthorchis viverrini infection in humans, Am J Trop Med Hyg 55 (1996) 295-301.

[15] B. Sripa, S. Kaewkes, Gall bladder and extrahepatic bile duct changes in Opisthorchis viverrini-infected hamsters, Acta Trop 83 (2002) 29-36.

[16] N. Okamura, M. Yoshida, A. Shibuya, H. Sugiura, I. Okayasu, M. Ohbu, Cellular and stromal characteristics in the scirrhous hepatocellular carcinoma: comparison with hepatocellular carcinomas and intrahepatic cholangiocarcinomas, Pathol Int 55 (2005) 724-731.

[17] G. Ramadori, B. Saile, Portal tract fibrogenesis in the liver, Lab Invest 84 (2004) 153-159.

[18] S. Ohira, K. Itatsu, M. Sasaki, K. Harada, Y. Sato, Y. Zen, A. Ishikawa, K. Oda, T. Nagasaka, Y. Nimura, Y. Nakanuma, Local balance of transforming growth factor-beta1 secreted from cholangiocarcinoma cells and stromal-derived factor-1 secreted from stromal fibroblasts is a factor involved in invasion of cholangiocarcinoma, Pathol Int 56 (2006) 381-389.

[19] S. Ohira, M. Sasaki, K. Harada, Y. Sato, Y. Zen, K. Isse, K. Kozaka, A. Ishikawa, K. Oda, Y. Nimura, Y. Nakanuma, Possible regulation of migration of intrahepatic cholangiocarcinoma cells by interaction of CXCR4 expressed in carcinoma cells with tumor necrosis factor-alpha and stromal-derived factor-1 released in stroma, Am J Pathol 168 (2006) 1155-1168.

[20] P. Micke, A. Ostman, Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy, Expert Opin Ther Targets 9 (2005) 1217-1233.

[21] C. Chuaysri, P. Thuwajit, A. Paupairoj, S. Chau-In, T. Suthiphongchai, C. Thuwajit, Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma, Oncol Rep 21 (2009) 957-969.

[22] O.W. Howell, H.E. Scharfman, H. Herzog, L.E. Sundstrom, A. Beck-Sickinger, W.P. Gray, Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells, J Neurochem 86 (2003) 646-659.

[23] R.L. Aycock, A.C. Bradshaw, E.H. Sage, B. Starcher, Development of UV-induced squamous cell carcinomas is suppressed in the absence of SPARC, J Invest Dermatol 123 (2004) 592-599.

[24] C. Duggan, S. Kennedy, M.D. Kramer, C. Barnes, P. Elvin, E. McDermott, N. O'Higgins, M.J. Duffy, Plasminogen activator inhibitor type 2 in breast cancer, Br J Cancer 76 (1997) 622-627.

[25] M. Osmak, D. Babic, M. Abramic, D. Milicic, I. Vrhovec, J. Skrk, Plasminogen activator inhibitor type 2: potential prognostic factor for endometrial carcinomas, Neoplasma 48 (2001) 462-467.

[26] B. Schmidt-Hansen, J. Klingelhofer, B. Grum-Schwensen, A. Christensen, S. Andresen, C. Kruse, T. Hansen, N. Ambartsumian, E. Lukanidin, M. Grigorian, Functional significance of metastasis-inducing S100A4(Mts1) in tumor-stroma interplay, J Biol Chem 279 (2004) 24498-24504.

[27] B.M. Steiglitz, D.R. Keene, D.S. Greenspan, PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1, J Biol Chem 277 (2002) 49820-49830.

[28] C.F. Singer, D. Gschwantler-Kaulich, A. Fink-Retter, C. Haas, G. Hudelist, K. Czerwenka, E. Kubista, Differential gene expression profile in breast cancer-derived stromal fibroblasts, Breast Cancer Res Treat 110 (2008) 273-281.

[29] K. Tomari, T. Kumagai, T. Shimizu, K. Takeda, Bone morphogenetic protein-2 induces hypophosphorylation of Rb protein and repression of E2F in androgen-treated LNCaP human prostate cancer cells, Int J Mol Med 15 (2005) 253-258.

[30] M. Zheng, D. Bocangel, R. Ramesh, S. Ekmekcioglu, N. Poindexter, E.A. Grimm, S. Chada, Interleukin-24 overcomes temozolomide resistance and enhances cell death by down-regulation of O6-methylguanine-DNA methyltransferase in human melanoma cells, Mol Cancer Ther 7 (2008) 3842-3851.

[31] J. Zou, J.H. Ren, D. Feng, H. Wang, J. Xu, Compensatory function of bradykinin B1 receptor in the inhibitory effect of captopril on cardiomyocyte hypertrophy and cardiac fibroblast proliferation in neonatal rats, Chin Med J (Engl) 121 (2008) 1220-1225.

[32] K. Saigusa, I. Imoto, C. Tanikawa, M. Aoyagi, K. Ohno, Y. Nakamura, J. Inazawa, RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest, Oncogene 26 (2007) 1110-1121.

[33] M.M. Mueller, N.E. Fusenig, Friends or foes - bipolar effects of the tumour stroma in cancer, Nat Rev Cancer 4 (2004) 839-849.

[34] S. Hirohata, L.W. Wang, M. Miyagi, L. Yan, M.F. Seldin, D.R. Keene, J.W. Crabb, S.S. Apte, Punctin, a novel ADAMTS-like molecule, ADAMTSL-1, in extracellular matrix, J Biol Chem 277 (2002) 12182-12189.

[35] M. Zeisberg, M. Khurana, V.H. Rao, D. Cosgrove, J.P. Rougier, M.C. Werner, C.F. Shield, 3rd, Z. Werb, R. Kalluri, Stage-specific action of matrix metalloproteinases influences progressive hereditary kidney disease, PLoS Med 3 (2006) e100.

[36] J. Castillo, E. Erroba, M.J. Perugorria, M. Santamaria, D.C. Lee, J. Prieto, M.A. Avila, C. Berasain, Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells, Cancer Res 66 (2006) 6129-6138.

[37] S. Morita, Y. Shirakata, A. Shiraishi, Y. Kadota, K. Hashimoto, S. Higashiyama, Y. Ohashi, Human corneal epithelial cell proliferation by epiregulin and its cross-induction by other EGF family members, Mol Vis 13 (2007) 2119-2128.

[38] B.W. Purow, R.M. Haque, M.W. Noel, Q. Su, M.J. Burdick, J. Lee, T. Sundaresan, S. Pastorino, J.K. Park, I. Mikolaenko, D. Maric, C.G. Eberhart, H.A. Fine, Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation, Cancer Res 65 (2005) 2353-2363.

[39] M. Gonzales, K. Haan, S.E. Baker, M. Fitchmun, I. Todorov, S. Weitzman, J.C. Jones, A cell signal pathway involving laminin-5, alpha3beta1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation, Mol Biol Cell 10 (1999) 259-270.

[40] Y. Shikada, Y. Yonemitsu, T. Koga, M. Onimaru, T. Nakano, S. Okano, S. Sata, K. Nakagawa, I. Yoshino, Y. Maehara, K. Sueishi, Platelet-derived growth factor-AA is an essential and autocrine

regulator of vascular endothelial growth factor expression in non-small cell lung carcinomas, Cancer Res 65 (2005) 7241-7248.

[41] C.G. Lin, S.J. Leu, N. Chen, C.M. Tebeau, S.X. Lin, C.Y. Yeung, L.F. Lau, CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family, J Biol Chem 278 (2003) 24200-24208.

[42] I.B. Lobov, P.C. Brooks, R.A. Lang, Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo, Proc Natl Acad Sci U S A 99 (2002) 11205-11210.

[43] R. Kirchmair, R. Gander, M. Egger, A. Hanley, M. Silver, A. Ritsch, T. Murayama, N. Kaneider, W. Sturm, M. Kearny, R. Fischer-Colbrie, B. Kircher, H. Gaenzer, C.J. Wiedermann, A.H. Ropper, D.W. Losordo, J.R. Patsch, P. Schratzberger, The neuropeptide secretoneurin acts as a direct angiogenic cytokine in vitro and in vivo, Circulation 109 (2004) 777-783.

[44] H. Le Pabic, D. Bonnier, U.M. Wewer, A. Coutand, O. Musso, G. Baffet, B. Clement, N. Theret, ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling, Hepatology 37 (2003) 1056-1066.

[45] K. Hashimoto-Torii, M. Torii, M.R. Sarkisian, C.M. Bartley, J. Shen, F. Radtke, T. Gridley, N. Sestan, P. Rakic, Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex, Neuron 60 (2008) 273-284.

[46] I.T. Tai, M. Dai, L.B. Chen, Periostin induction in tumor cell line explants and inhibition of in vitro cell growth by anti-periostin antibodies, Carcinogenesis 26 (2005) 908-915.

[47] L. Gillan, D. Matei, D.A. Fishman, C.S. Gerbin, B.Y. Karlan, D.D. Chang, Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility, Cancer Res 62 (2002) 5358-5364.

[48] Y. Kudo, I. Ogawa, S. Kitajima, M. Kitagawa, H. Kawai, P.M. Gaffney, M. Miyauchi, T. Takata, Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer, Cancer Res 66 (2006) 6928-6935.

[49] B.S. Siriwardena, Y. Kudo, I. Ogawa, M. Kitagawa, S. Kitajima, H. Hatano, W.M. Tilakaratne, M. Miyauchi, T. Takata, Periostin is frequently overexpressed and enhances invasion and angiogenesis in oral cancer, Br J Cancer 95 (2006) 1396-1403.

[50] R. Shao, S. Bao, X. Bai, C. Blanchette, R.M. Anderson, T. Dang, M.L. Gishizky, J.R. Marks, X.F. Wang, Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression, Mol Cell Biol 24 (2004) 3992-4003.

[51] H. Sasaki, M. Dai, D. Auclair, I. Fukai, M. Kiriyama, Y. Yamakawa, Y. Fujii, L.B. Chen, Serum level of the periostin, a homologue of an insect cell adhesion molecule, as a prognostic marker in nonsmall cell lung carcinomas, Cancer 92 (2001) 843-848.

[52] N. Fukushima, Y. Kikuchi, T. Nishiyama, A. Kudo, M. Fukayama, Periostin deposition in the stroma of invasive and intraductal neoplasms of the pancreas, Mod Pathol 21 (2008) 1044-1053.

[53] Y. Kikuchi, T.G. Kashima, T. Nishiyama, K. Shimazu, Y. Morishita, M. Shimazaki, I. Kii, H. Horie, H. Nagai, A. Kudo, M. Fukayama, Periostin is expressed in pericryptal fibroblasts and cancer-associated fibroblasts in the colon, J Histochem Cytochem 56 (2008) 753-764.

[54] H. Sasaki, C.Y. Yu, M. Dai, C. Tam, M. Loda, D. Auclair, L.B. Chen, A. Elias, Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer, Breast Cancer Res Treat 77 (2003) 245-252.

[55] R.O. Hynes, Integrins: a family of cell surface receptors, Cell 48 (1987) 549-554.

[56] E. Ruoslahti, M.D. Pierschbacher, New perspectives in cell adhesion: RGD and integrins, Science 238 (1987) 491-497.

[57] S. Bao, G. Ouyang, X. Bai, Z. Huang, C. Ma, M. Liu, R. Shao, R.M. Anderson, J.N. Rich, X.F. Wang, Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway, Cancer Cell 5 (2004) 329-339.

[58] P. Baril, R. Gangeswaran, P.C. Mahon, K. Caulee, H.M. Kocher, T. Harada, M. Zhu, H. Kalthoff, T. Crnogorac-Jurcevic, N.R. Lemoine, Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway, Oncogene 26 (2007) 2082-2094.

[59] G. Li, R. Jin, R.A. Norris, L. Zhang, S. Yu, F. Wu, R.R. Markwald, A. Nanda, S.J. Conway, S.S. Smyth, D.N. Granger, Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and alphavbeta5 and focal adhesion kinase (FAK) pathway, Atherosclerosis 208 (2010) 358-365.

[60] W. Yan, R. Shao, Transduction of a mesenchyme-specific gene periostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation, J Biol Chem 281 (2006) 19700-19708.

[61] R. Volpes, J.J. van den Oord, V.J. Desmet, Integrins as differential cell lineage markers of primary liver tumors, Am J Pathol 142 (1993) 1483-1492.

[62] M. Enjoji, H. Sakai, M. Nakashima, H. Nawata, Integrins: utility as cell type- and stage-specific markers for hepatocellular carcinoma and cholangiocarcinoma, In Vitro Cell Dev Biol Anim 34 (1998) 25-27.

[63] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673-687.

ผลงาน (OUTPUT) ของโครงการ

ผลการศึกษาวิจัยในโครงการนี้ได้รับการเผยแพร่ในรูปแบบต่างๆ ได้แก่ การนำเสนอผลงานในที่ประชุมระดับชาติและนานาชาติแบบ Poster presentation จำนวน 8 ครั้ง ได้รับรางวัล 2 ครั้ง การนำเสนอผลงานในที่ประชุมระดับชาติและนานาชาติแบบ Oral presentation จำนวน 2 ครั้ง ได้รับรางวัล 2 ครั้ง และการตีพิมพ์ในวารสารวิชาการระดับนานาชาติที่มี peer review และมี impact factor จำนวน 2 เรื่อง โดยมีค่า impact factor รวมเท่ากับ 6.95 กำลังอยู่ในระหว่างการจัดส่ง 1 เรื่อง และอยู่ในระหว่างการวิเคราะห์ข้อมูลดิบเพื่อการจัดเตรียม manuscript อีก 1 เรื่อง รวมทั้งหมดคาดว่าจะมีผลงานตีพิมพ์ในวารสารวิชาการจากโครงการนี้จำนวน 4 เรื่อง นอกจากนี้ผลงานตีพิมพ์หลักของโครงการนี้ได้รับการพิจารณาให้ได้รับรางวัล **Outstanding Preclinical Research Award** ประจำปี 2552 จากคณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล และนักศึกษาปริญญาเอกที่ทำผลงานดังกล่าวได้นำเสนอผลงานและได้รับรางวัลรองชนะเลิศอันดับ 1 ของ Merck Young Scientist Award ประจำปี 2553 ดังรายละเอียดด้านล่าง

1. นำเสนอผลงานแบบ Poster presentation

1.1 Utispan K, Thuwajit P, Abiko Y, **Thuwajit C** (2008). Profiling of secreted proteins from cholangiocarcinoma-associated fibroblast by protein array chip. In the Third Annual Symposium of Protein Society of Thailand: Frontier in Protein Research, Chulabhorn Research Institute Conference Center, August 28-29, Bangkok, Thailand

1.2 Utispan K, Abiko Y, Thuwajit P, **Thuwajit C** (2008). Genome-wide expression of cancer fibroblasts indicates roles of PN in cholangiocarcinoma. In the Commission on Higher Education Congress I University Staff Development Consortium, September 5-7, Ambassador City Jomtien, Chonburi, Thailand

1.3 Utispan K, Abiko Y, Thuwajit P, **Thuwajit C** (2008). Gene expression profile of cancer fibroblast indicates roles of PN in cholangiocarcinoma. In the Joint International Tropical Medicine Meeting 2008: Tropical Medicine in the –omics Era, October 13-14, Bangkok, Thailand **(รางวัลชมเชย)**

1.4 Utispan K, Chuaysri C, Thuwajit P, **Thuwajit C** (2008). Role of fibroblasts in cholangiocarcinogenesis: candidate genes and proteins as new therapeutic targets. In the Young Researchers Meet Senior Researchers, October 16-18, Petchaburi, Thailand

1.5 Utispan K, Abiko Y, Thuwajit P, **Thuwajit C** (2009). Periostin promotes cell proliferation and serves as apotential prognostic factor in cholangiocarcinoma. In the Second Biochemistry and Molecular Biology for Regional Sustainable Development. May 7-8, Khon Kaen, Thailand **(รางวัลนำเสนอดีเด่น รางวัลที่ 3)**

1.6 Utispan K, Abiko Y, Thuwajit P, **Thuwajit C** (2009). Cancer fibroblast-derived periostin reveals the potential prognostic factor in cholangiocarcinoma and promotes cancer cell invasion *in vitro*. In the 21st IUBMB International Congress of Biochemistry and Molecular Biology and 12th FAOBMB Congress. August 2-7, Shanghai, China.

1.7 Utispan K, Thuwajit P, Charngkaew K, Paupairoj A, Chau-in S, **Thuwajit C** (2009). Stromal fibroblast-derived periostin promotes cancer progression and serves as diagnostic and prognostic factors in cholangiocarcinoma. In the 5th International Conference on Tumor Microenvironment: Progression, Therapy & Prevention. October 20-24, Versailles, France.

1.8 **Thuwajit C**, Utispan K, Thuwajit P, Charngkaew K, Paupairoj A, Chau-in S (2010). Stromal fibroblast-derived periostin promotes cancer progression and serves as diagnostic and poor prognostic factors in cholangiocarcinoma. In the Mahidol-Kyoto Universities International Symposium 2010. December 15-17, Bangkok, Thailand

2. นำเสนอผลงานแบบ Oral presentation

2.1 **Thuwajit C**. Role of tumor-associated fibroblast in the promotion and progression of cholangiocarcinoma. In the 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine. October 15-17, 2009, Loutraki, Greece. **ร่างวัลการนำเสนอผลงาน Outstanding Achievement Award**

2.2 Utispan S. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. **The 2nd Running-up Merck Young Scientist Award 2010**, Merck Company Thailand

3. การตีพิมพ์ในวารสารระดับนานาชาติ

3.1 Chuaysri C, Thuwajit P, Paupairoj A, Chau-in S, Suthipongchai T, **Thuwajit C***. Alpha-smooth muscle actin positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma. ***Oncology Reports*** 2009, 21: 957-69 (**Impact factor 1.59**)

3.2 Utispan K, Thuwajit P, Abiko Y, Charngkaew K, Paupairoj A, Chau-in S, **Thuwajit C***. Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. ***Molecular Cancer*** 2010, 9:13 (**Impact factor 5.36**)

3.3 Utispan K, Sonongbua J, Thuwajit P, Chau-in S, Pairojkul C, **Thuwajit C***. Periostin activates integrin alpha5beta1 through AKT-dependent pathway in invasion of chaolangiocarcinoma. manuscript planned to submit to *European Journal of Cancer* (**Impact factor 4.47**)

3.4 **Thuwajit C***, Thuwajit P, Utispan K, Sonongbua J, Charngkaew K, Paupairoj A, Chau-in S. Fibroblast-derived angiogenic factors and the prediction value of microvascular density in CCA progression. manuscript will be submitted to *Modern Pathology* (**Impact factor 4.67**)

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รางวัลที่ได้รับ

1. The 4th **Outstanding Poster Award** on the topic "Reactive stroma in human cholangiocarcinoma: the increased expression of α-smooth muscle actin in cancer-associated fibroblast" in Annual Meeting 2008, Faculty of Medicine, Khon Kaen University, Thailand
2. The 4th **Outstanding Poster Award** on the topic "Gene expression profile of cancer fibroblast indicates roles of periostin in cholangiocarcinoma" in Joint International Tropical Medicine Meeting 2008: Tropical Medicine in the –omics Era, Bangkok, Thailand
3. The 3rd **Outstanding Poster Presentation Award** on the topic "Periostin promotes cell proliferation and serves as a potential prognostic factor in cholangiocarcinoma" In the Second Biochemistry and Molecular Biology for Regional Sustainable Development 2009, Khon Kaen, Thailand
4. The **Outstanding Award for Oral Presentation** on the topic "Role of tumor-associated fibroblast in the promotion and progression of cholangiocarcinoma" in the 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine 2009, Loutraki, Greece
5. The **Outstanding Preclinical Research Award** for the year 2009, Faculty of Medicine Siriraj Hospital, Mahidol University on the topic "Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker."
6. **The 2nd Running-up, Merck Young Scientist Award** for the year 2010 (to undersupervised Ph.D. student) to the publication entitled "Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker"

ภาคผนวก

Publication ที่ 1

(Reprint)

Alpha-smooth muscle actin positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma

Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma

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Abstract. Cancer-associated fibroblasts have been proposed to play a role in promoting carcinogenesis and tumor progression. To our knowledge, no direct evidence concerning fibroblasts in the genesis of cholangiocarcinoma (CCA) has previously been presented. This study aims to assess the value of activated fibroblasts with high alpha-smooth muscle actin (α-SMA) expression as an indicator for survival in CCA patients. The immunohistochemistry results indicated a high expression of α-SMA in CCA fibroblasts which had a statistically significant correlation with larger tumor size (P=0.009) and shorter survival time (P=0.013). The effect of CCA-associated fibroblasts (Cfs) on non-tumorigenic biliary epithelial cells (H-69) and CCA cell lines was investigated *in vitro* and compared to the effect of non-tumorigenic liver fibroblasts (Lfs). The increased proliferation effect of Cfs having high α-SMA on H-69 and 4 CCA cell lines compared to Lfs that expressed low α-SMA was observed. Cell cycle analysis indicated that Cf-derived conditioned-medium and direct Cf-epithelial cell contaction could drive epithelial cells into S+G2/M phases. These results indicate that fibroblasts in CCA stroma express high α-SMA and can be a prognostic indicator for poor patient survival. CCA fibroblasts have proliferative effects which may directly effect tumor promotion and progression of biliary epithelial cells. This warrants further investigation of fibroblasts as alternative therapeutic targets in CCA patients.

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Introduction

Carcinomas develop from cells of epithelial origin that have undergone genetic mutations and consequently result in the dysregulation of normal growth-controlling mechanisms. Research on the genesis of several carcinomas has been focused mainly on the study of tumor cells and considered the tumorigenesis as an independent process governed by the genes carried within the cancer cells themselves. However, changes in tumor stromal cells surrounding the epithelial malignancy have been observed (1). Tumor stroma is composed mainly of fibroblasts with a minority of inflammatory, smooth muscle and endothelial cells. Changes in these stromal cells have been postulated to enhance several tumorigenic phenotypes of the epithelial cells. It is well accepted that epithelial and stromal cells exchange a reciprocal molecular dialogue that ensures organ homeostasis for proper development and function (2,3). Malignant transformation of epithelial cells disrupts such homeostasis causing changes in tissue architecture, adhesion, cell death and proliferation.

Cholangiocarcinoma (CCA), a carcinoma of bile duct epithelium, is a serious health problem in South Asian countries including Thailand, Lao People's Democratic Republic, Vietnam, Cambodia and South China (4). Moreover, the incidence of CCA has been reported to be increasing in America and Europe (5,6). In Thailand the endemic area of CCA is in the Northeastern part of the country which strongly relates to the high incidence of a liver fluke, *Opisthorchis viverrini* infection (7). While in Korea or Japan, the risk factor for CCA is *Clornorchis sinesis* infection (8). In Western countries, the genesis of CCA does not correlate with a liver fluke infection but is associated with chronic inflammation of the bile duct from a variety of etiologies such as sclerosing cholangitis, choledochal cysts and congenital hepatic fibrosis (9-11). Despite the different causes of CCA, it is well recognized that CCA contains abundant fibrous stroma whereas hepatocellular carcinoma has little fibrous tissue (12). Fibrous stromal cells are α-smooth muscle actin (α-SMA) positive and their numbers in CCA

show a significant positive correlation with the degree of tumor fibrosis (13). However, there has been no direct evidence that the level of α-SMA expression in CCA fibroblasts has an impact on either the prognosis or the genesis of this cancer.

Studies of fibroblasts in several tumor types support a role of stromal cells in carcinogenesis (14-16). Fibroblasts with α-SMA expression can also be called activated fibroblasts or myofibroblasts which are the main cellular constituents of reactive stroma in solid tumors and metastases (17). These cancer-associated fibroblasts have been proposed to give a significant impact on the progression of adjacent malignant epithelia. Myofibroblasts in cancer are defined by not only the expression of α-SMA, but also fibroblast activated protein and many other proteins (18,19). Detection of α-SMA in cancer stroma has been investigated in many cancer types, and was proposed to have an association with an aggressive phenotype of cancer cells (20,21). Moreover, cumulative evidence suggests that the degree of activated fibroblasts influence the outcome of disease.

Epithelial-fibroblast interaction has been investigated in many carcinomas and revealed the important role of activated fibroblasts in the promoting action on tumor progression and metastasis (14,15,22-27). In particular, surrounding stromal cells can release growth factors, extracellular matrix proteins and angiogenic factors. Carcinogenic effects induced by cancer-associated fibroblasts have been demonstrated in *in vitro* models of a variety of carcinomas including prostate, breast, colon, head and neck cancers (22-25). A study by Olumi *et al* showed the prostate cancer cell proliferation induced by the capability of fibroblasts to produce growth factors (22). Stromal changes at the invasion front of cancers, mainly the appearance of myofibroblasts, drove tumor invasion (26). Moreover, fibroblast-induced angiogenesis by the production of angiogenic factors has been reviewed in several cancers (27). In cholangiopathic conditions, portal fibroblasts in ligation-induced bile duct-injured rats induced biliary epithelial cell proliferation (28). However, in our review of the literature, there was no direct evidence of the effects of fibroblasts in CCA stroma on human biliary epithelial cells.

Collectively, it is of great interest to study the characteristic of CCA-associated fibroblasts (Cfs) and their clinical relevance, especially in their ability to help determine the prognosis of CCA patients. In addition, the effect of Cfs on biliary epithelial cells is elaborated to explain the roles of fibroblasts in cholangiocarcinogenesis. In the present study, we investigated the expression of α-SMA in CCA tissues and studied the association of its level to the clinicopathological data. In this *in vitro* study, we isolated and characterized Cfs compared to normal liver fibroblasts (Lfs). The co-culture of Cfs with human biliary epithelial cell lines was performed to assess growth-modulating effects of the fibroblasts on biliary epithelial cells. The impact of activated fibroblasts with high α-SMA as a prognostic marker and their potential roles in the genesis of CCA is discussed.

Materials and methods

Cell lines. The H-69 cell line, was used as a representative of non-tumorigenic biliary epithelial cells. It is a SV40 large-T-antigen infected normal biliary epithelial cell line which could induce cells to be immortal. Four CCA cell lines KKU-M213 and KKU-OCA17, well differentiated; KKU-M214, moderately differentiated and KKU-100, poorly differentiated, were kindly donated by Dr Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. H-69 cells were maintained in enriched Dulbecco's minimum essential medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Invitrogen), 100 U/ml penicillin (Gibco, Invitrogen), 100 μg/ml streptomycin (Gibco, Invitrogen), 25 μg/ml adenine (Sigma, St. Louis, MO, USA), 5 μg/ml insulin (Gibco, Invitrogen), 1 μg/ml epinephrine (Sigma), 13.6 ng/ml T3T triiodo-L-thyronine (T3) (Sigma), 8.3 μg/ml holo-transferrin (Gibco, Invitrogen), 0.62 μg/ml hydrocortisone (Sigma) and 10 mg/ml epidermal growth factor (EGF; CytoLab Ltd., Rohovot, Israel). All CCA cell lines were grown in DMEM supplemented with 10% (v/v) FBS and incubated in a 5% CO_2 incubator at 37°C. Cells with passage of less than 10 and more than 90% viability measured by trypan blue staining were eligible to use in the experiments.

Patients and clinical data for α-SMA immunohistochemical analysis. From January 1998 to December 2002, resected specimens from patients with CCA attending the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand, were obtained. Necessary ethical clearance was obtained from the institute's ethics committee. Informed consent was obtained from each patient before surgery was performed.

Medical records of each patient were retrieved and 5-year survival was ascertained from these records. Patients who died within 1 month of surgical intervention were excluded from the study. The demographic, clinical, and macroscopic status documented during surgery in each patient were recorded. Definite diagnosis of CCA and metastasis staging were based on histopathological examination. Some lymph nodes were removed during surgery for staging determination. Tumor size was assessed as the largest diameter in the fresh specimen. Distance from the main tumor and lymph node metastases were recorded in surgical and pathological reports. Staging was defined using the TNM system and classified as stage I-IV (29,30). Stage IV was determined as the metastatic stage. Histological classification was based on the criteria of the WHO (29).

CCA tissue sections and immunohistochemistry for α-SMA. We randomly selected 55 tissues from CCA patients who underwent surgery between 1998 and 2002, without knowledge of clinicopathological features. Tissue sections of 4-μm thickness were prepared from formalin-fixed paraffin-embedded blocks. The sections were deparaffinized with xylene and hydrated through ethanol and water solutions. Antigen retrieval for α-SMA was done by boiling with high pressure for 5 min. After pretreatment, the sections were blocked for endogenous peroxidase activity by incubation in 0.5% (v/v) hydrogen peroxide in methanol on a shaker for 30 min and washed briefly in PBS. Non-specific binding was blocked with 5% (v/v) normal horse serum for 30 min in a

Table I. Primers for RT-PCR used in this study.

Gene names	Accession no.	Primers	Sequences (5'→3')	Product length (bp)
Vimentin	NM_003380	Forward	CAGGTGGACCAGCTAACCAA	152
		Reverse	TGCCAGAGACGCATTGTCA	
Cytokeratin 7	NM_005556	Forward	GATGCTGCCTACATGAGCAA	160
		Reverse	GGGAGCGACTGTTGTCCA	
α-SMA	NM_001613	Forward	CTGTCTTCCCGTCCATCGT	146
		Reverse	TCAGGGTCAGGATGCCTCT	
$ß_2m$	NM_004048	Forward	CATTCCTGAAGCTGACAGCA	150
		Reverse	GACTTTCCATTCTCTGCTGGA	

humidified chamber at room temperature. Mouse monoclonal anti-human α-SMA IgG (Sigma) was incubated overnight at 4°C at the dilution 1:200. HRP-rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody. Tissue sections were incubated with secondary antibody at the dilution of 1:500 for 30 min in a humidified chamber at room temperature. After being washed, the sections were reacted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.1% H_2O_2 in 50 mol/l Tris-HCl pH 7.8. For a negative control, PBS was applied on the section instead of the primary antibody. After development, the slides were counter-stained with Mayer's hematoxylin for 1 min. Finally, the sections were dehydrated through increasing concentrations of alcohol (70, 95 and 100% sequentially). The sections were cleared with xylene three times and were then mounted with permount.

Evaluation of immunohistochemical staining. The levels of α-SMA immunohistochemical staining in CCA tissues were evaluated without matching knowledge of any clinical data. The positive intensity of α-SMA in vascular smooth muscle cells was used as the reference staining value. The α-SMA staining in the fibroblasts within tumor stroma was qualitatively classified into 4 groups based on the assumption of the intensity of α-SMA in the fibroblasts when compared to that in vascular smooth muscle cells as follows: grade 0, no staining of α-SMA in CCA stromal fibroblasts; grade +1, α-SMA intensity was much lower than that in the vascular smooth muscle cells; grade +2, α-SMA intensity was lower than that in the vascular smooth muscle cells; grade +3, α-SMA intensity equaled to that in the vascular smooth muscle cells. For statistical analysis, the 0 and +1 were categorized as low expression, +2 and +3 as high expression.

Establishment of primary culture fibroblasts. Primary fibroblast cultures of three fibroblasts were established from each patient including, normal skin fibroblasts (Sfs) from leftover specimens at the abdominal incision site, non-tumorigenic liver fibroblasts (Lfs) and CCA-associated fibroblasts (Cfs). For Sf primary culture, tissues were minced into 2-3 mm^3 fragments and plated onto culture plates containing cultured medium under cover slips for 1 week. Then the cover glasses were removed when Sfs had grown and were observed around the explants. Cells were trypsinized and cultured in DMEM containing 10% FBS and penicillin/streptomycin as antibacterial agents and amphotericin B as an antifungal agent. Lfs and Cfs were cultured by the mincing technique (31). Briefly, tissues were washed thoroughly in serum-free Ham F-12 (Gibco, Invitrogen) containing 200 U/ml penicillin and 200 μg/ml streptomycin for 4 times to remove contaminating blood cells and prevent microbial contaminations. The tissues were then placed in sterile petri dishes and cut into ~1 mm^3 in size. Then the tissues were transferred to a new petri dish and minced into ~0.2 mm^3 in size. Tissues were minced thoroughly in 20% FBS containing Ham F-12 with 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml epidermal growth factor (CytoLab Ltd.), namely fibroblast-complete medium. The remaining large pieces of tissues were removed by centrifugation at 400 g for 5 min. The cell pellets were washed with 1X PBS once and resuspended in 5-10 ml of fibroblast-complete medium depending on the size of cell pellet and cultured in a 5% CO_2 incubator at 37°C. After three or four passages, the obtained cultured cells were confirmed as fibroblasts and non-epithelial cells, using the vimentin and cytokeratin 7 detection.

Semi-quantitative reverse transcription-PCR analysis. RNA extraction from the fibroblasts was performed once the cells had reached about 70% confluence. Total cellular RNA was prepared using TRIzol reagent (Gibco, Invitrogen) according to the manufacturer's directions. The cDNA was produced by the reverse transcription system using AMV reverse transcriptase (Promega, Madison, WI, USA). Two hundred and fifty micrograms of cDNA were used in the PCR reaction containing 1X PCR buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.08 U Taq polymerase, 0.5X SYBR-Green, 0.04 mM each forward primer and reverse primer. The sequences of PCR primers used are shown in Table I. To avoid the errors of the amount of input cDNA in each reaction, the expression of ß2-microglobulin ($ß_2m$) was measured as the internal control. The RT-PCR reactions were performed in ABI 7500 (Apply Biosystem, CA, USA). The CT obtained from the experimental samples were used to calculate the increases of altered expression level expressed as folds to that of negative control

by the relative quantification formula ($2^{-\Delta\Delta CT}$) as previously reported (32).

Western blot analysis of α-SMA. Western blot analysis of α-SMA in culture fibroblasts were performed as previously reported (33) with minor modifications. One thousand cells were lysed in a protein lysis buffer containing 62.5 mM Tris-HCl pH 6.8, 1.25% (w/v) SDS and 5% (v/v) glycerol. The concentration of total proteins extracted was determined for protein assay. The 30 mg of total proteins were separated on 10% (w/v) sodium dodecylsulfate-polyacrylamine gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) using the transfer buffer containing 25 mM Tris-HCl pH 8.5, 192 mM glycine and 20% (v/v) methanol in a semidry blotter (Semi-phor™, Hoefer Scientific Instrument) at 12 mA for 90 min. Membranes were then placed in the blocking solution containing 5% (w/v) skim-milk, 150 mM NaCl and 6 mM Tris, pH 7.4 at 4˚C overnight to prevent non-specific binding. The membrane was incubated overnight at 4˚C with mouse anti-α-SMA IgG (Sigma) (dilution 1:200) as the first antibody. Anti-mouse IgG conjugated with HRP (Zymed Labolatories, San Francisco, CA, USA) (dilution 1:500) as the secondary antibody was added to the membrane and incubated at room temperature for 1 h. The expression of proteins was detected by enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA). The detection of ß-actin expression was used as an internal control. The intensity of bands obtained from Cfs was compared to those from Sfs and Lfs. For re-probing the membranes, the first and second antibodies were removed by incubating the membranes for 1 h at 65˚C in stripping buffer containing 0.2 M glycine-HCl pH 2.5, 0.05% (v/v) Tween-20 and 0.1 M ß-mercaptoethanol.

Conditioned-medium cell proliferation assay. Conditioned-medium (CM) of all fibroblasts were collected by growing fibroblasts in fibroblast-complete medium for 2 days until cells reached about 80% confluency. Then cells were washed twice with 1X PBS and twice with 1% (v/v) FBS-Ham F-12 and incubated for 48 h. After that, CMs were collected, centrifuged at 1,000 g for 10 min to remove cell debris, sterile filtered and stored at -80˚C until used.

For the CM cell proliferation assay, either the H-69 cell line or CCA cell lines were plated in 96-well plates and then allowed to settle down in 10% (v/v) FBS-Ham F-12 for 2 days. A 1% (v/v) FBS-Ham F-12 was added and incubated for 12 h to keep them at the G1-arrest point. Then Cf-CM, Lf-CM and Sf-CMs were added and incubated for 2 days; 1% (v/v) FBS-Ham F-12 was added as a negative control. After incubation, viable epithelial cells were counted using the MTS assay according to the manufacturer's instructions (Promega). Briefly, 20 μl of MTS solution reagent was added into each well containing 100 μl of culture medium. Then, the plates were incubated for 2 h at 37˚C in a 5% CO_2 incubator. The amount of soluble formazan produced by cellular reduction of the MTS was measured by adding 25 μl of 10% (w/v) SDS to each well to stop the reaction. The absorbance at 490 nm using an ELISA reader was measured and converted to the number of viable cells by comparing to the standard curve. The number of viable epithelial cells after treatment with Cf-CM was compared to those of treatment with Lf-CM and Sf-CM. The number of viable cells cultured in 1% (v/v) FBS-medium was used as a reference value for comparison. The statistical significance was analyzed by t-test.

Cell cycle analysis of biliary epithelial cells treated with Cf-CM was performed under flow cytometry after propidium iodide (PI) staining (Gibco, Invitrogen). Epithelial cells treated with each type of CM were trypsinized and about 1 to $5x10^6$ cells were collected. Then the cell pellet was obtained by centrifugation at 400 g for 5 min and washed with 1X PBS. Cold 70% (v/v) EtOH was added and chilled at 4˚C for at least 4 h, or if needed, at -20˚C for long time storage. The suspension was centrifuged at 400 g for 5 min to remove all of the supernatant. One hundred microlitres of 1X PBS containing 0.2 μl of 50 mg/ml DNase-free RNaseA (Amresco, Solon, OH, USA) were added and incubated in a 37˚C CO_2 incubator for 30 min. Cell pellets were collected after centrifugation. Finally, 50 μl of 1 μg/ml PI solution was added and incubated with protection from light for at least 30 min. Cells were then analyzed for their distribution in the cell cycle by flow cytometry (Cytomic 500, Beckman Counter, Fullerton, CA, USA). The distribution of cells in each stage of the cell cycle was determined. Numbers of epithelial cells in S+G2/M phases of the cell cycle were measured in the condition of treatment with Cf-CM compared to those of treatment with Lf-CM and Sf-CM. The cell cycle status of cells treated in serum starved medium containing 1% (v/v) FBS was used as a negative control. These experiments were repeated three times in replicate culture dishes in the same experiments.

Contact co-culture of fibroblasts and biliary epithelial cells. To study the contact co-culture effect of fibroblasts on biliary epithelial cells, fibroblasts were grown in confluence, and biliary epithelial cells were then layered on top. A green fluorescent dye, CMFDA (5-chloromethyfluorescein diacetate, green fluorescent chloromethyl derivatives of fluorescein diacetate) (Gibco, Invitrogen), was used to stain the fibroblast population adhered on the culture plates prior to addition of the unstained biliary epithelial cells. Fluorescent microscopy as well as flow cytometry easily distinguished the CMFDA-stained fibroblasts from the unstained epithelial cells and allowed separation of populations which then could proceed to the analysis of the cell cycle distribution. This procedure was followed with minor modification of that previously reported (22). Briefly, 10^5 of each primary culture fibroblasts were grown to confluence in 6-well plates in fibroblast-complete medium for a 2-day period. CMFDA staining was accomplished by incubation in 1% (v/v) FBS-Ham F-12 containing 5 μM CMFDA, 100 U/ml penicillin, and 100 mg/ml streptomycin for 45 min at 37˚C. Subsequently, the medium was aspirated, and the fibroblasts were washed twice with serum-free Ham F-12 and further incubated with 10% (v/v) FBS-Ham F-12 for 1 h. Then, epithelial cells growing in serum-free Ham F-12 for 24 h to keep them in the G1-arrest condition, were released from the tissue culture plate by trypsinization, and 10^5 cells were reconstituted with 1% (v/v) FBS-Ham F-12 and then were plated on the CMFDA-stained fibroblasts. The co-culture between fibroblasts and biliary epithelial cells was performed for 4 days. Co-cultured fibro-

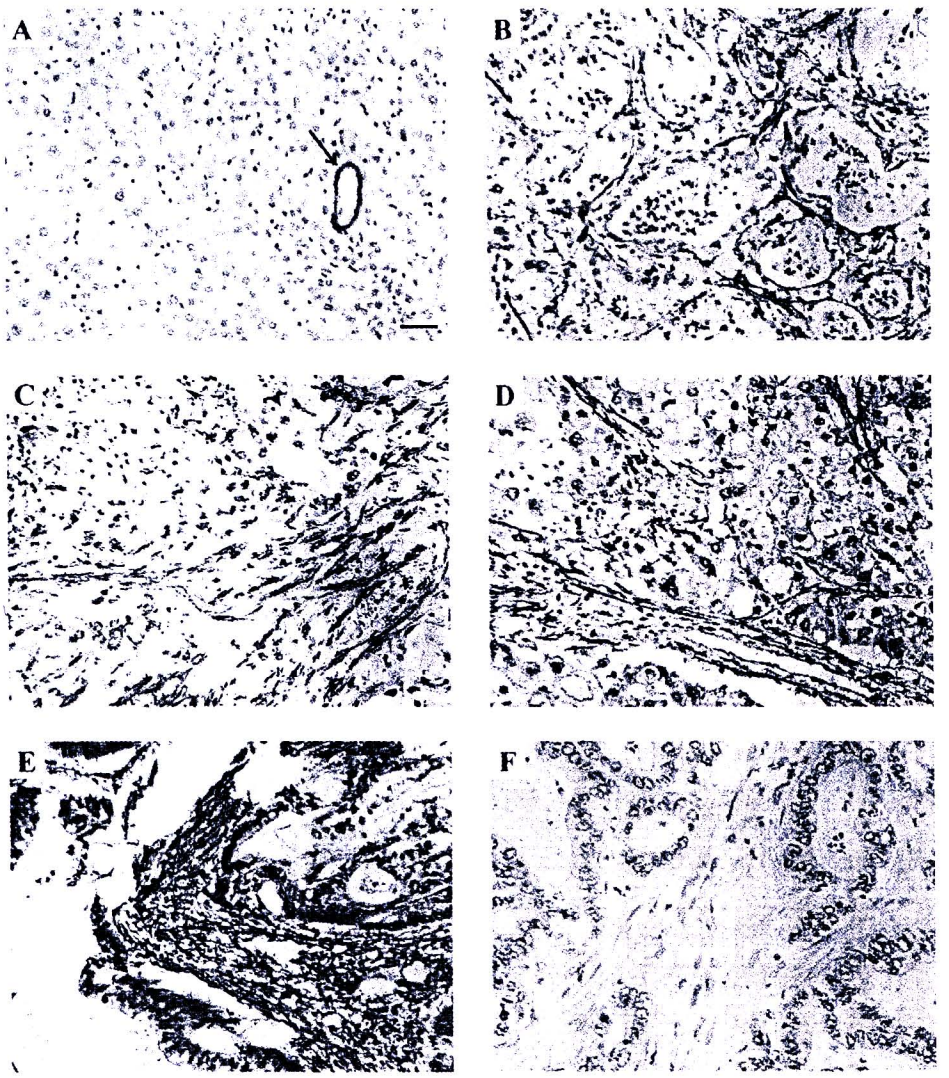

Figure 1. Immunohistochemical staining for α-SMA comparing a section of non-cancerous liver tissue (A) and CCA tissues (B-E). The staining of α-SMA classified as grade +3 in differently differentiated CCA tissues was demonstrated; well differentiated adenocarcinoma (B); moderately differentiated adenocarcinoma (C); poorly differentiated adenocarcinoma (D); papillary adenocarcinoma (E). A negative control without primary antibody is shown (F). Arrow represents the positive α-SMA staining of vascular smooth muscle cells (A). Bar = ~10 μm. (Magnification x200).

blasts and biliary epithelial cells were together trypsinized. Only PI-stained epithelial cells, but not dual dye-stained fibroblasts, were analyzed to determine the number of viable cells and their cycle distribution pattern under flow cytometry as was performed and mentioned earlier in CM cell proliferation assay.

Statistical analysis. Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Comparison of low- and high-grade expression of α-SMA was performed between stratified age groups (≤57 years and >57 years), gender, stage of tumor (stage I-III and stage IV), size of tumor (≤5 cm and >5 cm) and various histological types using Fisher's exact test. A Kaplan-Meier curve was plotted and the log-rank test was used to determine the statistical significance of 5-year survival. For statistical analysis of other experiments including viable cell counts and level of gene expression, t-test analysis was used. P-values of <0.05 were considered statistically significant.

Results

Increased expression of α-SMA in CCA tissues. The positive staining of α-SMA in non-cancerous liver tissue was identified in vascular smooth muscle cells but not stromal fibroblasts (Fig. 1). The expression of α-SMA was observed in fibroblasts of the cancerous area with no staining signals from CCA cells. Despite the different pattern of fibroblasts in the variously differentiated CCA tissues, almost all fibroblasts embedded in the CCA stroma were α-SMA positive. In well differentiated tissue, fibroblasts surrounded the relatively uniform CCA lobules, while α-SMA positive fibroblasts in moderately and poorly differentiated tissue sections appeared to invade nearly all of the CCA cells because of the distorted tubular patterns. Though nearly all stromal fibroblasts were α-SMA positive, the intensity of staining in fibroblasts of each section was compared to that of vascular smooth muscle cells and graded from 0-3 as described in Materials and methods. Despite the different intensities of staining, around

Table II. The correlation between α-SMA expression with clinical data including age, gender, tumor size, tumor staging, histological type, vascular invasion and lymph node metastasis.

Characteristics	Category	n	High	Low	P-value
Age (years)	≤57	28	17	11	1.000
	>57	27	16	11	
Gender	Male	34	21	13	0.782
	Female	21	12	9	
Tumor size (cm)	≤5	20	7	13	0.009*
	>5	35	26	9	
Tumor staging	I-III	10	6	4	1.000
	IV	45	27	18	
Histological type	WD	23	13	10	0.782
	MD	9	7	2	0.289
	PD	8	5	3	1.000
	Pap	15	8	7	0.553
Vascular invasion	Present	12	6	6	0.512
	Absent	43	27	16	
Lymph node metastasis	Present	14	12	2	1.000
	Absent	41	21	20	

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; Pap, papillary; *P<0.05.

98% of total cases were α-SMA positive. Hence, tissues with α-SMA intensity less than a positive control (vascular smooth muscle cells) (grade 0 to +1) were classified as low expression, whereas those with α-SMA intensity more than a positive control (grade +2 to +3) were classified as high expression.

Correlation of α-SMA expression with clinical presentation. Resected specimens from 55 consecutive patients, 34 were male and 21 were female with a male to female ratio of 1.6:1 (Table II). Their median age was 57 years (range, 28-72 years). The comparisons of expression of α-SMA between age groups, gender, tumor size, tumor staging, histological type, vascular invasion and lymph node metastasis are shown in Table II. The expression of α-SMA was similar between, age groups, sexes, tumor staging, various histological types, vascular invasion and lymph node metastasis (P>0.05). However, high α-SMA expression was documented significantly more often in tumor sizes of >5 cm (74%, 26 of 35 cases) than of tumors ≤5 cm (35%, 7 of 20 cases) (P=0.009).

Expression of α-SMA correlates with the survival time. Survival analysis was performed in 52 patients (out of a total of 55 cases) with 5-year follow-up data and excluding the 3 patients with 1-month postoperative period deaths possibly caused by surgical complications. The results showed that 31 patients (60%) had high expression levels of α-SMA, whereas the other 21 patients (40%) had low expression levels (Fig. 2). Five-year survival in the former and latter was 6% (2/31) and 29% (6/21) respectively (P=0.013). Analysis for the correlation

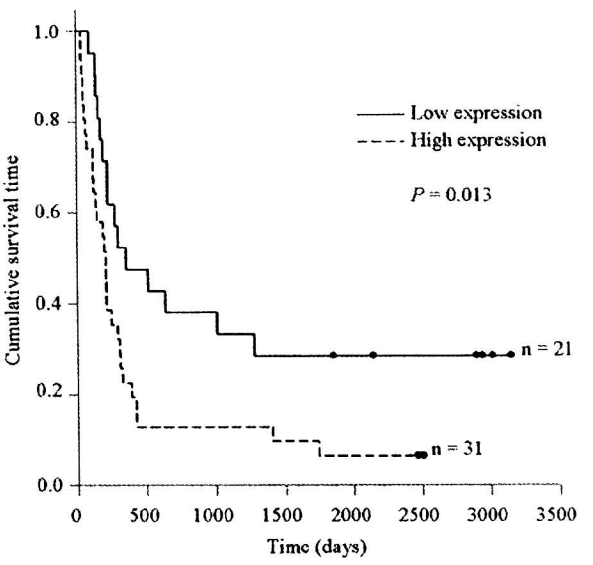

Figure 2. The survival curve using Kaplan-Meier method. Survival time of patients with tumors having a high expression of α-SMA as opposed to patients having low expression tumors is shown. The dots indicate patients who survived >5 years.

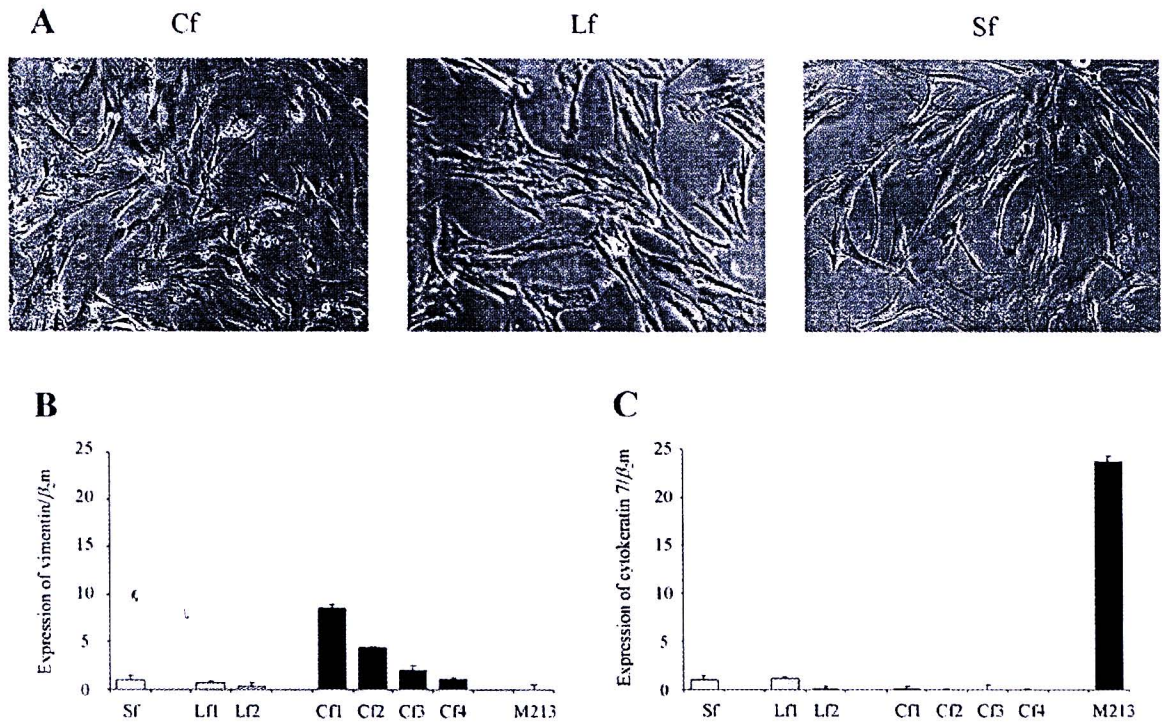

Figure 3. Primary culture fibroblasts from CCA patients including CCA-associated fibroblasts (Cf), non-tumorigenic liver fibroblast (Lf), and skin fibroblast (Sf) (A). (Magnification x100). The spindle-like shape was observed in all three types of fibroblasts. Semi-quantitative RT-PCR of cell specific markers including vimentin (B) and cytokeratin 7 (C), in primary culture fibroblasts is shown. The same number represents the identical patients from whom the primary culture fibroblasts were harvested. The expression of β_2m was used to normalize the amount of cDNA template in each sample. The expression levels in Sf and M213 CCA cell line were used as the positive controls for vimentin and cytokeratin 7, respectively. The values shown represent the mean ± SD of three experiments.

between α-SMA expression and survival time showed that 31 (60%) of the patients who had high expression had a median survival of 205 days and the remaining 21 (40%) with low expression had a median survival of 358 days.

Primary culture fibroblasts expressed a high level of α-SMA and vimentin. We established primary culture fibroblasts from three tissue sources of CCA patients; Sfs from the abdominal incision areas of the patients having undergone hepatectomy, Lfs from a distant area of CCA mass embedded in the hepatectomized liver; and Cfs from tumorigenic area of CCA tissues. The morphology of all primary culture fibroblasts was a spindle-like shape (Fig. 3A).

Real-time PCR for vimentin, a fibroblast marker, demonstrated that all of the primary culture fibroblasts had a certain level of expression more than that of the M213 CCA cell line used as a negative control (Fig. 3B). The expression of vimentin in Cfs showed at a higher level than that observed in Lfs and Sfs and even in different Cfs. We also confirmed the absence of epithelial biliary cell contamination in the primary fibroblast culture by measuring the expression of the cytokeratin-7 epithelium-specific marker. The result revealed a slight expression of cytokeratin-7 in all primary culture fibroblasts compared to that of M213 CCA cell lines used as a positive control (Fig. 3C). To characterize whether these primary culture fibroblasts were activated fibroblasts as detected in CCA tissues, real-time PCR of α-SMA was performed. The results revealed that Cfs had significantly higher expression level of α-SMA than those of Lfs, whereas

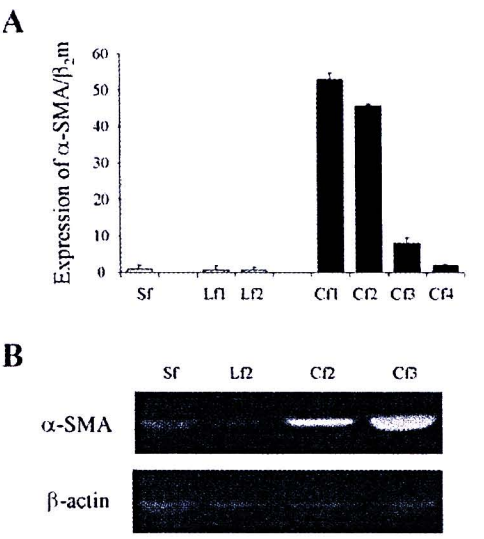

Figure 4. Detection of α-SMA in different primary culture fibroblasts by real-time RT-PCR (A) and Western blot analysis (B). Cfs and Lfs from different CCA patients was studied. The same number represents the identical patients from whom the primary culture fibroblasts were harvested. The values shown represent the mean ± SD of three experiments. In Western blot analysis, the expression of ß-actin was used as an internal control of a housekeeping gene expression level.

the expression levels of α-SMA in Lfs were similar to those of Sfs (Fig. 4A). Western blot analysis revealed higher expression of α-SMA in Cfs than in non-tumorigenic fibroblasts (Fig. 4B).

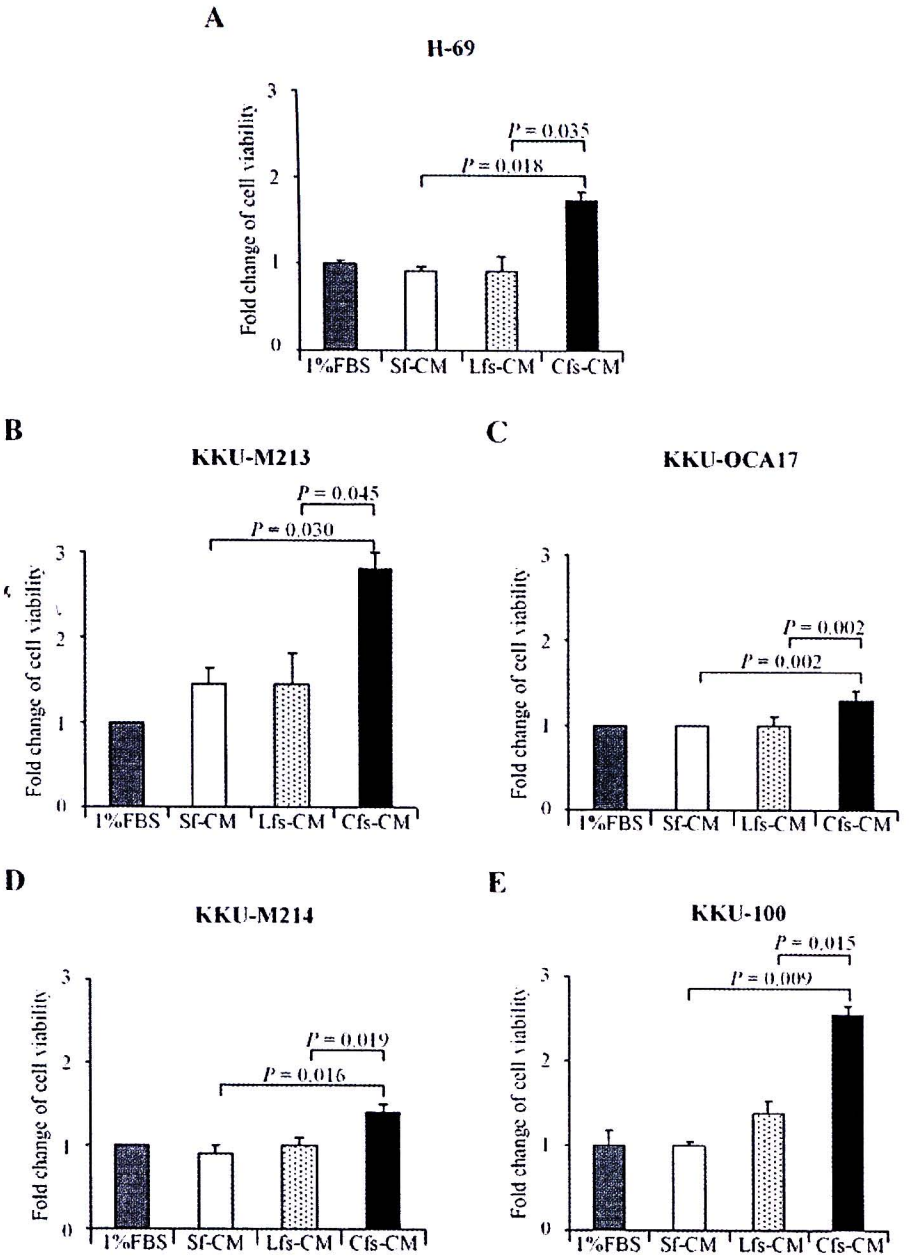

Figure 5. Proliferation effect of fibroblast-derived conditioned-media on different epithelial cells; non-tumorigenic biliary epithelial cells (H-69) (A); and differently differentiated CCA cell lines: well differentiated cell line (KKU-M213) (B), well differentiated cell line (KKU-0CA17) (C), moderately differentiated cell line (KKU-M214) (D), poorly differentiated cell line (KKU-100) (E). Results were indicated as fold change in viable cell number as compared to control medium containing 1% (v/v) FBS. Bars represent the mean ± SD of triplicate experiments.

Cf conditioned-media significantly induced proliferation of biliary epithelial cells. To characterize the fibroblast cultures and compare their putative effect on cancer cells, the biliary epithelial cells of both non-tumorigenic and tumorigenic origin were incubated with different CMs and effects on their proliferation and cell cycle distributions were analyzed. The results revealed a growth-promoting effect on biliary cells with Cf-CM treatment more than Lf-CM and Sf-CM treatments (Fig. 5). The potential of cell proliferation induction as measured by the MTS assay occurred in the following order: Cf-CM > Lf-CM ≥ Sf-CM. This proliferation effect could be seen both in non-tumorigenic biliary epithelial cells; H-69, and tumorigenic biliary epithelial cells or CCA cell lines with different differentiations.

Cell cycle analysis of biliary epithelial cells after being exposed to Cf-CM indicated increased number of cells in S+G2/M phases of the cell cycle in both the H-69 cell line (Fig. 6) and KKU-M213 CCA cell line compared to those either treated with Lf-CM or starved in 1% (v/v) FBS containing media. The number of cells in treatments with Lf-CM and Sf-CM showed quite similar distribution patterns of cells in the cell cycle. This result showed the potential of Cf-CM to induce cell proliferation by driving both non-tumorigenic and tumorigenic biliary epithelial cells into

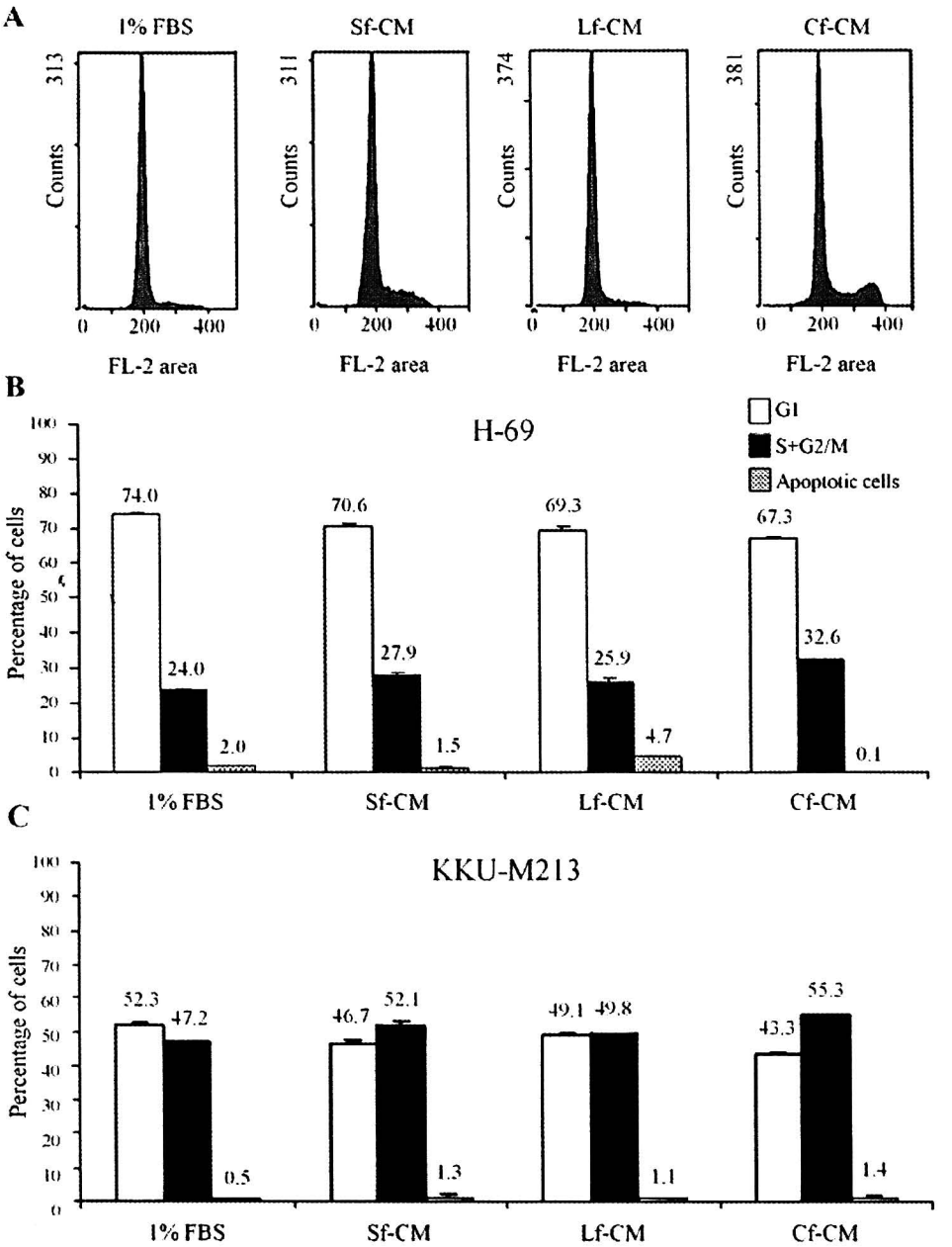

Figure 6. Cell cycle analysis of PI-stained biliary epithelial cells after treatment with different CMs. The cell populations were analyzed for DNA content after PI staining using flow cytometry. The raw data as provided by the FACS machine, which showed the FACS profiles (x-axis, DNA content; y-axis, cell number) of H-69 cell line are shown (A). The cell cycle distribution of the profiles was quantitated and is shown in bar graphs including G1, S+G2/M-phases for H-69 cell line (B) and KKU-M213 cell line (C). G1-arrest cells by culturing in 1% (v/v) FBS were used as the negative control. A higher percentage of biliary epithelial cells in the S+G2/M phases after treatment with Cf-CM was observed compared to those treated with Lf-CM and Sf-CM. Results are expressed as the means ± SD of triplicate experiments.

active proliferative stages of cell cycle. Moreover, the result of Cf-CM treated H-69 showed less apoptotic activity than the H-69 cells treated with Sf-CM or Lf-CM (Fig. 6B). However, this result was not seen in the response of the KKU-M213 CCA cell line (Fig. 6C).

Contact co-culture of Cf and biliary epithelial cells increased biliary cell proliferation and activated cells into the active stages of the cell cycle. Primary culture fibroblasts and biliary epithelial cells were cultured together in the cell-to-cell contact mode. The epithelial cells were plated on-top and were not stained with CMFDA. Cell cycle analysis of biliary epithelial cells activated by direct contact with Cfs revealed more numbers of cells in the proliferative stages of the cell cycle (S+G2/M) than those contact co-cultured with Lfs and Sfs. This phenomenon could be observed in both H-69 and KKU-100 CCA cell lines (Fig. 7).

Discussion

Stromal-epithelial interactions can affect tumor progression from pre-neoplasia to neoplasia. Changes in the stromal

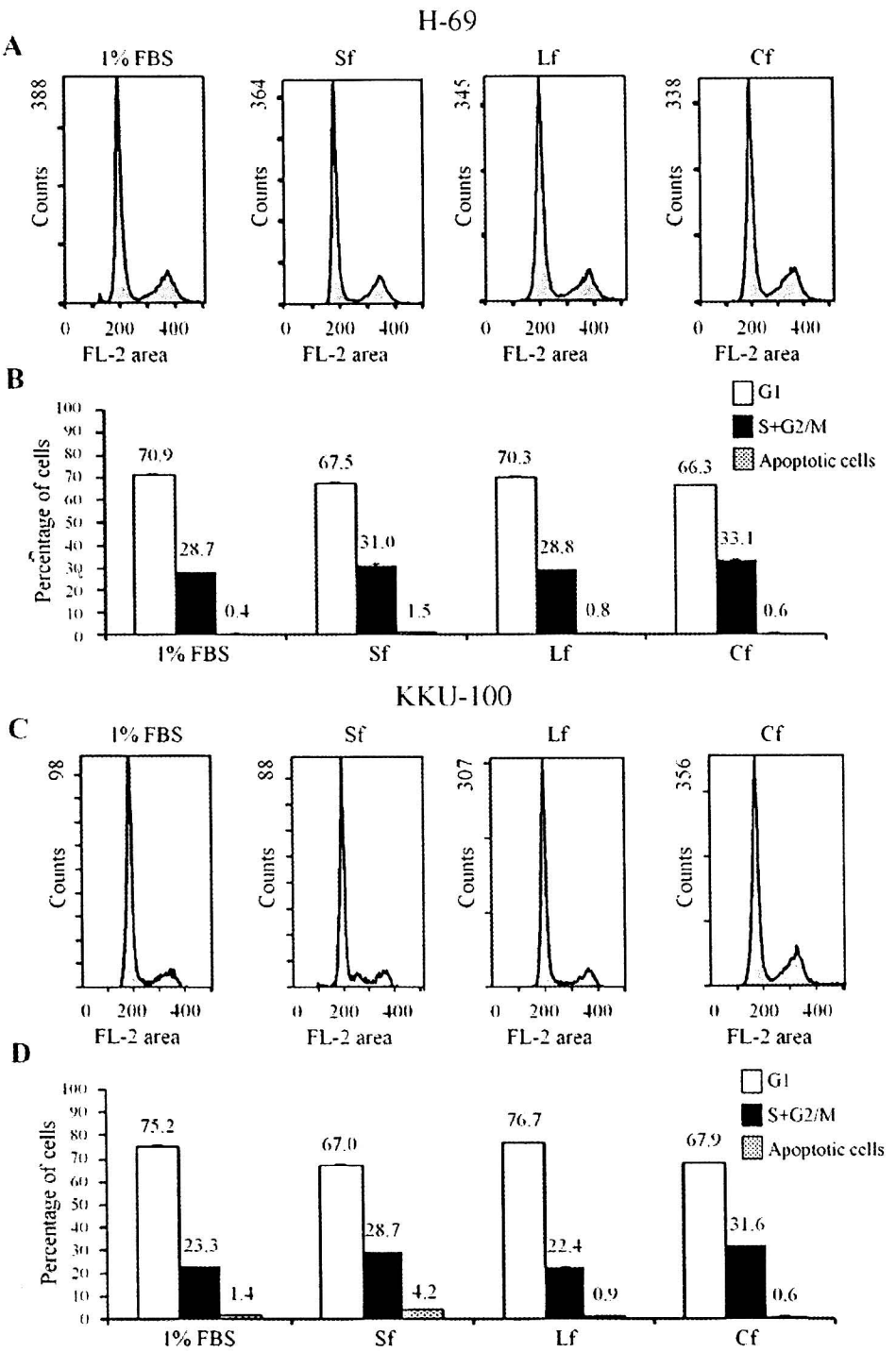

Figure 7. Cell cycle analysis of PI-stained biliary epithelial cells after contact co-culture with different fibroblasts including Sf, Lf and Cf. The cell populations were analyzed for DNA content after PI staining using flow cytometry. The raw data as provided by the FACS machine, which showed the FACS profiles (x-axis, DNA content; y-axis, cell number), are shown for H-69 (A) and KKU-100 (C). The cell cycle distribution was quantitated and is shown in a bar graph including G1, S+G2/M-phases for a non-tumorigenic biliary epithelial cell line (H-69) (B) and KKU-100 (D). Cells cultured in 1% (v/v) FBS containing medium were used as a negative control. Results are expressed as the means ± SD of triplicate experiments.

microenvironment, including changes in fibroblasts, endothelial cells, lymphocytes and extracellular matrix, can potentiate tumor promotion and progression in many cancer types (22-27). Though there have been reports on the detection of activated fibroblasts and the consequence of marked fibrosis in CCA (13,34), the possible effect of activated stromal fibroblasts or myofibroblasts and their impact on the genesis and progression of CCA is not well defined. In this study, we demonstrated the presence of myofibroblasts in CCA tissues by immunohistochemistry and its correlation with patient clinicopathological data. The results of α-SMA expression indicated a high level of this

protein only in fibroblasts embedded in CCA stroma (Fig. 1). No α-SMA expression was observed in fibroblasts embedded in the adjacent area to the tumor mass; not even in the CCA cells. This result confirmed other previous studies showing that fibroblasts in CCA stroma were α-SMA immunoreactive (13,34) and were prominent in the sinusoids surrounding cancer nodules and in the cancerous stroma but not in sinusoids remote from cancer masses (13). Moreover, the number of intratumoral α-SMA positive stromal cells showed a significant positive correlation with the amount of cancerous fibrous stroma in CCA (13). However, the correlation of α-SMA and the prognosis of CCA patients has not been previously reported. We demonstrated that the level of α-SMA expression in CCA fibroblasts correlated with tumor size (Table II) and 5-year survival of the patients with statistical significance (Fig. 2). Patients with high α-SMA expression in the stromal fibroblasts tend to have shorter survival times than patients with low α-SMA. This may be useful in identifying CCA patients with a poor prognosis. Moreover, in regard to our data, the level of α-SMA in CCA fibroblasts correlated with large tumor size with statistical significance (Table II). This implies that CCA fibroblasts may act as a tumor stromal progressing agent in CCA likely by secreting growth promoting factors (22). We conclude that the present data support the notion of CCA fibroblasts as a useful indicator of patient survival, probably by inducing a highly malignant property of cancer cells i.e. increased cell proliferation and decreased apoptosis.

To demonstrate the effect of activated fibroblasts in CCA, we isolated primary culture fibroblasts from fresh CCA tissues, then characterized and studied their biological roles on biliary epithelial cells *in vitro*. From the review of literature, it was found that some cancer cells responded to fibroblasts by direct physical contact between the cells (23,35) whereas some responded to the secreted substances released from the cancer fibroblasts (22,24) and some responded in both modes (22). Herein, we did the experiments in both contact and non-contact co-cultures between CCA fibroblasts and human biliary epithelial cells. The proliferation, distribution in the cell cycle and apoptotic effects of fibroblast-treated epithelial cells were observed. Firstly, the primary culture fibroblasts including Cfs, Lfs and Sfs were confirmed to be without epithelial cell contamination by the presence of vimentin and the absence of cytokeratin-7 (Fig. 3). Cytokeratin-7 has been proven to be one of the important proteins specifically expressed not only in normal biliary epithelium (36), but also in CCA and liver metastases of extrahepatic bile duct cancer (37). For vimentin, it is ubiquitously expressed by cells of mesenchymal origin including fibroblasts, endothelial cells, smooth muscle cells and some other cells (38). Even though Cfs and Lfs were both fibroblasts, our data showed that the expression of vimentin in Cfs was much higher than in Lfs (Fig. 3B). Moreover, the expression level of vimentin in different Cfs was also observed in the same fashion as that of α-SMA expression (Fig. 4A). Since cancer fibroblasts with up-regulation of vimentin were associated with poor patient survival (39), the increased vimentin in Cfs as compared to Lfs found in our study may imply that CCA fibroblasts exhibited a certain phenotype different from that of non-tumorigenic liver fibroblasts. This supports the possibility that Cfs have more tumorigenic functions than Lfs. In concert with the finding in colorectal cancer where vimentin expression was claimed to reflect a higher malignant potential of the tumor, these findings may be useful as a predictive marker for disease recurrence and as a marker of poor prognosis (40). It may imply that CCA patients having cancer fibroblasts with different levels of vimentin may have different prognoses. With the support of further experiments to determine the correlation of vimentin and the prognosis of CCA patients, our findings may suggest the potential to use vimentin expression in CCA stroma fibroblasts as a prognostic indicator.

Portal fibroblasts with the α-SMA positive phenotype have been demonstrated to regulate the proliferation of bile duct epithelial cells in cholangiopathology (28). However, no direct evidence on the effect of CCA fibroblasts on biliary epithelial cells has been reported. In our study, primary culture Cfs showed that the activated phenotype had a higher expression of α-SMA than that in Lfs and Sf (Fig. 4). In the *in vitro* non-contact co-culture used herein, the results showed that Cf-CM significantly promoted biliary cell proliferation to a greater extent than that of either Lfs or Sfs (Fig. 5). Cf-CM treatment to the non-tumorigenic biliary epithelial cells (H-69) dramatically stimulated cell proliferation. In addition, the same effect was seen when CCA cell lines were exposed to Cf-CM. This is in agreement with findings in other cancers where substances released from cancer fibroblasts induced epithelial cell proliferation (22,39). Cancer fibroblasts are capable of modulating the phenotypes of nearby epithelial cells through paracrine signaling mechanisms. They have been documented to secrete a variety of growth factors including transforming growth factor-ß, platelet-derived growth factor, insulin-like growth factor I and II, hepatocyte growth factor/epithelial scatter factor, and fibroblast growth factor (41-46). Most of these factors are predominantly stimulators of proliferation which play an important part in promoting the carcinogenic process. For example, the increases in transforming growth factor-ß expression in breast cancer correlated with the accumulation of fibrotic desmoplastic tissue (47) and increased rate of tumor progression (48). H-69 and CCA cell lines used in this study represent the epithelial cell at different stages of carcinogenesis as follows: H-69 represents cells at the promotion step whereas CCA cell lines represent cells at the progression step. Hence, it may be proposed that Cfs can produce secreted substances which have a proliferative effect on biliary cells of both non-tumorigenic and tumorigenic types, which thus can govern the cholangiocarcinogenesis at both promotion and progression steps. Moreover, the results revealed that Cf-CM exhibited the anti-apoptotic effect on the H-69 cell line when compared to that treatment with either Lf-CM or Sf-CM (Fig. 6B). However, this anti-apoptotic effect could not be seen in CCA cell lines treated with Cf-CM (Fig. 6C). This observation may be supported with the previous finding that prostate cancer progression was associated with suppression of the apoptosis pathway via the activation of PI(3)K substrate (49). This suppression of apoptosis is an early event occurring at the transition from histological normal epithelium to prostate intraepithelial neoplasia.

In addition to the conditioned-medium assays, without direct interaction between biliary epithelial cells and fibroblasts, we demonstrated that culturing biliary epithelial cells on the CCA fibroblasts could influence the biliary cell proliferation compared to those cultured on the normal fibroblasts (Fig. 7). Though, this can not exclude the effects of a paracrine effect from fibroblast-derived substances, the role of cell-cell contact to induce some signal transduction pathway-mediated cell proliferation may be considered. The importance of such signals which govern cell proliferation was published in demonstrating serum-activated fibroblasts promoting clonogenic growth of human breast cancer cells (35). In regard to our study, the CM influence on epithelial cells without any contact seems to show more increased biliary epithelial cell proliferation than that of the contact experiment. This may be due to the fact that even fibroblast-epithelial cell contact could induce cell proliferation but at the same time, may elaborate a cell-cell contact inhibition phenomenon that will retard cell proliferation capacity.

In summary, α-SMA positive fibroblasts induced proliferation of both non-tumorigenic biliary epithelial cells and CCA cells via both secreted substances and cell-cell contact. It is tempting to speculate that CCA fibroblasts may directly promote and influence progression of cholangiocarcinogenesis. In the early event, fibroblasts may induce the non-tumorigenic epithelial cells to have uncontrolled growth by not only stimulating the entering of cells into the cell cycle, but also by inhibiting apoptosis. In the late stage of carcinogenesis, when biliary epithelial cells are completely transformed to CCA cells, induction of cell proliferation seems to have more effect. The activated fibroblasts have been identified to have the ability to produce many soluble factors and reported to modulate various aspects of tumor progression including proliferation or invasion (44,50), angiogenesis (51) or inhibition of cell death (52).

In light of the evidence presented by us, α-SMA expression level should prove beneficial as a predictive marker for the 5-year survival potential of CCA patients. These activated fibroblasts have a critical role to play in the induction of uncontrolled tumor growth and in helping cancer cells to exhibit aggressive malignant behavior that finally attenuates the patient survival time. Regarding the belief that targeting the tumor as an organ would be more effective than targeting the tumor alone, stromal therapy has been proposed to be more flexible and applicable to a wider range of disease stages, as its target is dynamic (53). Furthermore, in hepatocellular carcinoma, chemotherapy was demonstrated to be more effective if therapies against the underlying fibrosis were also employed (54,55). These present experiments support the possibility to further use the fibroblast as a therapeutic target in treatment of CCA patients.

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References

1. Ronnov-Jessen L, Peterson OW and Bissell MJ: Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 76: 69-125, 1996.
2. Cunha GR, Alarid ET, Turner T, Donjacour AA, Boutin EL and Foster BA: Normal and abnormal development of the male urogenital tract: role of androgens, mesenchymal-epithelial interactions and growth factor. J Androl 13: 465-475, 1992.
3. Haffen K, Kedinger M, Simon-Assmann PM and Lacroix B: Mesenchyme-dependent differentiation of intestinal brush-border enzymes in the gizzard endoderm of the chick embryo. Prog Clin Biol Res 85: 261-270, 1982.
4. Vatanasapt V, Sripa B, Sithithaworn P and Mairiang P: Liver fluke and liver cancer. Cancer Surv 33: 313-343, 1999.
5. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P and Thomas HC: Changing international trends in a mortality rates for liver, biliary, and pancreatic tumors. J Hepatol 37: 806-813, 2002.
6. Taylor-Robinson SD, Toledano MB, Arora S, *et al*: Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. Gut 48: 816-820, 2001.
7. Jongsuksuntigul P and Imsomboon T: Opisthorchiasis control in Thailand. Acta Trop 88: 229-232, 2003.
8. Choi D, Lim JH, Lee KT, *et al*: Cholangiocarcinoma and *Clonorchis sinensis* infection: a case-control study in Korea. J Hepatol 44: 1066-1073, 2006.
9. Broome U, Olsson R, Loof L, *et al*: Natural history and prognostic factors in 305 Swedish patients with primary sclerosing cholangitis. Gut 38: 610-615, 1996.
10. Scott J, Shousha S, Thomas HC and Sherlock S: Bile duct carcinoma: a late complication of congenital hepatic fibrosis: case report and review literature. Am J Gastroenterol 73: 113-119, 1980.
11. Lipsett PA, Pitt HA, Colombani PM, Boitnott JK and Cameron JL: Choledochal cyst disease: a changing pattern of presentation. Ann Surg 220: 644-652, 1994.
12. The liver cancer study group of Japan: Primary liver cancers in Japan. Cancer 54: 1747-1755, 1984.
13. Terada T, Makimoto K, Terayama N, Suzuki Y and Nakanuma Y: Alpha-smooth muscle-positive stromal cells in cholangiocarcinomas, hepatocellular carcinomas and metastatic liver carcinomas. J Hepatol 24: 706-712, 1996.
14. Adam L, Crepin M, Lelong JC, Spanakis E and Israel L: Selective interactions between mammary epithelial cells and fibroblasts in co-culture. Int J Cancer 59: 262-268, 1994.
15. Dong-Le Bourhis X, Berthois Y, Millot G, Degeorges A, Sylvi M, Martin PM and Calvo F: Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. Int J Cancer 71: 42-48, 1997.
16. Skobe M and Fusenig NE: Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. Proc Natl Acad Sci USA 95: 1050-1055, 1998.
17. Micke P and Ostman A: Exploring the tumor environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets 9: 1217-1223, 2005.
18. Ronnov-Jessen L, Peterson OW, Koteliansky VE and Bissell MJ: The origin of the myofibroblasts in breast cancer: recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 95: 859-873, 1995.
19. Garrin-Chesa P, Old LJ and Rettig WJ: Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc Natl Acad Sci USA 87: 7235-7239, 1990.
20. Tsujino T, Seshimo I, Yamamoto H, *et al*: Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res 13: 2082-2090, 2007.
21. Gregoire M and Lieubeau B: The role of fibroblasts in tumor behavior. Cancer Metastasis Rev 14: 339-350, 1995.
22. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD and Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 59: 5002-5011, 1999.

23. Sadlonova A, Novak Z, Johnson MR, *et al*: Breast fibroblasts modulate epithelial cell proliferation in three-dimensional *in vitro* co-culture. Breast Cancer Res 7: R46-R59, 2005.
24. Nakagawa H, Liyanarachchi S, Davuluri RV, Auer H, Martin EW Jr, De la Chapelle A and Frankel WL: Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profile. Oncogene 23: 7366-7377, 2004.
25. Tokumaru Y, Fujii M, Otani Y, Kameyama K, Imanishi Y, Igarashi N and Kanzaki J: Activation of matrix metalloproteinase-2 in head and neck squamopus cell carcinoma: studies of clinical samples and *in vitro* cell lines co-cultured with fibroblasts. Cancer Lett 150: 15-21, 2000.
26. De Wever O and Mareel M: Role of tissue stroma in cancer cell invasion. J Pathol 200: 429-447, 2003.
27. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD and Rowley DR: Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. Cancer Res 65: 8887-8895, 2005.
28. Jhandier MN, Kruglov EA, Lavoie EG, Sevigny J and Dranoff JA: Portal fibroblasts regulate the proliferation of bile duct epithelia via expression of NTPDase2. J Biol Chem 280: 22986-22992, 2005.
29. Hirohashi S, Blum HE, Ishak KG, *et al*: Tumours of the liver and intrahepatic bile ducts. In: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. Hamilton SR and Aaltonen LA (eds). IARC press, Lyon, pp157-180, 2000.
30. Yamasaki S: Intrahepatic cholangiocarcinoma: macroscopic type and stage classification. J Hepatobiliary Pancreat 10: 288-291, 2003.
31. Rattanasinganchan P, Leelawat K, Treepongkaruna SA, Tocharoentanaphol C, Subwongcharoen S, Suthipongchai T and Tohtong R: Establishment and characterization of a cholangiocarcinoma cell line (RMCCA-1) from a Thai patient. World J Gastroenterol 12: 6500-6506, 2006.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. Methods 25: 402-408, 2001.
33. Thuwajit C, Thuwajit P, Kaewkes S, Sripa B, Uchida K, Miwa M and Wongkham S: Increased cell proliferation of mouse fibroblast NIH-3T3 *in vitro* induced by excretory/secretory product(s) from *Opisthorchis viverrini*. Parasitology 129: 455-464, 2004.
34. Okamura N, Yoshida M, Shibuya A, Sugiura H, Okayasu I and Ohbu M: Cellular and stromal characteristics in the scirrhous hepatocellular carcinoma: comparison with hepatocellular carcinomas and intrahepatic chaolangiocarcinomas. Pathol Int 55: 724-731, 2005.
35. Samoszuk M, Tan J and Chorn G: Clonogenic growth of breast cancer cells co-cultured in direct contact with serum-activated fibroblasts. Breast Cancer Res 7: R274-R283, 2005.
36. Van Eyken P, Sciot R and Desmet VJ: Immunohistochemistry of cytokeratins in primary human liver tumors. APMIS (Suppl) 23: 77-85, 1991.
37. Fischer HP, Altmannsberger M, Weber K and Osborn M: Keratin polypeptides in malignant epithelial liver tumors. Am J Pathol 127: 530-537, 1987.
38. Dulbecco R, Allen R, Okada S and Bowman M: Functional changes of intermediate filaments in fibroblastic cells revealed by a monoclonal antibody. Proc Natl Acad Sci USA 80: 1915-1918, 1983.
39. Lebret SC, Newgreen DF, Thompson EW and Ackland ML: Induction of epithelial to mesenchymal transition in PMC42-LA human breast carcinoma cells by carcinoma-associated fibroblasts secreted factors. Breast Cancer Res 9: R19, 2007.
40. Ngan CY, Yamamoto H, Seshimo I, *et al*: Quantitative evaluation of vimentin expression in tumor stroma of colorectal cancer. Br J Cancer 96: 986-992, 2007.
41. San Francisco IF, DeWolf WC, Peehl DM and Olumi AF: Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. Int J Cancer 112: 213-218, 2004.
42. Ellis MJ, Singer C, Hornby A, Rasmussen A and Cullen KJ: Insulin-like growth factor mediated stromal-epithelial interactions in human breast cancer. Breast Cancer Res Treat 31: 249-261, 1994.
43. Frazier KS and Grotendorst GR: Expression of connective tissue growth factor mRNA in the fibrous stroma of mammary tumors. Int J Biochem Cell Biol 29: 153-161, 1997.
44. Nakamura T, Matsumoto K, Kiritoshi A and Tano Y: Induction of hepatocytye growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: *in vitro* analysis of tumor-stromal interactions. Cancer Res 57: 3305-3301, 1997.
45. Ponten F, Ren Z, Nister M, Westermark B and Ponten J: Epithelial-stromal interactions in basal cell cancer: the PDGF system. J Investig Dermatol 102: 304-309, 1994.
46. Yan G, Fukabori Y, McBride G, Nikolaropolous S and McKeeham WL: Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptors genes in prostate epithelial cells accompany stromal independence and malignancy. Mol Cell Biol 13: 4513-4522, 1993.
47. Lohr M, Schmidt C, Ringel J, Kluth M, Muller P, Nizze H and Jesnowski R: Transforming growth factor-ß1 induces desmoplasia in an experimental model of human pancreatic carcinoma. Cancer Res 61: 550-555, 2001.
48. Akhurst RJ: TGF-ß antagonists: why suppress a tumor suppressor? J Clin Invest 9: 1533-1536, 2002.
49. Paweletz CP, Charboneau L, Bichesel VE, *et al*: Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene 20: 1981-1989, 2001.
50. Vogetseder W, Feichtinger H, Schulz TF, *et al*: Expression of 7F7-antigen, a human adhesion moleucle identical to intercellular adhesion molecule-1 (ICAM-1) in human carcinomas and their stromal fibroblasts. Int J Cancer 43: 768-773, 1989.
51. Orimo A, Tomioka Y, Shimizu Y, *et al*: Cancer-associated myofibroblasts possess various factors to promote endometrial tumor progression. Clin Cancer Res 7: 3097-3105, 2001.
52. Olumi AF, Dazin P and Tlsty TD: A novel coculture technique demonstrates that nomal human prostatic fibroblasts contribute to tumour formation of LNCaP cells by retarding cell growth. Cancer Res 58: 4524-4530, 1998.
53. Liotta LA and Kohn EC: The microenvironment of the tumour-host interface. Nature 411: 375-379, 2001.
54. Friedman SL, Maher JJ and Bissell DM: Mechanisms and therapy of hepatic fibrosis: report of the AASLD single topic basic research conference. Hepatology 32: 1403-1408, 2000.
55. Bilimoria MM, Lauwers GY, Doherty DA, *et al*: Underlying liver disease, not tumour factors, predicts long-term survival after resection of hepatocellular carcinoma. Arch Surg 136: 528-535, 2001.

Publication ที่ 2

(Reprint)

Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker

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Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker

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Abstract

Background: Fibroblasts play important roles in several cancers. It was hypothesized that cholangiocarcinoma (CCA)-associated fibroblasts (Cfs) differ from non-tumorigenic liver fibroblasts (Lfs) in their gene expression profiles resulting in the capability to promote cancer. Periostin (PN) is a multi-functional protein and has emerged as a promising marker for tumor progression. The role of PN in CCA, however, has not yet been explored.

Results: In this study, the gene expression profile of Cfs in comparison to Lfs was performed using oligonucleotide microarrays. The common- and unique-expressed genes in Cfs and the promising roles in cancer promotion and progression were determined. PN was markedly over-expressed in Cfs confirmed by real time RT-PCR and western blot analysis. Immunohistochemistry examination of a number of patients with intrahepatic CCA showed the expression of PN solely in stromal fibroblasts, but was expressed neither in cancer cells nor immune cells. Low to no expression of PN was observed in tissues of benign liver disease and hepatocellular carcinoma. CCA patients with high levels of PN had significantly shorter survival time than those with low levels ($P = 0.026$). Multivariate analysis revealed high levels of PN ($P = 0.045$) and presence of lymph node metastasis ($P = 0.002$) as independent poor prognostic factors. The *in vitro* study revealed that recombinant PN induced CCA cell proliferation and invasion. Interestingly, interference RNA against integrin α_5 significantly reduced the cellular response to PN-stimulated proliferation and invasion.

Conclusion: The gene expression profile of fibroblasts in CCA is apparently explored for the first time and has determined the genes involving in induction of this cancer progression. High PN can be used to distinguish CCA from other related liver diseases and is proposed as a prognostic factor of poor survival. Regulation of fibroblast-derived PN in CCA proliferation and invasion may be considered as an alternative therapeutic approach.

Background

Cholangiocarcinoma (CCA) originates from biliary epithelial cells and is a unique cancer in northeastern Thailand where the prevalence of a liver fluke, *Opisthorchis viverrini* infection is higher than elsewhere in the country. A recent study showed a strong positive correlation of CCA incidence and the prevalence of *O. viverrini* infection [1]. In other countries, CCA has been shown to correlate with *Clonorchis sinesis* [2,3], and chronic biliary diseases [4]. Even though CCA is caused from the different etiologies, it is well recognized to contain an abundant fibrous stroma that is mainly composed of α-smooth muscle actin (SMA) positive fibroblasts [5,6]. In addition, the degree of α-*SMA* expression has been shown to correlate with the survival of patients, in part, via the ability of these cancer-associated fibroblasts to induce proliferation of bile duct epithelial and cancer cells [6].

The ability of stromal fibroblasts to generate a favorable microenvironment for cancer cells leading to cancer

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development, invasion and metastasis has been summarized [7,8]. Mitotic substances have been produced from stromal fibroblasts to promote tumor growth in many cancers [9,10]. In addition, some matrix metalloproteinases which are often mentioned as proteolytic substances of the extracellular matrix (ECM) and have been reported to show increased production from cancer stromal fibroblasts. These proteolytic substances appear to help to promote cancer cell invasion and metastasis [11]. Specifically, in CCA, stromal-derived factor 1 (SDF-1) has been secreted from stromal fibroblasts into the microenvironment in which it was located at the edge of cancer masses and was proposed to play important role in induction of CCA cell invasion and metastasis [12].

To understand the roles of fibroblasts in carcinogenesis, cancer promotion and progression, gene profiling of cancer fibroblasts have been studied in many cancers [13-15]. Stromal cancer fibroblasts from breast cancer with invasion were compared with the expression profiles of fibroblasts in benign breast disorders. *HYL* (Csk-homologous kinase CHK) involving in regulation of Src kinase, *GM CSF-1* (granulocyte monocyte colony stimulating factor-1) and *osteopontin* were up-regulated which may result in induction of tumor growth and metastasis [13]. Among genes encoded for secreted proteins over-expressed in fibroblasts of human basal cell carcinoma [14], genes including *CTSK* (cathepsin K), *SFRP2* (secreted frizzled-related protein 2), *PDGFRL* (platelet-derived growth factor receptor-like protein), and *DCN* (decorin) were shown to be up-regulated in non-epithelial cells of breast cancer [16]. In contrast, these genes could not be detected in fibroblasts isolated from cancers of pancreas [17], and liver metastases of colon cancer [18]. Taken together, it is possible to say that differential gene expression profile of cancer fibroblasts is partly similar but actually unique for each cancer type. This supports the importance of specific recognition of the concerted performance between fibroblasts and epithelial cells in carcinogenesis and progression in different organs of origin. So it is of great value to investigate the specific gene expression profile of the CCA-derived fibroblasts to help us better understand the molecular mechanisms that fibroblasts use to promote cancer.

Periostin (PN) is a secreted protein which was first identified in bone and implicated in regulating adhesion and differentiation of osteoblasts. The cancer biology role of PN has been investigated in a wide range of cancers including cell proliferation [19,20], migration [21], invasion/metastasis [22,23], and angiogenesis [23,24]. When not regarding the specific sources, either from cancer cells or fibroblasts, secreted PN has been reported to induce tumorigenic properties of epithelial cells via the activation of integrins (ITGs) receptors [21].

Even though substantial evidence has shown that cancer-associated fibroblasts are involved in tumor promotion and with the evidence that fibroblasts in CCA induce more aggressive tumorigenic properties of cancer cells [6], the role of CCA-derived fibroblasts in this cancer is yet to be determined. In the present study, fibroblasts isolated from CCA tissues or CCA-associated fibroblasts (Cfs) which were already characterized by the present group [6], were explored. The genome wide expressions of these Cfs were determined and compared to non-tumorigenic liver fibroblasts (Lfs). The altered expression of genes focusing on the impact of soluble products from Cfs on the promotion and progression of CCA was investigated. Interestingly, PN, which has never been reported in CCA was found at a high level whereas no-to-low PN was detected in non-tumor liver tissues and cancer of hepatocytes. The overexpression of PN in CCA tissues was detected solely in fibroblasts and associated with poor prognosis and short survival of the patients. The effect of PN to induce cell proliferation and invasion has been examined.

Results

Gene expression analysis of Cf and validation by real time RT-PCR

To reduce the genetic background of different patients, the gene expression profile of Cfs was compared to those of two Lfs namely Lf1 and Lf2. Lf1 was isolated from non-tumorigenic liver tissues of hepatectomized liver from the CCA patient who Cfs were originated from. The Lf2 was isolated from the other CCA patient. Genes with differential expressed levels in Cf compared to Lf1 were 3,560 for 2-fold or more up-regulation and 2,339 for 0.5-fold or less down regulation (Fig 1A and 1B). The comparison of the Cf to Lf2 was 4,579 and 3,348 for up- and down-regulation. The common differential genes which are genes altered in their expressions in Cfs when compared to both Lf1 and Lf2 (Cf/Lfs), were 1,466 for up-regulation and 495 for down-regulation. Arylacetamide deacetylase (*DAC*), procollagen C endopeptidase enhancer 2 (*PCPE2*), serpin peptidase inhibitor (*PAI*) and S100 calcium binding protein A4 (*S100A4*) were predominantly over-expressed at high levels in Cfs whereas bone morphogenic protein 2 (*BMP2*), matrix-remodeling associated 5 (*DKFZp564I1922*), bradykinin receptor B1 (*BRADYB1*), response gene to complement 32 (*RGC32*) and interleukin 24 (*IL-24*) were down-regulated with a high array intensity (Table 1).

Most of common differentially expressed genes in Cfs play roles in controlling cellular metabolism (Table 2 and Table 3). The up-regulated genes encoded for secreted proteins were mostly classified in groups of extracellular region, proteolysis, and ECM organization/biosynthesis which took up to 11% of total genes (Table 2). Among

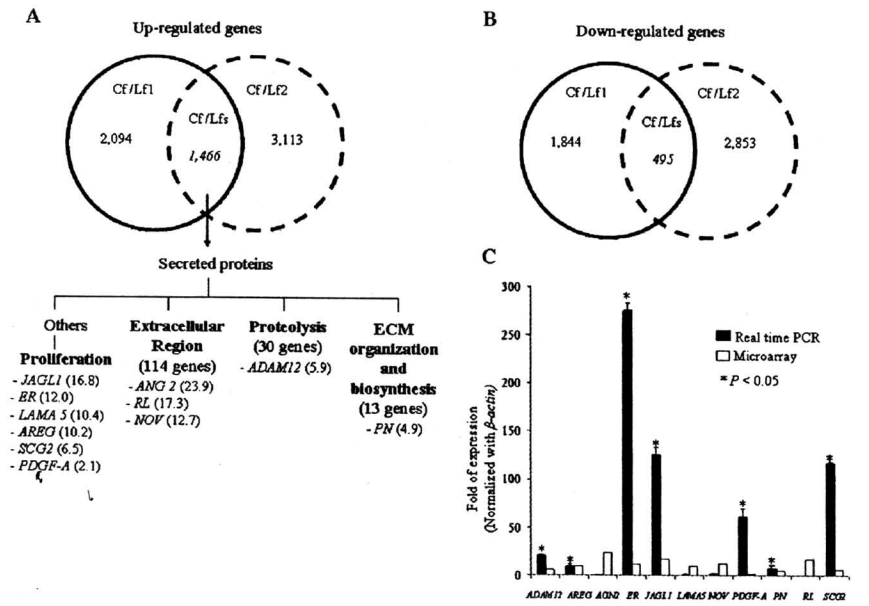

Figure 1 Genome wide expression analysis of Cf and Lfs and gene validation by real time PCR. A Vane diagram showed common up-regulated genes (A) and common down-regulated genes (B) in Cf (Cf/Lfs). In this study, eleven genes encoded secreted proteins involved in induction of epithelial cell tumorigenesis including proliferation, invasion, metastasis and angiogenesis were selected from 4 main different groups of biological functions (A). The numbers in the parentheses represent folds of gene expression level of Cf over those in Lfs. The results show the comparison of gene expression levels measured by real time PCR and oligonucleotide microarray (C). Folds of expression represent fold changes of gene expression level in Cfs as compared to that in Lfs.

these genes in addition to the secreted protein encoding genes that act in cell proliferation and motility, 11 genes having several tumorigenic functions were selected for further exploration including a disintegrin and matrix metalloproteinase 12 (*ADAM12*), amphiregulin (*AREG*), angiopoietin (*AGN2*), epiregulin (*ER*), jagged1 (*JAGL1*), laminin alpha 5 (*LAMA5*), nephroblastoma over expressed (*NOV*), platelet-derived growth factor-α (*PDGF-A*), periostin (*PN*), reelin (*RL*), and secretogranin 2 (*SCG2*) (Fig 1A).

The up-regulated levels of these genes in Cfs were verified by relative quantification using real time RT-PCR. In concordance with microarray data, real time RT-PCR results revealed that *ADAM12*, *AREG*, *ER*, *JAGL1*, *PDGF-A*, *PN* and *SCG2* had significant up-regulations in Cfs compared to Lfs, but that of *NOV* was not statistically significantly increased (Fig 1C). *ANG2*, *LAMA5*, and *RL*, however, showed the opposite direction to the microarray results.

Detection of PN expression in Cf and CCA tissues

Using different biological preparation lots of Cfs from those used in microarray analysis, both real time RT-PCR and western blot analysis confirmed that Cfs had higher expressions of *PN* than Lfs with statistical significance (Fig 2A and 2B). The expression of *PN* in KKU-100, KKU-M055, KKU-M156 and KKU-M213 CCA cell lines was detected at a very low level compared to the high expression in Cfs (Fig 2C).

To check whether the increased expression of *PN* mRNA can be found in CCA tissues, real time PCR was performed using total RNA extracted from pieces of CCA mass. Using *β-actin* and *gapdh* as the internal controls, the results showed the median of *PN* mRNA expression was higher in CCA tissues (4.347 and 2.449 using *β-actin* and *gapdh* respectively) than in benign liver tissues (1.064 and 1.625, respectively) (Fig 2D). This increased up-regulation was not statistically significant. In addition, to achieve the aim to use a rapid method such as real time PCR in place of immunohistochemical detection of PN in CCA tissues, the *PN* mRNA level was related to the intensity of PN immunoreactivity detected by immunohistochemistry. The results indicated the positive correlation of *PN* mRNA level and the encoding protein found in CCA tissues with statistical significance ($P = 0.045$) (Fig 2E).

Expression of PN in CCA tissues and clinicopathological relevance

Immunohistochemistry revealed that the expression of PN was exclusively localized in fibroblasts but not cancer cells (Fig 3). Of all 52 cases, 43 cases or 83% were PN

Table 1 List of top 20 common up-regulated genes and top 20 common down-regulated genes.

Gene Common up-regulated genes	Abbreviation	Intensity of Cf	Mismatch binding	Ratio Cf/Lfs
arylacetamide deacetylase (esterase)	*DAC*	115.92	P	956.45
sparc/osteonectin (testican 3)	*SPOCK3*	16.70	P	669.24
neuropeptide Y receptor Y1	*NPYR*	20.86	P	416.71
collagen, type XIV, alpha 1 (undulin)	*COL14A1*	47.74	P	245.03
growth associated protein 43	*B-50*	23.52	P	232.10
procollagen C-endopeptidase enhancer 2	*PCPE2*	117.81	P	224.83
sorbin and SH3 domain containing 2	*SORB2*	10.65	P	192.25
myozenin 2	*MYOZ2*	6.53	P	139.50
serpin peptidase inhibitor, clade B (ovalbumin), member 2 transcript variant 2	*PAI2*	260.13	P	133.32
doublecortin-like kinase 1	*DCLK*	19.11	P	112.57
formyl peptide receptor-like 2	*FPRL2*	3.24	P	111.02
contactin associated protein-like 3	*CASPR3*	5.09	P	106.75
integrin, beta-like 1 (with EGF-like repeat domains)	*ITGBL1*	13.35	P	82.92
collagen, type IV, alpha 6	*COL4A6*	76.71	P	77.06
myc target 1	*MYCT1*	28.15	P	73.88
S100 calcium binding protein A4	*S100A4*	103.88	P	72.30
phosphodiesterase 1A, calmodulin-dependent	*HSPDEA1*	12.50	P	71.51
neurofilament, light polypeptide 68 kDa	*NEFL*	5.71	P	69.33
ADAMTS-like 1	*ADAMTSR1*	3.24	P	68.55
early B-cell factor 1	*EBF*	4.57	P	60.80
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	*SIAT7E*	0.02	P	3943.41
fibrillin 2 (congenital contractural arachnodactyly)	*FBN2*	0.05	P	1035.17
fibroblast growth factor receptor 2	*FGFR2*	0.04	P	684.03
pregnancy specific beta-1-glycoprotein 5	*PSG*	0.07	P	349.77
Sal-like 1 (Drosophila)	*SALL1*	0.03	P	312.29
membrane metallo-endopeptidase	*MME*	0.05	P	274.25
odz, odd Oz/ten-m homolog 2 (Drosophila)	*TEN-M2*	0.93	P	134.10
R-spondin 3 homolog (*Xenopus laevis*)	*RSPO3*	0.4	P	70.16
bone morphogenetic protein 2	*BMP2A*	1.19	P	68.96
neuroligin 4, Y-linked	*NLGN4Y*	0.09	P	68.62
matrix-remodelling associated 5	*DKFZp564I1922*	1.31	P	57.89
collagen, type IV, alpha 4	*COL4A4*	0.67	P	49.71
bradykinin receptor B1	*BRADYB1*	1.61	P	48.96
microfibrillar-associated protein 4	*MFAP4*	0.3	P	32.40
matrix metallopeptidase 3 (stromelysin 1, progelatinase)	*MMP-3*	0.85	P	30.01
chromosome 13 open reading frame 15	*RGC32*	1.69	P	28.40
fibroblast growth factor 13	*FGF13*	0.38	P	28.15
ephrin receptor A5	*EPHA5*	0.40	P	25.68
interleukin 24	*IL-24*	1.68	P	25.27

Only those having transcripts, not EST or clones in cDNA library are listed.
P = presence to detectable intensity

positive (Table 4). Among these positive cases, 58% of them showed high expression levels. High expression of PN was observed in well- (Fig 3A), moderately- (Fig 3B) and poorly-differentiated malignant tissues (Fig 3C). For PN-negative CCA tissues, only 17% (9/52) were in this group in which no PN was detected in either fibroblasts or cancer cells (Fig 3D). In contrast, benign liver tissues showed no (2/8) to slight (6/8) PN expression. Similar to benign liver tissues, hepatocellular carcinoma revealed low PN expression in their stromal cells (Fig 3E and 3F). Moreover, double immunofluorescence staining revealed co-localization of α-SMA and PN in the fibroblasts within cancerous area (Fig 3G).

Cumulative survival of CCA patients with low or high PN expression in cancer stromal fibroblasts was analyzed using the Kaplan-Meier method. The patients with

Table 2 Gene ontology of common up-regulated genes. Only genes in the top-five ranking of each group are shown.

Gene ontology (%)	Accession no.	Description	Ratio
Cellular metabolism (23.7%)	NM_000909	neuropeptide Y receptor Y1	416.71
	NM_004734	doublecortin-like kinase 1	112.57
	NM_002961	S100 calcium binding protein A4	72.30
	AF208502	early B-cell factor 1	60.80
	AW004016	ST6 beta-galactosamide alpha-2,6 sialyltransferase 2	59.04
Protein binding (20.8%)	BF449063	collagen, type XIV, alpha 1 (undulin)	245.03
	NM_002045	growth associated protein 43	232.10
	AI659533	sorbin and SH3 domain containing 2	192.25
	BF939176	myozenin 2	139.5
	AF333769	contactin associated protein-like 3	106.75
Signal transduction (10.9%)	AW026543	formyl peptide receptor-like 2	111.02
	NM_004791	integrin, beta-like 1	82.92
	NM_005019	phosphodiesterase 1A	71.51
	AF159570	regulator of G-protein signalling 5	49.64
	W67461	angiopoietin-like 1	49.16
Extracellular region (7.8%)	NM_001086	arylacetamide deacetylase (esterase)	956.45
	AI808090	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	669.24
	NM_013363	procollagen C-endopeptidase enhancer 2	224.83
	AI889941	collagen, type IV, alpha 6	77.06
	NM_052866	a disintegrin and metalloproteinase with thrombospondin motif-like 1	68.55
Transcription factor (7.5%)	AF332197	sine oculis homeobox homolog 2	44.20
	AI681917	iroquois homeobox protein 3	35.34
	NM_020639	receptor-interacting serine-threonine kinase 4	29.41
	AK023792	PBX/knotted 1 homeobox 2	29.40
	AF208967	paternally expressed 3	26.74
Protein modification (5.5%)	AW975934	Titin	32.01
	NM_020639	receptor-interacting serine-threonine kinase 4	29.41
	NM_000222	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	25.21
	BF446673	hemicentin 1	18.96
	NM_002848	protein tyrosine phosphatase, receptor type, O	14.58
Receptor (4.8%)	BF941499	G protein-coupled receptor 116	35.62
	L35594	ectonucleotide pyrophosphatase/phosphodiesterase 2	32.11
	NM_002820	parathyroid hormone-like hormone	30.44
	U61276	jagged 1 (Alagille syndrome)	19.60
	AK022548	integrin, alpha 7	15.62
Cell differentiation (4.3%)	AA343027	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	67.06
	NM_000216	Kallmann syndrome 1 sequence	27.91
	AL560266	Fc receptor-like A	19.61
	AA127691	neuropilin 2	19.35
	NM_002506	nerve growth factor, beta polypeptide	19.11
Cell adhesion (3.5%)	NM_006727	cadherin 10, type 2 (T2-cadherin)	55.43
	NM_000072	CD36 molecule (thrombospondin receptor)	40.71
	AL573851	endothelial cell adhesion molecule	22.52
	N69091	protocadherin 17	22.32
	AA489646	protocadherin beta 13	19.36
Cell cycle (2.4%)	NM_003914	cyclin A1	34.8
	NM_015714	G0/G1switch 2	26.62
	NM_001759	cyclin D2	16.45
	NM_001992	coagulation factor II (thrombin) receptor	13.90

Table 2: Gene ontology of common up-regulated genes. Only genes in the top-five ranking of each group are shown. *(Continued)*

	AK024082	Tousled-like kinase 2	11.09
Cell motility (2.2%)	NM_005045	reelin	17.30
	NM_003062	slit homolog 3 (Drosophila)	5.12
	M21121	chemokine (C-C motif) ligand 5	5.06
	NM_014795	zinc finger E-box binding homeobox 2	4.85
	D45864	protein kinase, cGMP-dependent, type I	4.74
Proteolysis (2.0%)	NM_001870	carboxypeptidase A3 (mast cell)	20.01
	NM_024539	ring finger protein 128	12.68
	AL574912	protease, serine, 35	11.59
	NM_001873	carboxypeptidase E	9.45
	NM_000892	kallikrein B, plasma (Fletcher factor) 1	6.40
Cell proliferation (2.0%)	U77914	jagged 1 (Alagille syndrome)	16.76
	NM_004624	vasoactive intestinal peptide receptor 1	13.07
	BF514079	Kruppel-like factor 4 (gut)	12.89
	NM_001432	epiregulin	11.98
	BC003355	laminin, alpha 5	10.36
Apoptosis (1.6%)	NM_002575	serpin peptidase inhibitor	133.32
	NM_000557	growth differentiation factor 5	14.79
	NM_003728	unc-5 homolog C (C. elegans)	9.60
	BF432648	tumor necrosis factor receptor superfamily	8.73
	NM_003551	non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	6.80
ECM organization and biosynthesis (0.9%)	BC001186	protocadherin beta 5	15.90
	M25813	tenascin XB	12.46
	NM_002380	matrilin 2	4.89
	AY140646	periostin, osteoblast specific factor 2	4.89
	NM_004612	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53 kDa	4.56

survival time under 14 d were identified as peri-operative deaths (n = 1) and excluded from the analysis. Median survival time was 395 ± 157 d for patients with low and 179 ± 35 d for patients with high PN expression. We found that the patients with high PN positive fibroblasts had statistically significantly shorter survival times than those with low PN positive fibroblasts (P = 0.026) (Fig 4). The prognostic value of PN expression and other clinicopathological factors among CCA patients was analyzed using multivariate Cox Proportional Hazard Regression model. The results revealed that high PN expression (HR = 2.02, P = 0.045), and the presence of lymph node metastasis (HR = 3.13, P = 0.002) were the independent risk factors for the overall survival of CCA patients after hepatectomy (Table 5). However, lymph node metastasis and other clinical data showed no association with PN expression (Table 6).

PN promotes proliferation and invasion of CCA cells

PN could induce proliferation of KKU-M156, KKU-M213 and KKU-M055 CCA cell lines (Fig 5A-C), but not KKU-100 (Fig 5D). In addition, KKU-M156, KKU-M213 and KKU-M055 responded to the proliferative effect of optimal PN concentration in a time dependent manner with statistical significance at the 24 h-treatment for all cell types (Fig 5E). To reinforce the proliferation effect of PN on CCA cell lines, colony formation assay with and without soft agar were performed and the result indicated the increased numbers of colonies in the condition of PN treatment in comparison to the negative control without PN stimulation (Fig 5F). In addition, flow cytometric analysis indicated an increased number of KKU-M213 and KKU-M156 cells distributed in S+G2/M when exposed to PN (Fig 6A and 6B).

To address the invasion effect of PN on CCA cells, the invasion assay of cell lines with high $ITG\alpha_5$ expression was performed in a Boyden chamber. The results showed that exogenous PN could markedly induce invasion of KKU-M156 and KKU-M213 CCA cell lines up to around 210% and 230% of cells without PN treatment (Fig 6C).

Knockdown of ITGα5 attenuates PN-induced proliferation and invasion

Treatment of CCA cells with si$ITG\alpha_5$ and lipofectamine (mock) for 6 h did not affect cell viability (Fig 7A). The

Table 3 Gene ontology of common down-regulated genes.

Gene ontology (%)	Accession no.	Description	Ratio
Cellular metabolism (31.6%)	NM_030965	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	3943.41
	NM_022969	fibroblast growth factor receptor 2	684.03
	AU152837	Sal-like 1 (Drosophila)	312.29
	NM_007287	membrane metallo-endopeptidase	73.38
	BF589322	R-spondin 3 homolog (Xenopus laevis)	70.16
Signal transduction (17.8%)	NM_001200	bone morphogenetic protein 2	68.96
	NM_000710	bradykinin receptor B1	48.96
	R72286	microfibrillar-associated protein 4	32.40
	NM_004114	fibroblast growth factor 13	28.15
	BE218107	EPH receptor A5	25.68
Transcription factor (11.5%)	AJ277914	LIM homeobox 9	25.01
	NM_001452	forkhead box F2	18.08
	AA705845	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	17.16
	BG261252	ecotropic viral integration site 1	11.58
	NM_020327	activin A receptor, type IB	9.32
Protein modification (7.9%)	AF245505	matrix-remodelling associated 5	57.89
	AA725644	ubiquitin specific peptidase 42	18.73
	NM_001982	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	11.34
	AV727260	protein tyrosine phosphatase, receptor type, D	10.93
	NM_002570	proprotein convertase subtilisin/kexin type 6	9.83
Cell differentiation (6.2%)	NM_000641	interleukin 11	17.51
	BC006454	growth arrest-specific 7	15.20
	M69148	midkine (neurite growth-promoting factor 2)	14.03
	NM_003991	endothelin receptor type B	10.85
	AI758962	EPH receptor A4	7.15
Cell adhesion (5.9%)	NM_001999	fibrillin 2	1035.17
	NM_014893	neuroligin 4, Y-linked	68.62
	AI694562	collagen, type IV, alpha 3	22.21
	NM_005864	embryonal Fyn-associated substrate	8.44
	AU146651	collagen, type XII, alpha 1	4.89
Cell cycle (5.9%)	NM_014059	chromosome 13 open reading frame 15	28.40
	M19701	retinoblastoma 1 (including osteosarcoma)	4.93
	NM_002009	fibroblast growth factor 7	4.44
	NM_014703	Vpr (HIV-1) binding protein	3.92
	AI983033	DEAD/H box polypeptide 12	3.91
Cell motility (4.7%)	NM_002784	pregnancy specific beta-1-glycoprotein 9	69.22
	X99268	twist homolog 1	5.45
	NM_015180	spectrin repeat containing, nuclear envelope 2	3.81
	AI990816	laminin, alpha 1	3.59
	N90777	neuropilin 2	3.57
Cell proliferation (4.5%)	NM_016931	NADPH oxidase 4	5.79
	AF064826	glypican 4	4.31
	NM_004525	low density lipoprotein-related protein 2	3.85
	NM_001963	epidermal growth factor (beta-urogastrone)	3.70
	AF064103	CDC14 cell division cycle 14 homolog A	3.67
Apoptosis (4.0%)	NM_006850	interleukin 24	25.27
	NM_002135	nuclear receptor subfamily 4, group A, member 1	5.48

Table 3: Gene ontology of common down-regulated genes. *(Continued)*

NM_003823	tumor necrosis factor receptor superfamily	4.59
AJ301610	glutamate receptor, ionotropic, kainate 2	3.92
NM_005809	peroxiredoxin 2	3.90

Only genes in the top-five ranking of each group are shown.

reduction of $ITG\alpha_5$ expression was observed to be 88% of that expressed in both KKU-M213 and KKU-M156 CCA cells without transient knockdown of this gene (Fig 7B). The knockdown effect could be detected up to 72 h after si$ITG\alpha_5$ treatment (data not shown). Thus, the subsequent investigations of cell proliferation and invasion were done within 72 h after transient knockdown with si$ITG\alpha_5$.

The reduction of $ITG\alpha_5$ expressions in both KKU-M213 and KKU-M156 CCA cells resulted in a significant decreased response of cells to PN-induced cell proliferation and invasion (Fig 7C and 7D). A 104% of KKU-M213 cell proliferation induction was detected in si$ITG\alpha_5$-treated cells exposed to PN, whereas cell proliferation could increase up to 130% in cells with intrinsic $ITG\alpha_5$ expression (Fig 7C). In the same manner, KKU-M156 showed 118% and 98% of cell proliferation induction observed in cells untreated and treated with si$ITG\alpha_5$. Both $ITG\alpha_5$-knockdown CCA cell lines did not respond to PN-activated cell invasion whereas PN dramatically induced invasion of both cell lines having normal intrinsic $ITG\alpha_5$ expression (168% for KKU-M156 and 172% for KKU-M213) (Fig 7D). Finally, cells with high $ITG\alpha_5$ expressions were more susceptible to PN stimulation to proliferate and invade than cells without or minimal $ITG\alpha_5$ expression.

Discussion

Cancer-associated fibroblasts have been recognized for their impact in the genesis, promotion and progression of many carcinomas and highlighted in several reviews [8,25]. CCA is notoriously associated with dense

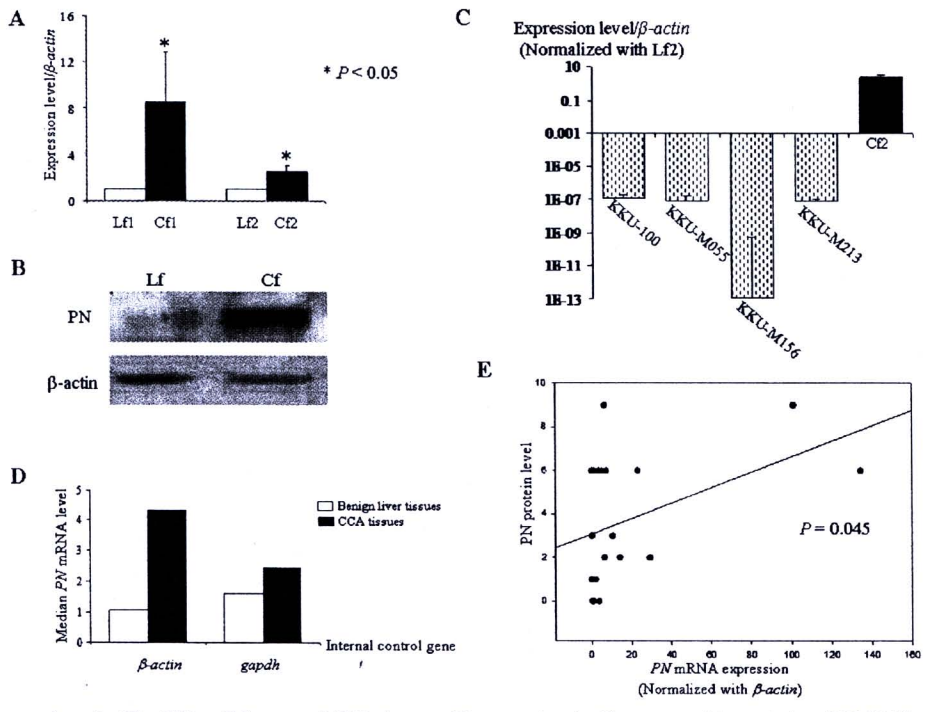

Figure 2 PN expressions in Cfs, CCA cell lines and CCA tissues. *PN* expression in Cfs measured by real time RT-PCR (A) and western blot analysis (B) using different biological preparations of Cfs and Lfs from those used in microarray. The expression of *PN* in CCA cell lines and fibroblasts extracted from CCA tissues is graphically depicted (C). Results are expressed as mean ± SD of three independent experiments. Means of *PN* mRNA expression levels were measured in 20 CCA cases and compared to 5 cases of benign liver diseases using both *β-actin* and *gapdh* as internal controls (D). The positive correlation of mRNA and protein levels of PN in CCA tissues is shown with statistical significance by Spearman correlation analysis (E).

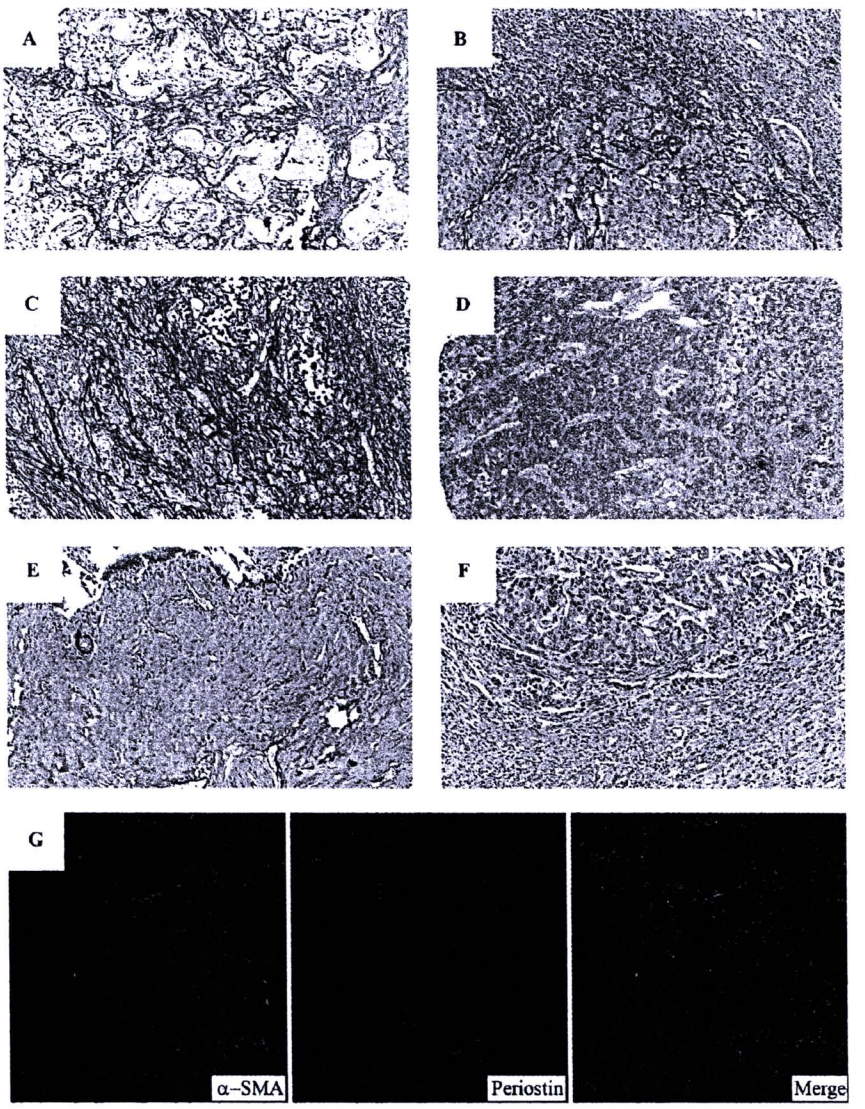

Figure 3 Immunohistochemical staining of PN in CCA tissues. The expression of PN was localized in fibroblasts but not cancer cells. High expression of PN was observed in well- (A), moderately- (B) and poorly-differentiated tissues (C), whereas PN negative staining CCA tissue was demonstrated (D). Benign liver tissue (E) and hepatocellular carcinoma (F) showed no to slight expression. Magnification, 100×. Double immunofluorescence staining showed co-expression of PN and α-SMA in CCA stromal fibroblasts (G). Magnification, 200×.

desmoplastic stroma with activated fibroblasts [5,6]. Relatively little, however, is known about the contribution of the stromal fibroblasts to CCA. The authors in the present group have recently shown that CCA stromal fibroblasts, with and without direct interaction with cancer cells could induce cancer cell proliferation [6]. Herein, this study focused on the gene expression profile of CCA-derived fibroblasts in order to investigate the molecular mechanism of how fibroblasts induce a favorable microenvironment to promote cancer. Even though the current study is limited to a single cancer fibroblast line isolated from a single CCA patient, the validity of array results was strengthened by comparing gene expression levels in cancer fibroblasts to the two lines of normal fibroblasts; one isolated from the same CCA patient and the other from a second patient. Only genes in cancer fibroblasts altered from both normal fibroblast lines were investigated as the common up- or down-regulated genes. This is to provide evidence that the fibroblasts used in our study are valid representatives of fibroblasts found in CCA.

By comparing gene profiles in fibroblasts from CCA with those of other tumor types, it is suggested that CCA fibroblasts display not only common genotypes for

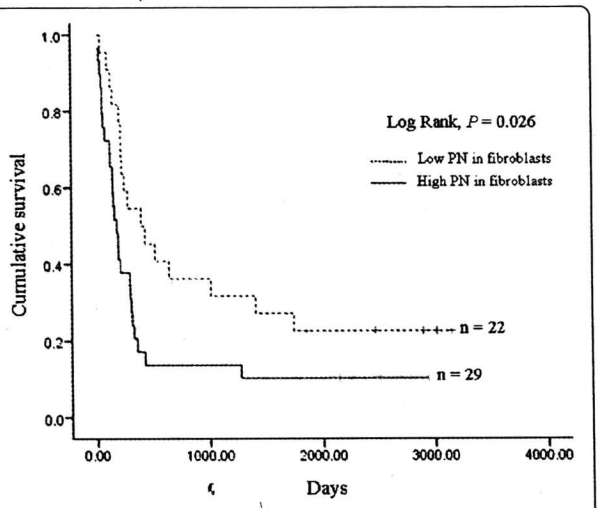

Figure 4 Multivariate analysis using Kaplan-Meier method. Cumulative survival analysis showed significantly shorter survival time of the patients with high PN expression in fibroblasts when compared to those who had low PN expression in fibroblasts (P = 0.026).

activated cells but also unique characteristics. Genes involved in metabolism of cells needed to be up-regulated in order to support the active function of CCA stromal fibroblasts to produce many supporting proteins in the cancer environment. Neuropeptide Y receptor Y1 has been indicated to receive the activation signal to induce neuroproliferation [26] and doublecortin-like kinase 1, a microtubule-associated active protein kinase expressed in growth cones of postmitotic neurons [27] may help facilitate fibroblast proliferation. In similar to human basal cell carcinoma fibroblasts [14], *SPARC* or osteonectin, was also over-expressed in CCA-derived fibroblasts. *SPARC*-null mice were recently demonstrated to resist UV-induced squamous cell carcinoma, suggesting a tumor-promoting role of *SPARC* [28]. In contrast to the cancer-associated fibroblasts in metastatic colon cancer to the liver which showed down-regulation of *SDF-1* [18], CCA-derived fibroblasts had up-regulated *SDF-1* (data not shown).

The highly up-regulated genes in CCA-derived fibroblasts showed several interesting functions involved in cancer progression. Serpin peptidase inhibitor, clade B member 2 (*SERPINE2*) or plasminogen activator inhibitor type 2 (*PAI2*) is involved in cancer invasion and metastasis by controlling serine protease urokinase plasminogen activator. In a recent review, several studies led to the suggestion that the significance of *PAI2* expression on prognosis of cancers is organ context-dependent [29]. In breast cancer, *PAI2* was expressed in both stromal and tumor cells and associated with prolonged disease-free survival [30]. In contrast, high levels of *PAI2* in endometrial cancer were reported to correlate with the invasion potential of the cancer [31]. S100 calcium binding protein A4 (*S100A4*) has been revealed as the metastasis-inducing protein [32]. Genes such as procollagen C-endopeptidase enhancer 2 (*PCPE2*) were also detected which may involve in collagen synthesis [33]. These results support the function of fibroblasts in CCA to promote a desmoplastic reaction. For down-regulated genes, bone morphogenetic protein 2 (*BMP2A*), a multi-functional growth factor belonging to the transforming growth factor-β superfamily was decreased in CCA fibroblasts as reported in breast cancer-derived fibroblasts [13]. *BMP2A*-encoding protein has been elucidated to induce hypophosphorylation of retinoblastoma protein causing cell cycle arrest [34]. Hence, decreased BMP2A in the CCA microenvironment may promote cancer cells to enter the cell cycle. Moreover, a decreased level of interleukin 24 (*IL-24*), an apoptotic inducible cytokine [35], in cancer tissues, attenuates cancer cells from undergoing apoptosis. The response gene to complement 32 (*RGC32*), a novel p53-inducible gene, and bradykinin receptor B1 (*BRADYB1*) decreased expression in CCA-derived fibroblasts. Being intracellular protein and membrane receptor, respectively, *RGC32* and *BRADYB1* have elucidated the function of inhibition of fibroblast cell proliferation [36,37]. It seems possible to conclude that down-regulated genes in fibroblasts encode proteins, if acting in the intracellular region, can inhibit the proliferation of fibroblasts themselves, but if they exist in the extracellular region, they may involve inhibition of cancer cell proliferation. This evidence strengthens the roles of fibroblast-derived pro-

Table 4 PN expression in CCA tissues compared to benign liver tissues and hepatocellular carcinoma.

Tissues	Total cases (n)	PN expression in fibroblasts		
		Negative	Positive	
			Low	High
CCA	52	9 (17%)	13 (25%)	30 (58%)
Benign liver tissue	8	2 (25%)	6 (75%)	0 (0%)
Hepatocellular carcinoma	4	1 (25%)	3 (75%)	0 (0%)

Table 5 Multivariate analysis by Cox proportional hazard regression model for the evaluation of prognostic factors.

Variable (No. of patients)	No. of dead patients (5-yr survival cut-off)	Hazard ratio (HR)	95% confidence interval (CI)	*P*
Age in years				
≤ 57 (25)	21	1		
>57 (26)	22	1.25	0.62-2.48	0.533
PN expression				
Low (22)	17	1		
High (29)	26	2.02	1.02-4.02	0.045*
Lymph node metastasis				
Absence (36)	28	1		
Presence (15)	15	3.13	1.54-6.35	0.002*
Histological type				
Well-differentiated (20)	16	1		
Moderately-differentiated (8)	8	2.77	1.10-6.98	0.031*
Poorly-differentiated (8)	7	1.64	0.63-4.29	0.310
Papillary (15)	12	0.60	0.25-1.44	0.254
Tumor size (cm)				
≤ 5 (28)	23	1		
>5 (23)	20	1.49	0.76-2.94	0.251

* *P* value of equal or less than 0.05 means statistical significance

teins released into a tumor environment to induce a high proliferative capability of cancer cells.

Fibroblasts have been proposed the bipolar effects in cancers [38]. In our microarray results, *ADAMTS-like 1* (*ADAMTSR1*) was over-expressed in CCA-derived fibroblasts. The ADAMTS-like proteins have been discussed as the enhancers of ADAMTS proteases [39]. Since some ADAMTS have been proven to be anti-angiogenic factors [40] partly via the trapping of vascular endothelial growth factor by thrombospondin motifs of ADAMTS [41]. So up-regulation of *ADAMTSR1* in fibroblasts may inhibit angiogenesis. Moreover, *stromelysin-1* or *MMP-3* which can degrade ECM and induce cancer invasion and metastasis, showed the decreased expression in fibroblasts. Taken together, the increased expression of *ADAMTSR1* and the decreased expression of *MMP-3* may highlight fibroblasts in term of suppressing CCA progression.

Table 6 Correlation between PN expression level and clinicopathological parameters.

Variable	n	PN expression (%)		Univariate analysis	Multivariate analysis	
		Low	High	*P*	HR	*P*
Age in years				0.575		
≤ 57	26	10 (38.5)	16 (61.5)		1	
>57	26	12 (46.2)	14 (53.8)		0.899	0.870
Sex				0.375		
Female	20	10 (50.0)	10 (50.0)		1	
Male	32	12 (37.5)	20 (62.5)		1.638	0.452
Histological type				0.083		
Well-differentiated	21	8 (38.1)	13 (61.9)		1	
Moderately-differentiated	8	1 (12.5)	7 (87.5)		3.720	0.271
Poorly-differentiated	8	6 (75.0)	2 (25.0)		0.184	0.86
Papillary	15	7 (46.7)	8 (53.3)		0.611	0.505
Tumor size (cm)				0.123		
≤ 5	29	15 (51.7)	14 (48.3)		1	
>5	23	7 (30.4)	16 (69.6)		2.493	0.161
Lymph node metastasis				0.830		
Absence	37	16 (43.2)	21 (56.8)		1	
Presence	15	6 (40.0)	9 (60.0)		1.459	0.590

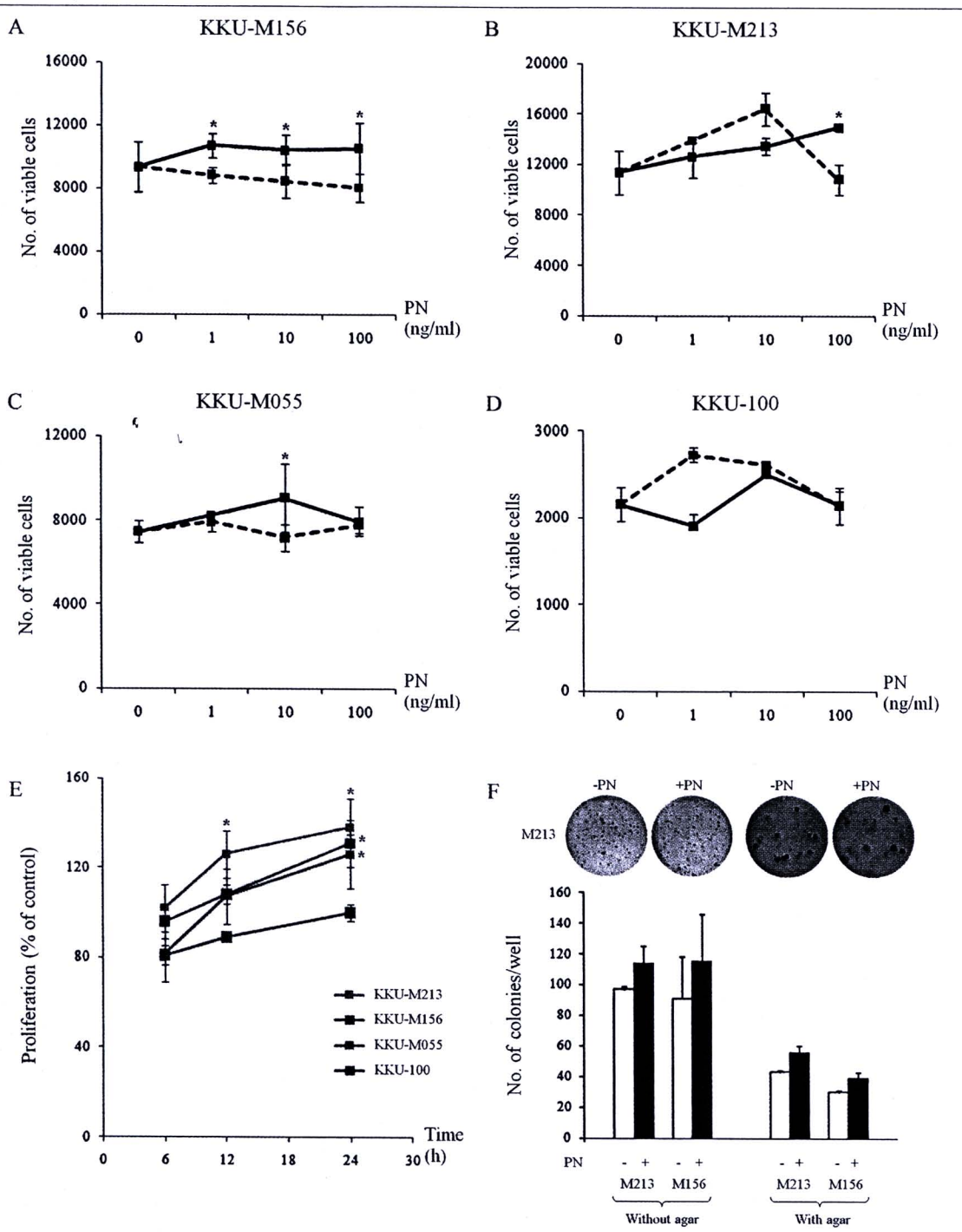

Figure 5 PN promotes cell proliferation of CCA cell lines. KKU-M156, KKU-M213 and KKU-M055 showed significantly induced proliferation with different concentrations of PN (A-C) whereas KKU-100 was unresponsive to PN (D). With an optimal dose of PN for each cell line, KKU-M213, KKU-M156, and KKU-M055 increased cell proliferation in a time-dependent manner (E). Triplicate experiments were performed for each assay. Results are expressed as mean ± SD and an asterix represents a *P* value less than 0.05 when compared to the negative controls without PN treatment. Black and dashed lines represent cells with and without PN treatment, respectively. Colony formation assay with and without agar was performed (F). Colony of more than 30 cells was counted under inverted microscope. Numbers of colonies/well in 6-well plate of both KKU-M213 and KKU-M156 CCA cell lines were higher in condition of PN treatment than those without PN. Results are expressed as mean ± SD of duplicate experiments. Pictures of crystal violet-stained cells are of KKU-M213 CCA cells in comparison between with and without PN.

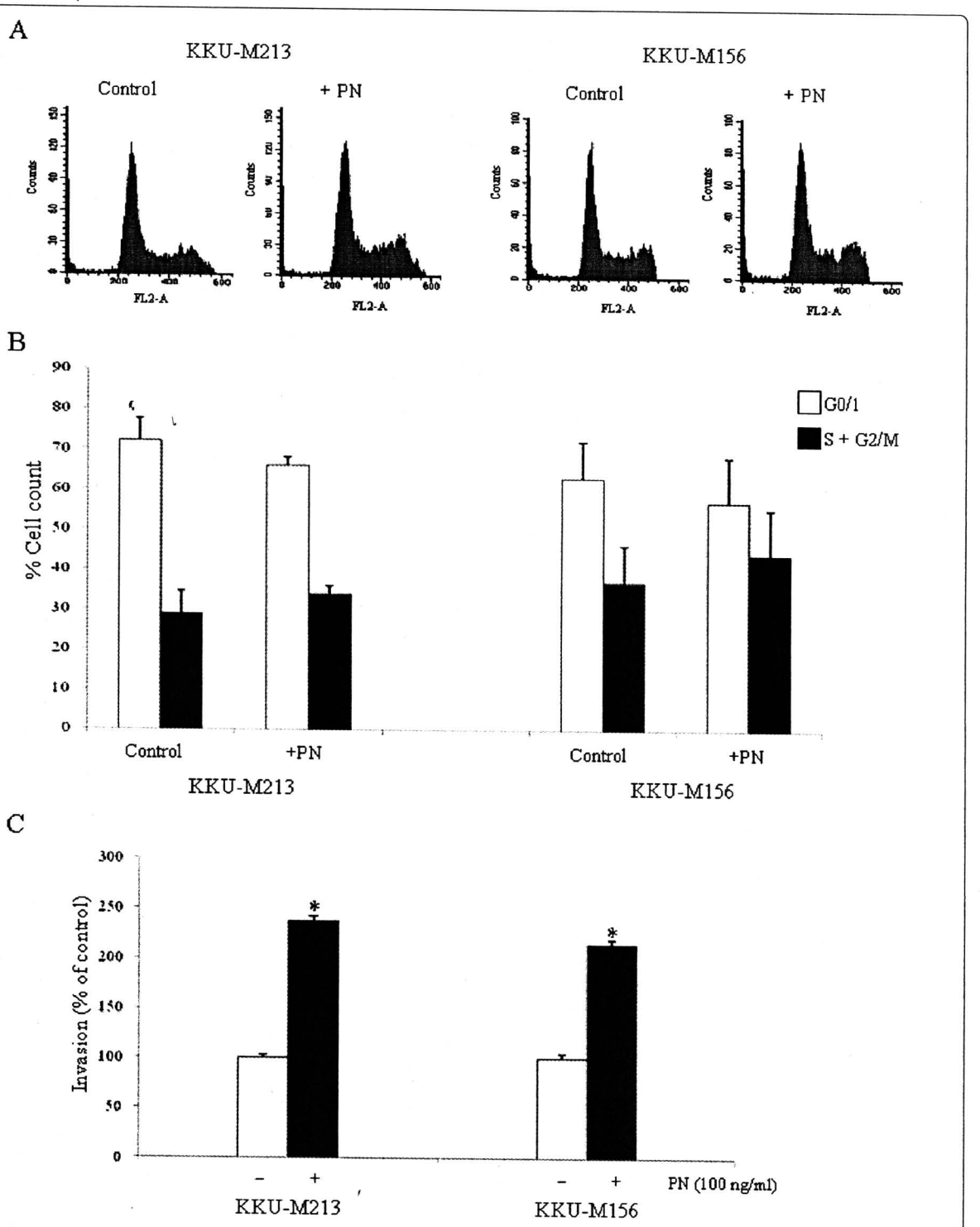

Figure 6 Cell cycle distribution and invasion analysis of cancer cells with and without stimulation by PN. Cell cycle analysis of KKU-M213 and KKU-M156 induced by PN (A). PN could drive cells from G1 into S and G2/M phases of the cell cycle when compared to control cells without PN treatment (B). Invasion induction by PN on KKU-M213 and KKU-M156 CCA cell lines is shown (C). Numbers of invaded cells when no PN was used served as control and were adjusted to be 100% (white bar). The increase of invaded cells induced by PN is observed and shown by a black bar. Each bar graph represents mean ± SD of three independent experiments. An asterix represents a *P* value of less than 0.05.

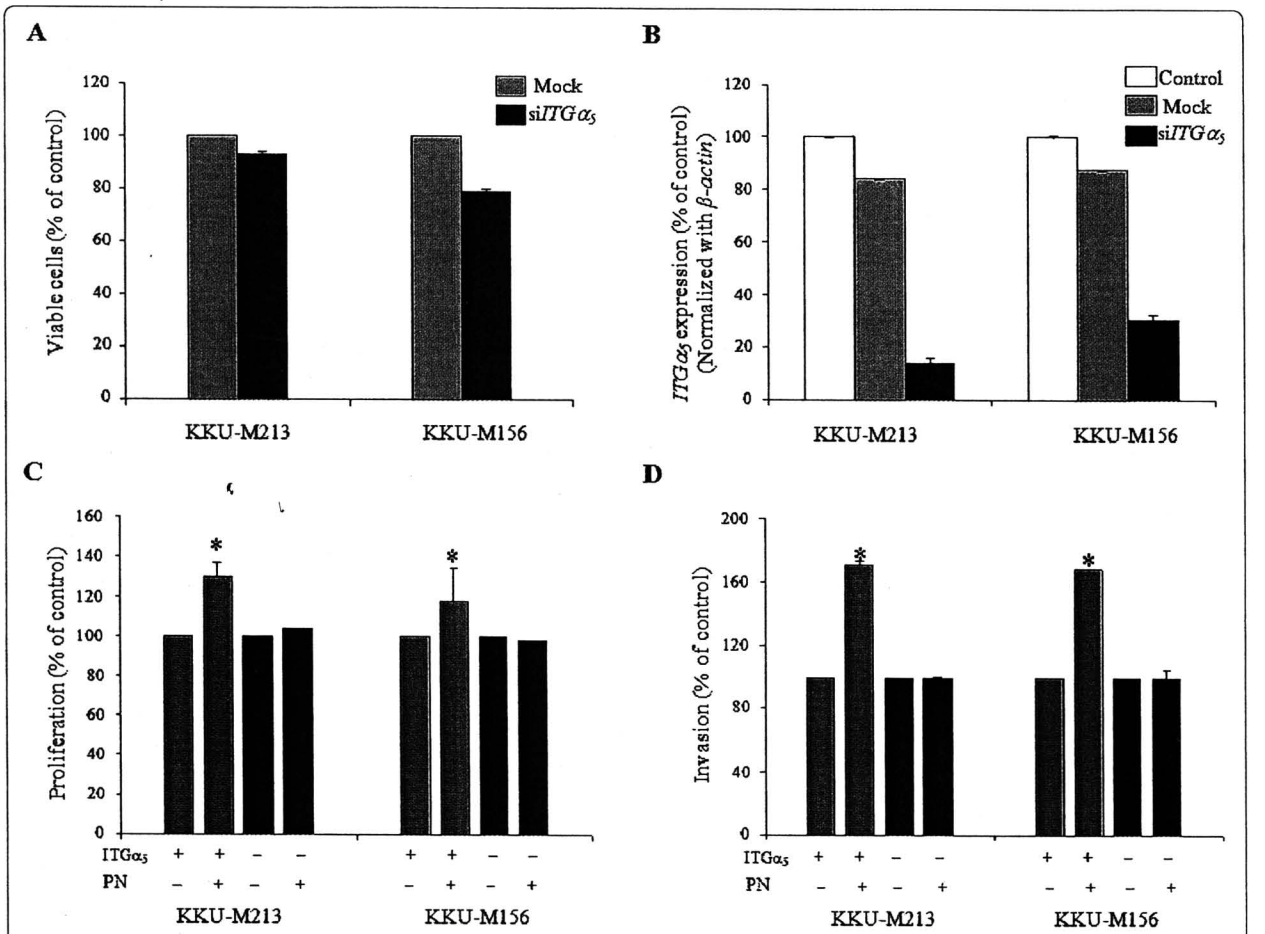

Figure 7 Effect of si*ITGα*$_5$ on PN-induced proliferation and invasion of CCA cells. Lipofectamine-treated cells (mock) had nearly the same viability as si*ITGα*$_5$-treated cells (A). KKU-M213 and KKU-M156 showed dramatically decreased expression of *ITGα*$_5$ detected by real time RT-PCR after exposure to si*ITGα*$_5$ (B). Percentage of proliferation and invasion compared to control mock cells is shown. The si*ITGα*$_5$-treated cells (negative ITGα$_5$) could not respond to PN in induction of proliferation as much as that detected in cells without exposure to si*ITGα*$_5$ (positive ITGα$_5$) (C). A similar effect was observed in PN-induced invasion of CCA cell lines (D). Results represent mean ± SD of three independent experiments and an asterix represents significantly increased cell proliferation and invasion by PN compared to mock cells without PN treatment.

Theoretically, proteins secreted from fibroblasts having interplay with cancer cells could be detected in the extracellular region and be involved in ECM organization and biosynthesis. Within these 2 groups of genes, we focused our interest on genes encoded secreted proteins and their products have been previously reported of their tumorigenic effects. *ADAM12, AREG, AGN2, ER, JAGL1, LAMA5, NOV, PDGF-A, PN, RL,* and *SCG2* were selected to explore. *AREG, ER, JAGL1,* and *LAMA5* are predominantly reported for proliferation induction in cancer cells [42-45]. *PDGF-A, NOV, AGN2,* and *SCG2* are involved in angiogenesis [46-49], whereas *ADAM12* and *RL* play an important role in cell motility, invasion and metastasis [50,51]. For *PN,* many carcinogenic functions including cell proliferation, invasion, metastasis and angiogenesis have been demonstrated [20-24]. This study employed real time PCR to verify the up-regulation of these genes, and found that only *ADAM12, AREG, ER, JAGL1, PDGF-A, PN* and *SCG2* were significantly increased in their expression levels in CCA-derived fibroblasts and may promote CCA progression through activation of cancer growth, invasion and angiogenesis.

Herein PN was chosen to deeply explore since its well accepted multifunction in cancer as mentioned above. Moreover, the result from our group about the expressions of *ADAM12, AREG, ER, JAGL1, PDGF-A, PN* and *SCG2* in whole CCA tissues (n = 20) showed that only *AREG, PDGF-A* and *PN* had higher level in cancer than those in benign liver tissues with statistical significance (data not shown). *AREG* and *PDGF-A* could be detected in not only fibroblasts but also in cancer and endothelial

cells, however *PN* expressed exclusively in CCA fibroblasts. In order to demonstrate role of fibroblast-derived proteins in CCA, we determined that *PN* should be the first target to explore.

The strong evidence using different biological preparations of CCA-derived fibroblasts and CCA tissues confirmed the increased levels of PN at both mRNA and protein. Most of CCA tissues of all differentiated types had high levels of PN and expressed exclusively in α-SMA positive fibroblasts. In the same direction, the findings showed no expression of PN in CCA cell lines when compared to the high level expressed in the fibroblasts. This may strengthen the results of the absence of PN in cancer cells in CCA tissues. From these results taken together, it can be concluded that PN detected in CCA tissues is only of fibroblast origin as reported in some cancers [52-54]. In cancers of head and neck, ovary, and colon, PN was found in cancer cells and has been proposed to induce tumorigenic properties of cancer cells via an autocrine mechanism [21,22]. Hence results from the present study allow the speculation to propose a phenomenon that fibroblast-derived PN in CCA may affect cancer cells by a paracrine mode and has a promising role in cancer promotion. These results revealed that a high PN level in fibroblasts was an independent risk factor in CCA patients and those having high PN had significantly low cumulative survival time after surgery. PN might therefore be used as a poor prognostic marker in patients suffering from CCA. Detections of PN at both mRNA and encoding protein in CCA tissues are in the same direction to distinguish CCA from non-cancer syndromes of bile ducts. In addition, most benign liver tissues and hepatocellular carcinoma showed no to only a minimal expression of PN when compared to the high level detected in CCA tissues. Hence, serum PN may help to distinguish CCA from benign conditions and closely-related liver cancer and may use as the prognostic or predictive marker as previously reported [52,55].

To show the tumorigenic impacts of PN on CCA cells, recombinant PN was employed as extracellular PN to mimic the paracrine effect of PN produced from cancer stromal fibroblasts to induce CCA cell proliferation and invasion. Though receptors ITG$\alpha_v\beta_3$ and ITG$\alpha_v\beta_5$ have been shown to be the receptors for PN in several cancer cells [21], PN promoted invasiveness of pancreatic cancer cells via the β_4 integrin [56]. This suggests the cell type dependent on a specific ITG responded to PN. The study herein reveals that PN-induced cell proliferation and invasion could be inhibited by RNAi against *ITG*α_5. Hence, ITGα_5 is a potentially promising receptor for PN in CCA cells. As the well known receptor for fibronectin, the apparent reason for ITGα_5 production in CCA cells is to support the abundance of fibronectin found in CCA [57]. In addition, ITGα_5 can only be from dimerization with the β_1 subunit and activation of ITG$\alpha_5\beta_1$ has been revealed to support cell survival [58] and induce invasion and angiogenesis [59,60]. Though further studies need to be performed before such a conclusion is valid in CCA, this work highlights the PN-induced-ITGα_5 pathway as one of the activated pathways to induce an aggressive CCA.

TGF-β has been proposed to induce the expression of *PN* [61]. *O. viverrini* excretory/secretory product has also recently been shown to be the stimulator of fibroblast proliferation via the TGF-β-mediated signal transduction pathway [62] and this pathway seems likely to be the cause of *PN* expression in CCA-derived fibroblasts. The authors' laboratory has checked the effect of parasitic product-treated fibroblasts and found that these fibroblasts increased *PN* expression compared to the normal liver fibroblasts without exposure (unpublished data). It is interesting to propose that in CCA cells; the expression of fibroblast-derived PN could be induced by TGF-β produced from infected parasites since the early stage of carcinogenesis and may be in concert with TGF-β produced from CCA cells in a late stage of cancer [12]. Though *in vivo* experiments are needed to confirm, fibroblast-derived PN may influence *O. viverrini*-associated CCA at the early stage of cancer as well as to promote cancer progression in the later time. With this information, targeting the stroma in CCA may not only be effective in treatment of primary, invasive and metastatic tumors, but may also play role in prevention of tumor development.

Conclusions

To the authors' knowledge, this study is the first to describe the gene expression profile of CCA-derived fibroblasts. Molecular understanding of fibroblasts in CCA by the functions of certain up- and down-regulated genes has been revealed and has suggested certain groups of genes in controlling cancer cell proliferation, invasion, metastasis and angiogenesis (Fig 8). These findings provide evidence that fibroblasts are important sources of tumorigenic substances, particularly PN, when produced into the microenvironment of CCA. High levels of PN are found in most CCA patients and can be used as a poor prognostic marker. In addition, the level of PN can be used to distinguish CCA from other benign liver conditions and hepatocellular carcinoma. The interaction of fibroblast-derived PN and CCA cells helps to promote cell proliferation and invasion probably via ITGα_5. Though further investigations are needed, this study suggests promising evidence of the value of using serum PN as a prognostic marker of poor survival in CCA patients. Moreover, targeting fibroblasts or fibroblast-derived-PN-stimulated pathways in cancer cells to attenuate the tumorigenic induction of PN is a further challenge to inhibit CCA progression in the patients.

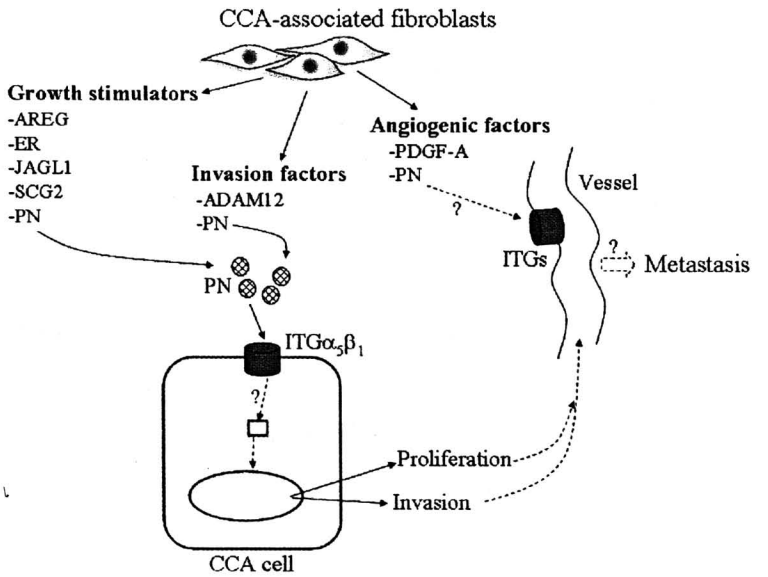

Figure 8 Proposed impacts of CCA-associated fibroblasts revealed in this study. A schematic representation of the main alterations in CCA-associated fibroblasts revealed in this study. The biological functions of protein products from the up-regulated genes in fibroblasts are represented. Tumorigenic effects of PN on CCA cancer cells are also proposed.

Methods

Cells and culture condition

Cfs and Lfs were established and characterized in this lab as previously reported [6]. CCA cell lines including KKU-M213; KKU-M156; KKU-M055; and KKU-100 were received as a kind gift from Associate Professor Dr. Banchob Sripa (Khon Kaen University). Cfs and Lfs were grown in the complete media which is 10% FBS containing DMEM with 20% epidermal growth factor (CytoLab Ltd., Rehovot, Israel). CCA cells were cultured in 10% FBS containing Ham-F12 (Invitrogen, Carlsbad, CA) supplemented with antibiotics and an anti-fungal agent at 37°C and in 5% CO_2 incubator.

Gene expression profiling study and data analysis

Total RNA was extracted using RNeasy Micro Kit (Qiagen, Valencia, CA) using the manufacturer's instructions. The quality of the RNA was assessed by an Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). Affymetrix GeneChip Human Genome U133 plus 2.0 Array containing 38,500 human genes (Affymetrix, Santa Clara, CA) was used following Affymetrix's instruction. The array data were scanned by a GeneChip Scanner 3000 (Affymetrix) and analysed by Affymetrix microarray suite, version 5.0. Raw data from the GeneChips were used to analyze expression levels and expressed as fold changes and gene ontology was categorized by Gene Spring G.X.7.3 software (Agilent Technologies). Average fold change of gene expression was determined by intensity comparison between Cf and Lf1 and Lf2.

Semi-quantitative real time PCR

Complementary DNA was synthesized from 1 μg of total RNA using the first strand cDNA synthesis kit (AMV) (Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions. Relative expression levels in genes of Cfs and Lfs were determined by SYBR Green-based real time PCR using ABI 7500 (Applied Biosystem, Foster City, CA) and calculated by the $2^{-\Delta\Delta C}{}_T$ equation. In this case, $\Delta C_T = C_T$ (Cf)-C_T (Lf). *β-actin* served as an internal control to adjust the amount of starting cDNA. The sequences of genes tested in this study were retrieved from PubMed http://www.ncbi.nlm.nih.gov and the primers were designed by Primer 3 (Table 7).

Human CCA tissues and immunohistochemistry

Fifty-two cases of CCA tissues were obtained from patients who had undergone hepatectomy using the protocol approved by the Human Research Ethics Committee, Khon Kaen University (HE490143). The age, sex, tumor size, histological type and staging data were derived from the medical charts and pathological records. Benign liver tissues were characterized as chronic inflammation by other causes rather than CCA.

Paraffin-embedded tissues were used and antigens were retrieved in 10 mM citrate buffer pH 6.0 at 95°C for 40 min and endogenous peroxidase was blocked in 3% H_2O_2 for 5 min. After blocking non-specific binding with 2% bovine serum albumin for 20 min, 1:10,000 rabbit anti-human PN (Biovendor, Heidelberg, Germany)

Table 7 Primer sequences for real time PCR.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size (bp)	Accession no.
ADAM12	tttgggggtcaacagttttc	agagctgggttccctttgt	191	NM_003474
AREG	tggggaaaagtccatgaaaa	tttcgttcctcagcttctcc	174	NM_001657
AGN2	ccacctgaggaactgtctcg	ggtcttgctttggtccgtta	191	NM_001147
ER	catatgggagaaggggagt	aagtgcaattacagagtgcaaaa	166	NM_001432
JAGL1	gcctgccttaagtgaggaaa	gccaagaacaacacatcaaaga	169	U77914
LAMA5	gtgatgaaaagcgggaatgt	acctccacagagcgagtcat	221	BC003355
NOV	tgcaattccaagaaaatatcactg	cttggatttggagcttggaa	167	NM_002514
PDGF-A	acacgagcagtgtcaagtgc	tctggttggctgctttaggt	250	X03795
PN	cactctttgctcccaccaat	tcaaagactgctcctcccata	157	AY140646
RL	tgctgaatttggggctactt	gggagatagggtcttcatcca	198	NM_005045
SCG2	cccgaagaatgatgataccc	aaatgttgggatttgcttgg	195	NM_003469
ITGα_5	agttgcatttccgagtctgg	ccaaacaggatggctaggat	223	NM_002205
β-actin	cacactgtgcccatctacga	ctccttaatgtcacgcacga	162	X00351
gapdh	ctcctcctgttcgacagtca	gttaaaagcagccctggtga	140	NM_002046

Note: *ADAM12*, a disintegrin and matrix metalloproteinase 12; *AREG*, amphiregulin; *AGN2*, angiopoietin 2; *ER*, epiregulin; *JAGL1*, jagged soluble form; *LAMA5*, laminin alpha 5; *NOV*, nephroblastoma over expressed; *PDGF-A*, platelet-derived growth factor alpha; *PN*, periostin; *RL*, reelin; *SCG2*, secretogranin 2; *ITGα5*, integrin alpha 5; *β-actin*, beta-actin; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase

was applied to the sections at room temperature overnight, followed by anti-rabbit Envision$^+$ System-HRP labeled polymer (Dako, Carpinteria, CA) for 30 min at room temperature. The immunoreactive signal was developed by diaminobenzidine (DAB; Sigma, St Louis, MO) and counterstained with hematoxylin. The signal was checked under light microscope.

PN expression of intratumoral fibroblasts on the histologic sections was semi-quantitatively scored on the basis of PN-positive fibroblasts percentage and the immunostaining intensity. The number of positive fibroblast cells were classified as < 10% (negative); 10-25% (+1); 26-50% (+2); and >50% (+3). The intensity of PN expression in fibroblasts was scored no staining, 0; weak staining, 1; intermediate or focal weak and focal intense staining, 2; intense staining, 3. The interpretation of PN expression was performed by summarization the scores of the percent positive cell (0-3) and the scores of staining intensity (1-3) to reach the total final score of 0-6. The results were then categorized as follows; low expression, score ≤ 4; and high expression, score > 4. All samples were anonymized and independently scored by one pathologist (KC) and 2 investigators (PT and CT). In case of disagreement, the slides were reexamined and a consensus was reached by at least 2 observers.

Double immunofluorescence staining of α-SMA and PN

In order to localize the expression of PN and α-SMA in CCA tissues, double immunofluorescence staining was performed. The 1:200 mouse anti-human α-SMA antibody (Sigma) and 1:500 rabbit anti-human PN antibody (Biovendor) were used as primary antibodies. Anti-mouse IgG-Alexa 488 and anti-rabbit IgG-Cy3 (Invitrogen) were used as the second antibodies. Nucleus was stained with Hoechst (Invitrogen). The signal was observed under the LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany) at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

Protein extraction and western blot analysis

Twenty μgs of total protein from the cell lysate were separated in 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA). For PN detection, 1:500 rabbit anti-human PN (Biovendor) and 1:1,000 goat anti-rabbit conjugated HRP (Abcam, Cambridge, MA) were used. The signal was visualized by ECL (Pierce, Rockford, IL). The expression of β-actin was used as an internal control to determine an equal amount of loading proteins.

Cell proliferation assay

CCA cells with or without treatment with si*ITGα$_5$* cells were arrested in HAM-F12 without serum supplement for 12 h. Different concentrations of recombinant PN (Biovendor) prepared in 1% FBS containing HAM-F12 were incubated with cells for 6, 12 and 24 h. The viable cells in each condition were determined using an MTS assay (Promega, Madison, WI) according to the manufacturer's instruction.

Cell cycle analysis by flow cytometry

Cell cycle distribution analysis used cells stained with propidium iodide (Invitrogen) as previously described [63]. The distribution of cells in each stage of the cell cycle was quantitated in a flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Numbers of CCA cell lines in S+G2/M phases of the cell cycle were measured and compared between conditions with and without recombinant PN treatment. These experiments were repeated two times using replicate culture dishes in the same experiment.

Colony formation assay

CCA cell lines were cultured in 6-well plate. After 24-h culture, recombinant PN diluted in 1% FBS containing medium was added and the plate was incubated in CO_2 incubator. Soft agar colony formation assay was also performed using 0.5% and 0.35% MetaPhor® agarose (Cambrex Bio Science, Rockland, ME) as lower and upper layers, respectively. After 12 d, cells were fixed with 5% v/v glutaraldehyde and stained with 0.5% w/v crystal violet in 40% v/v methanol. Cell growth was estimated by counting numbers of colonies with more than 30 cells under inverted microscope and compared between those of treat and untreated with PN. The experiment was performed in duplicate.

Invasion assay

KKU-M213 and KKU-M156 CCA cells were seeded in PN (100 ng/ml) containing medium into the Matrigel invasion chamber (BD Biosciences, San Jose, CA) and incubated for 24 h. Invaded cells were fixed with 5% v/v glutaraldehyde and stained with 0.5% w/v crystal violet in 40% v/v methanol for 30 min each. The number of invaded cells was counted under a microscope by two independent investigators using 100× magnification fields. The assays were done in replicate and three independent experiments were performed.

Small interfering RNA against receptor integrin α_5

Two hundred thousand CCA cells were seeded into a 6-well plate for 24 h before transfection of si*ITG*α_5 (Santa Cruz Biotechnology, Santa Cruz, CA) by Lipofectamine 2000 (Invitrogen). Three siRNA strands (5'-gucagaauuucgagacaaa-3', 5'-caccaacaagagagccaaa-3', and 5'-ccacugaccagaacuagaa-3') were used to target *ITG*α_5 mRNA. The efficiency of knock down was tested by real time PCR using *β-actin* as an internal control.

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL). The correlation of PN expression and pathological parameters of CCA patients was analyzed by the χ^2-test and binary logistic multivariate analysis. Patient survival was calculated from the time of surgical resection to death and the survival curves were constructed according to Kaplan-Meier, with a Log-Rank test. A multivariate analysis was performed by the Cox proportional hazard regression model. The significance of the different data was determined by the Student's t-test. A *P* value of equal to or less than 0.05 was defined as statistically significant.

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Authors' contributions

KU performed most of the experiments and helped to draft the manuscript. YA contributed to the microarray experiment. PT performed real time PCR of some genes and helped KC and AP in immunohistochemical scoring. SC contributed to the patient clinicopathological data and samples collection. CT contributed to the design of the entire study, data analysis and preparation of the manuscript.
All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Sriamporn S, Pisani P, Pipitgool V, Suwanrungruang K, Kamsa-ard S, Parkin DM: **Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand.** *Trop Med Int Health* 2004, **9**:588-594.
2. Lim MK, Ju YH, Franceschi S, Oh JK, Kong HJ, Hwang SS, Park SK, Cho SI, Sohn WM, Kim DI, Yoo KY, Hong ST, Shin HR: ***Clonorchis sinensis* infection and increasing risk of cholangiocarcinoma in the Republic of Korea.** *Am J Trop Med Hyg* 2006, **75**:93-96.
3. Choi D, Lim JH, Lee KT, Lee JK, Choi SH, Heo JS, Jang KT, Lee NY, Kim S, Hong ST: **Cholangiocarcinoma and *Clonorchis sinensis* infection: a case-control study in Korea.** *J Hepatol* 2006, **44**:1066-1073.
4. Lazaridis KN, Gores GJ: **Primary sclerosing cholangitis and cholangiocarcinoma.** *Semin Liver Dis* 2006, **26**:42-51.
5. Okamura N, Yoshida M, Shibuya A, Sugiura H, Okayasu I, Ohbu M: **Cellular and stromal characteristics in the scirrhous hepatocellular carcinoma: comparison with hepatocellular carcinomas and intrahepatic cholangiocarcinomas.** *Pathol Int* 2005, **55**:724-731.
6. Chuaysri C, Thuwajit P, Paupairoj A, Chau-In S, Suthiphongchai T, Thuwajit C: **Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma.** *Oncol Rep* 2009, **21**:957-969.
7. Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD: **Cancer-associated stromal fibroblasts promote pancreatic tumor progression.** *Cancer Res* 2008, **68**:918-926.

8. Micke P, Ostman A: **Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy.** *Expert Opin Ther Targets* 2005, **9**:1217-1233.
9. Augsten M, Hagglof C, Olsson E, Stolz C, Tsagozis P, Levchenko T, Frederick MJ, Borg A, Micke P, Egevad L, Ostman A: **CXCL14 is an autocrine growth factor for fibroblasts and acts as a multi-modal stimulator of prostate tumor growth.** *Proc Natl Acad Sci USA* 2009, **106**:3414-3419.
10. Tuxhorn JA, Ayala GE, Rowley DR: **Reactive stroma in prostate cancer progression.** *J Urol* 2001, **166**:2472-2483.
11. Rosenthal EL, McCrory A, Talbert M, Carroll W, Magnuson JS, Peters GE: **Expression of proteolytic enzymes in head and neck cancer-associated fibroblasts.** *Arch Otolaryngol Head Neck Surg* 2004, **130**:943-947.
12. Ohira S, Itatsu K, Sasaki M, Harada K, Sato Y, Zen Y, Ishikawa A, Oda K, Nagasaka T, Nimura Y, Nakanuma Y: **Local balance of transforming growth factor-beta1 secreted from cholangiocarcinoma cells and stromal-derived factor-1 secreted from stromal fibroblasts is a factor involved in invasion of cholangiocarcinoma.** *Pathol Int* 2006, **56**:381-389.
13. Singer CF, Gschwantler-Kaulich D, Fink-Retter A, Haas C, Hudelist G, Czerwenka K, Kubista E: **Differential gene expression profile in breast cancer-derived stromal fibroblasts.** *Breast Cancer Res Treat* 2008, **110**:273-281.
14. Micke P, Kappert K, Ohshima M, Sundquist C, Scheidl S, Lindahl P, Heldin CH, Botling J, Ponten F, Ostman A: **In situ identification of genes regulated specifically in fibroblasts of human basal cell carcinoma.** *J Invest Dermatol* 2007, **127**:1516-1523.
15. Fromigue O, Louis K, Dayem M, Milanini J, Pages G, Tartare-Deckert S, Ponzio G, Hofman P, Barbry P, Auberger P, Mari B: **Gene expression profiling of normal human pulmonary fibroblasts following coculture with non-small-cell lung cancer cells reveals alterations related to matrix degradation, angiogenesis, cell growth and survival.** *Oncogene* 2003, **22**:8487-8497.
16. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K: **Molecular characterization of the tumor microenvironment in breast cancer.** *Cancer Cell* 2004, **6**:17-32.
17. Fukushima N, Sato N, Prasad N, Leach SD, Hruban RH, Goggins M: **Characterization of gene expression in mucinous cystic neoplasms of the pancreas using oligonucleotide microarrays.** *Oncogene* 2004, **23**:9042-9051.
18. Nakagawa H, Liyanarachchi S, Davuluri RV, Auer H, Martin EW Jr, de la Chapelle A, Frankel WL: **Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles.** *Oncogene* 2004, **23**:7366-7377.
19. Erkan M, Kleeff J, Gorbachevski A, Reiser C, Mitkus T, Esposito I, Giese T, Buchler MW, Giese NA, Friess H: **Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity.** *Gastroenterology* 2007, **132**:1447-1464.
20. Tai IT, Dai M, Chen LB: **Periostin induction in tumor cell line explants and inhibition of in vitro cell growth by anti-periostin antibodies.** *Carcinogenesis* 2005, **26**:908-915.
21. Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY, Chang DD: **Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility.** *Cancer Res* 2002, **62**:5358-5364.
22. Kudo Y, Ogawa I, Kitajima S, Kitagawa M, Kawai H, Gaffney PM, Miyauchi M, Takata T: **Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer.** *Cancer Res* 2006, **66**:6928-6935.
23. Siriwardena BS, Kudo Y, Ogawa I, Kitagawa M, Kitajima S, Hatano H, Tilakaratne WM, Miyauchi M, Takata T: **Periostin is frequently overexpressed and enhances invasion and angiogenesis in oral cancer.** *Br J Cancer* 2006, **95**:1396-1403.
24. Shao R, Bao S, Bai X, Blanchette C, Anderson RM, Dang T, Gishizky ML, Marks JR, Wang XF: **Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression.** *Mol Cell Biol* 2004, **24**:3992-4003.
25. De Wever O, Demetter P, Mareel M, Bracke M: **Stromal myofibroblasts are drivers of invasive cancer growth.** *Int J Cancer* 2008, **123**:2229-2238.
26. Howell OW, Scharfman HE, Herzog H, Sundstrom LE, Beck-Sickinger A, Gray WP: **Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells.** *J Neurochem* 2003, **86**:646-659.
27. Burgess HA, Reiner O: **Doublecortin-like kinase is associated with microtubules in neuronal growth cones.** *Mol Cell Neurosci* 2000, **16**:529-541.
28. Aycock RL, Bradshaw AC, Sage EH, Starcher B: **Development of UV-induced squamous cell carcinomas is suppressed in the absence of SPARC.** *J Invest Dermatol* 2004, **123**:592-599.
29. Croucher DR, Saunders DN, Lobov S, Ranson M: **Revisiting the biological roles of PAI2 (SERPINB2) in cancer.** *Nat Rev Cancer* 2008, **8**:535-545.
30. Duggan C, Kennedy S, Kramer MD, Barnes C, Elvin P, McDermott E, O'Higgins N, Duffy MJ: **Plasminogen activator inhibitor type 2 in breast cancer.** *Br J Cancer* 1997, **76**:622-627.
31. Osmak M, Babic D, Abramic M, Milicic D, Vrhovec I, Skrk J: **Plasminogen activator inhibitor type 2: potential prognostic factor for endometrial carcinomas.** *Neoplasma* 2001, **48**:462-467.
32. Schmidt-Hansen B, Klingelhofer J, Grum-Schwensen B, Christensen A, Andresen S, Kruse C, Hansen T, Ambartsumian N, Lukanidin E, Grigorian M: **Functional significance of metastasis-inducing S100A4(Mts1) in tumor-stroma interplay.** *J Biol Chem* 2004, **279**:24498-24504.
33. Steiglitz BM, Keene DR, Greenspan DS: **PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1.** *J Biol Chem* 2002, **277**:49820-49830.
34. Tomari K, Kumagai T, Shimizu T, Takeda K: **Bone morphogenetic protein-2 induces hypophosphorylation of Rb protein and repression of E2F in androgen-treated LNCaP human prostate cancer cells.** *Int J Mol Med* 2005, **15**:253-258.
35. Zheng M, Bocangel D, Ramesh R, Ekmekcioglu S, Poindexter N, Grimm EA, Chada S: **Interleukin-24 overcomes temozolomide resistance and enhances cell death by down-regulation of O6-methylguanine-DNA methyltransferase in human melanoma cells.** *Mol Cancer Ther* 2008, **7**:3842-3851.
36. Zou J, Ren JH, Feng D, Wang H, Xu J: **Compensatory function of bradykinin B1 receptor in the inhibitory effect of captopril on cardiomyocyte hypertrophy and cardiac fibroblast proliferation in neonatal rats.** *Chin Med J (Engl)* 2008, **121**:1220-1225.
37. Saigusa K, Imoto I, Tanikawa C, Aoyagi M, Ohno K, Nakamura Y, Inazawa J: **RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest.** *Oncogene* 2007, **26**:1110-1121.
38. Mueller MM, Fusenig NE: **Friends or foes - bipolar effects of the tumour stroma in cancer.** *Nat Rev Cancer* 2004, **4**:839-849.
39. Hirohata S, Wang LW, Miyagi M, Yan L, Seldin MF, Keene DR, Crabb JW, Apte SS: **Punctin, a novel ADAMTS-like molecule, ADAMTSL-1, in extracellular matrix.** *J Biol Chem* 2002, **277**:12182-12189.
40. Vazquez F, Hastings G, Ortega MA, Lane TF, Oikemus S, Lombardo M, Iruela-Arispe ML: **METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity.** *J Biol Chem* 1999, **274**:23349-23357.
41. Luque A, Carpizo DR, Iruela-Arispe ML: **ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165.** *J Biol Chem* 2003, **278**:23656-23665.
42. Castillo J, Erroba E, Perugorria MJ, Santamaria M, Lee DC, Prieto J, Avila MA, Berasain C: **Amphiregulin contributes to the transformed phenotype of human hepatocellular carcinoma cells.** *Cancer Res* 2006, **66**:6129-6138.
43. Morita S, Shirakata Y, Shiraishi A, Kadota Y, Hashimoto K, Higashiyama S, Ohashi Y: **Human corneal epithelial cell proliferation by epiregulin and its cross-induction by other EGF family members.** *Mol Vis* 2007, **13**:2119-2128.
44. Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J, Sundaresan T, Pastorino S, Park JK, Mikolaenko I, Maric D, Eberhart CG, Fine HA: **Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation.** *Cancer Res* 2005, **65**:2353-2363.
45. Gonzales M, Haan K, Baker SE, Fitchmun M, Todorov I, Weitzman S, Jones JC: **A cell signal pathway involving laminin-5, alpha3beta1 integrin, and mitogen-activated protein kinase can regulate epithelial cell proliferation.** *Mol Biol Cell* 1999, **10**:259-270.
46. Shikada Y, Yonemitsu Y, Koga T, Onimaru M, Nakano T, Okano S, Sata S, Nakagawa K, Yoshino I, Maehara Y, Sueishi K: **Platelet-derived growth factor-AA is an essential and autocrine regulator of vascular endothelial growth factor expression in non-small cell lung carcinomas.** *Cancer Res* 2005, **65**:7241-7248.

47. Lin CG, Leu SJ, Chen N, Tebeau CM, Lin SX, Yeung CY, Lau LF: **CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family.** *J Biol Chem* 2003, **278**:24200-24208.
48. Lobov IB, Brooks PC, Lang RA: **Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo.** *Proc Natl Acad Sci USA* 2002, **99**:11205-11210.
49. Kirchmair R, Gander R, Egger M, Hanley A, Silver M, Ritsch A, Murayama T, Kaneider N, Sturm W, Kearny M, Fischer-Colbrie R, Kircher B, Gaenzer H, Wiedermann CJ, Ropper AH, Losordo DW, Patsch JR, Schratzberger P: **The neuropeptide secretoneurin acts as a direct angiogenic cytokine in vitro and in vivo.** *Circulation* 2004, **109**:777-783.
50. Le Pabic H, Bonnier D, Wewer UM, Coutand A, Musso O, Baffet G, Clement B, Theret N: **ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling.** *Hepatology* 2003, **37**:1056-1066.
51. Hashimoto-Torii K, Torii M, Sarkisian MR, Bartley CM, Shen J, Radtke F, Gridley T, Sestan N, Rakic P: **Interaction between reelin and notch signaling regulates neuronal migration in the cerebral cortex.** *Neuron* 2008, **60**:273-284.
52. Sasaki H, Dai M, Auclair D, Fukai I, Kiriyama M, Yamakawa Y, Fujii Y, Chen LB: **Serum level of the periostin, a homologue of an insect cell adhesion molecule, as a prognostic marker in nonsmall cell lung carcinomas.** *Cancer* 2001, **92**:843-848.
53. Fukushima N, Kikuchi Y, Nishiyama T, Kudo A, Fukayama M: **Periostin deposition in the stroma of invasive and intraductal neoplasms of the pancreas.** *Mod Pathol* 2008, **21**:1044-1053.
54. Kikuchi Y, Kashima TG, Nishiyama T, Shimazu K, Morishita Y, Shimazaki M, Kii I, Horie H, Nagai H, Kudo A, Fukayama M: **Periostin is expressed in pericryptal fibroblasts and cancer-associated fibroblasts in the colon.** *J Histochem Cytochem* 2008, **56**:753-764.
55. Sasaki H, Yu CY, Dai M, Tam C, Loda M, Auclair D, Chen LB, Elias A: **Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer.** *Breast Cancer Res Treat* 2003, **77**:245-252.
56. Baril P, Gangeswaran R, Mahon PC, Caulee K, Kocher HM, Harada T, Zhu M, Kalthoff H, Crnogorac-Jurcevic T, Lemoine NR: **Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway.** *Oncogene* 2007, **26**:2082-2094.
57. Chen CY, Lin XZ, Tsao HC, Shiesh SC: **The value of biliary fibronectin for diagnosis of cholangiocarcinoma.** *Hepatogastroenterology* 2003, **50**:924-927.
58. Zhang Z, Vuori K, Reed JC, Ruoslahti E: **The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression.** *Proc Natl Acad Sci USA* 1995, **92**:6161-6165.
59. Zeng ZZ, Yao H, Staszewski ED, Rockwood KF, Markwart SM, Fay KS, Spalding AC, Livant DL: **Alpha(5)beta(1) integrin ligand PHSRN induces invasion and alpha(5) mRNA in endothelial cells to stimulate angiogenesis.** *Transl Oncol* 2009, **2**:8-20.
60. Tuomi S, Mai A, Nevo J, Laine JO, Vilkki V, Ohman TJ, Gahmberg CG, Parker PJ, Ivaska J: **PKCepsilon regulation of an alpha5 integrin-ZO-1 complex controls lamellae formation in migrating cancer cells.** *Sci Signal* 2009, **2**:ra32.
61. Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, Ozawa H, Toyama Y, Bonewald LF, Kudo A: **Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta.** *J Bone Miner Res* 1999, **14**:1239-1249.
62. Thuwajit C, Thuwajit P, Uchida K, Daorueang D, Kaewkes S, Wongkham S, Miwa M: **Gene expression profiling defined pathways correlated with fibroblast cell proliferation induced by *Opisthorchis viverrini* excretory/secretory product.** *World J Gastroenterol* 2006, **12**:3585-3592.
63. Thuwajit C, Thuwajit P, Kaewkes S, Sripa B, Uchida K, Miwa M, Wongkham S: **Increased cell proliferation of mouse fibroblast NIH-3T3 in vitro induced by excretory/secretory product(s) from *Opisthorchis viverrini*.** *Parasitology* 2004, **129**:455-464.

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Publication ที่ 3

(Manuscript draft)

Periostin activates integrin alpha5beta1 through AKT-dependent pathway in invasion of chaolangiocarcinoma

Periostin activates integrin α5β1 through AKT-dependent pathway in invasion of cholangiocarcinoma

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ABSTRACT

Cholangiocarcinoma (CCA) is a progressive tumor with high metastatic potency. Recently, our group has found that extracellular matrix protein periostin (PN) mainly produced from stromal activated fibroblasts of CCA shows strong effect to induce cancer cell invasion. This work aims to investigate specific integrins (ITGs) utilized by PN to drive invasion of CCA cells. The activated intracellular signal transduction pathway is also explored. Seven Thai patients-derived CCA cell lines were investigated their ITG expression patterns by real time PCR. The results indicated high level of ITGα6 in almost all cell lines. In addition, ITGα5 were detected in not only CCA cell lines but in immortalized non-tumorigenic biliary epithelial cell. For β-subunits, the result revealed high expression levels of β1 and β4 ITGs in cancer and non-tumorigenic cells. These results imply high level of ITGs α5β1 and α6β4 in CCA cells. Immunocytochemistry and flow cytometry analysis confirmed intact α5β1 and α6β4 ITGs on the membrane of M213 CCA cells. Cell adhesion on PN-coated surface was decreased after ITGα5β1 inhibition by neutralizing antibody in similar way to the decreased PN-induced invasion. Furthermore, the ITGα5-knocked down cells had decreased level of intracellular pAKT compared to negative control cells after activation with PN. Taken these results together, it is likely to suggest that PN-activated CCA cell invasion through ITGα5β1 and AKT-dependent signal transduction pathway. This study explain the underlying mechanism of the stromal cell-derived PN in promotion of CCA progression. Importantly, the work highlights the potential of using this mechanism as alterative therapeutic target in CCA patients.

Key words: Fibroblast, Cholangiocarcinoma, Periostin, Integrin, Invasion, AKT

Introduction

Cholangiocarcinoma (CCA) is a major public health problem causing by liver fluke infection in the Northeastern and Northern parts of Thailand. It is a slow growing cancer with rapid metastatic and mortality rate [1]. The incidence of CCA has increased worldwide with the highest incidence in Thailand where around 93-100 per 100,000 have been diagnosed [2]. The pathogenesis of CCA has been demonstrated mainly by the genetic mutations in bile duct epithelial cells as the results of endogenous and exogenous mutagens exposure. However, several works indicate the crucial roles of stromal cells or cells in the tumor microenvironment as being involve in the genesis and progression of this cancer [3,4].

The most abundant cells in CCA stroma, like other epithelium-derived cancers, are cancer fibroblasts. Almost of stromal fibroblasts in CCA tissues were α-SMA positive [3,5]. The immunohistochemistry was carried out for α-SMA staining in CCA, hepatocellular carcinoma and metastatic liver carcinoma tissues [5]. The result showed that α-SMA positive stromal cells showed a significant positive correlation with the degree of tumor fibrosis. The authors suggested that in CCA and metastatic liver carcinomas, α-SMA-positive cells transformed into activated fibroblasts or myofibroblasts, are incorporated into the tumor and produce extracellular matrix proteins that may lead to tumor fibrosis. The recent work from our research group confirmed the previous finding that CCA stromal fibroblasts were α-SMA positive and importantly the presence of α-SMA positive fibroblasts in CCA stroma correlated with poor patient survival [3]. In addition, these fibroblasts isolated from CCA mass have recently been investigated the certain gene expression pattern that facilitate the production of several tumorigenic proteins. One of these proteins is periostin (PN) and its production has been confirmed to express exclusively from activated or CCA-associated fibroblasts [4].

PN is an extracellular matrix (ECM) protein with multifunctional roles in tumorigenesis and tumor progression. It has been proposed as a marker-associated cancer aggressiveness in pancreatic cancer [6,7], gastric cancer [8], breast cancer [9], thyroid carcinoma [10], and non-small cell lung cancer [11]. The potential role of PN in regulating at each step of the transformation of normal into malignant cells and metastatic tumors has recently been well concluded [12]. PN has recently been found in CCA and the major source of production is from CCA-associated fibroblasts. The impact of fibroblast-derived PN is convinced by its ability to activate cancer cell proliferation and invasion [4].

To activate biological functions of cells, PN has been investigated its ability to bind to integrin (ITG) receptors. ITGs are belonging to the large family of transmembranous glycoproteins that were initially identified as cation-dependent receptors for components of ECM [13,14] that serve as fundamental components of an 'integral membrane complex' linking the cytoskeleton to ECM. ITGs are transmembrane heterodimers that consist of non-covalently bound α- and β-glycoprotein subunits. In human, there are 24 different ITGs, which arise from the association between one of each 18 α-subunits and 8 β-subunits. Importantly, some subunits can combine with several different partners leading to different active ITGs on cell membrane. In epithelial ovarian carcinoma, PN was secreted from cancer cells and proposed as a ligand for αvβ3 and αvβ5 ITGs and promotes cell motility [15]. In CCA, we have found that after transient knockdown ITGα5, the invasive property of cancer cells was reduced [4].

For signaling pathways activated by PN, in colon cancer, PN potently promotes metastatic growth of colon cancer by augmenting cell survival via the AKT/PKB pathway [16]. Similarly, PN from pancreatic cancer cells activated ITGβ4 through PI3K pathway [17]. But in vascular smooth muscle cells, PN was demonstrated to induce cell migration through ITGs αvβ3 and αvβ5 and focal adhesion kinase (FAK) pathway [18]. In breast cancer, PN

enhanced angiogenesis in part from the up-regulation of the VEGF receptor FLK-1/KDR by endothelial cells through ITG αvβ3-FAK-mediated signaling pathway [19]. In contrast to pancreatic cancer, α6β4 integrin complex acts as the cellular receptor for PN and is shown to promote the invasiveness of tumor cells through phosphorylation of FAK and PI3K/AKT kinase pathway [17]. In addition, PN could induce epithelial-mesenchymal transition (EMT) characteristic resulting tumor metastasis by requirement of the cross-talk between ITGαvβ5 and EGFR signaling pathways [20]. It seem to happen that PN can activate specific ITGs and signal pathway depends on cell context. Hence it is interesting whether of these pathways can be activated in CCA cells after exposure to fibroblast-derived PN.

ITGs α- and β-subunits contribute the extracellular domain where the ligands can bind and transduce signal inside the cells and finally regulates cell adhesion, proliferation, migration, and apoptosis [21]. Several studies reveal various types of ITGs expressed on bile duct epithelium and its cancer cells [22,23]. The ITGs α2, α3, α6, and β4 have been demonstrated to express in both normal and proliferating bile duct epithelium, whereas ITGs α1 is not expressed [22]. Some of these ITGs are also found in normal bile duct epithelial cells including ITGs α3, α5, α6, and β4 [22]. In cell lines derived from CCA tissues, ITGs α2, α3, α6, β1, and β4 have been detected high in well differentiated tumor types but low level in poorly differentiated ones [22]. In support to this, ITGs α2, α3, β1, and β4, but not α6 are expressed in ETK-1 cells derived from sarcomatoid CCA tissues [23]. Moreover, some ITGs was highlighted as the cellular phenotype of CCA distinguish from hepatocellular carcinoma including ITGβ4 [22,23] and ITGα6 [24]. Interestingly, αvβ6 integrin has been recently reported in human CCA but not in HCC tissues and may considered as a specific immunohistochemical marker for differential diagnosis of primary liver cancers [25].

The present study focuses on specific ITGs which can be activated by PN and mediated the tumorigenic functions of CCA cell in particular cancer cell adhesion and invasion. The underlying signaling pathway activated by PN-ITG ligation is also investigated. Here, the ITGs expression in CCA cell lines is studied. The abundances of ITGs α5β1 and α6β4 are demonstrated and the adhesion assay shows that CCA cells favor to use ITGα5β1 in response to exogenous PN. Using cells with low expression of intact ITGα5β1, by either si*ITGα5β1* or neutralizing anti-ITGα5 antibody treatments, the results show that AKT, but not ERK, signaling pathway involves in PN-stimulated CCA cell invasion.

Materials and methods

1. CCA cell line culture

Human CCA cell lines KKU-M055, KKU-100, KKU-M139, KKU-M156, KKU-M213, and KKU-M214 were kindly donated from Associated Professor Banchop Sripa, Khon Kaen University, Thailand. The non-tumorigenic immortalized bile duct epithelial cell MMNK1 was kindly provided by Professor Naoya Kobayashi, Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama Japan. CCA cells and MMNK1 were cultured in Ham F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), and 5 µg/ml amphotericin B (Advanced Remedies Pvt. Ltd, Solapur, India). Cells were cultured in 5% CO_2 incubator at 37°C. Cells were passaged by 0.25% trypsin-EDTA and those of more than 90% viability were used in further experiments.

2. Measurement of ITGs expression pattern in CCA cell lines by real time PCR

Total RNA was extracted from all CCA cell lines and MMNK1 using PerfectPure RNA Cultured Cell Kit (5Prime, Maryland, USA). Complementary DNA was synthesized from 1 µg of total RNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (M-MLV RT) (Invitrogen, California, USA) according to the instructions. Expression levels of *ITGs* including *ITGs αv*, *α5*, and *α6*; and *ITGs β1*, *β3*, *β4*, and *β5* were determined by SYBR Green-based real time PCR in Light Cycler® 480 II machine (Roche Diagnostics Ltd., Rolkreuz, Switzerland). The *β-actin* was served as an internal control to adjust the amount of starting cDNA. The expression of each *ITG* was calculated by the $2^{-\Delta Cp}$ equation. In this case, $\Delta C_p = C_p(ITG) - C_p(\beta\text{-}actin)$. The sequences of all genes in this study were retrieved from PubMed (www.ncbi.nln.nih.gov) and the primers were designed by Primer 3 (Table 1).

3. Flow cytometry analysis of ITGs expression

For detection of the actual ITGα5 level in biliary epithelial cells, cell pellet of around 1 x 10^6 was fixed with 2% formaldehyde for 15 min at room temperature. The fixed cells were incubated with 1:50 goat-anti human ITGα5 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 100 µl washing solution which was HAM/F-12 containing 2% (v/v) FBS, 1% (w/v) BSA and 10 mM NaN_3 for 2 h at room temperature. Cells were centrifuged in 1 ml washing solution at 400 g for 3 times and 5 min each to get rid of the excess primary antibody and followed by staining with 1:2,000 donkey-anti goat IgG-Alexa 488 (Invitrogen, Carlsbad, CA) diluted in 100 µl washing solution for 1 h at room temperature with light protection. For ITGβ4 detection, using the same process as above, 1:100 mouse anti-integrin ITGβ4 monoclonal antibody (MAB2060) (Chemicon, Millipore, California, USA) diluted in 100 µl washing solution was incubated with cells for 1 h at 4°C and followed by 1:100 FITC-conjugated polyclonal rabbit anti-mouse antibody (F0261) (Dako, Glostrup, Denmark) in 100 µl washing solution for 30 minutes at 4 °C with light protection.

Cells were centrifuged in 1 ml washing solution at 400 g for 2 times and 5 min each to get rid of the excess secondary antibody. The ITGα5 and ITGβ4 signals were determined in FL-1 channel of Becton Dickinson FACSort (Becton Dickinson, Franklin Lakes, NJ) and data analysis was performed by CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The relative Mean Fluorescence Intensity (MFI) of CCA cell lines was normalized to that of the negative control stained with only secondary antibody. Two independent experiments were performed.

4. Immunocytochemistry of ITGα5β1 and α6β4 in CCA cell lines

Immunocytochemistry was employed to localize ITGα5 on the cell membrane. KKU-M213 (2 x 10^4 cells) were cultured on sterile cover slip placed in 24-well plate for 48 h. Cells were fixed with 4% paraformaldehyde for 15 min and blocked with 1% BSA for 30 min at room temperature. Then cells were incubated with 1:50 goat anti-human ITGα5 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 1% BSA for 2 h at room temperature and subsequently stained with 1:500 donkey-anti goat IgG-Alexa 488 (Invitrogen, Carlsbad, CA) diluted in 1% BSA for 1 h at room temperature with light protection.

For localizing the position of ITGβ4 receptor, cells were plated onto glass cover slips at a density of 4 x 10^4 cells in 24-well plate. After 48 hours in culture, cells were washed twice with PBS and then blocked with blocking solution (10% FBS in PBS) for 30 minutes at room temperature. Cells were further incubated in 1:500 mouse anti-integrin ITGβ4 monoclonal antibody diluted in blocking solution for 2 h at room temperature. After three washes with PBS, cells were incubated in 1:2,000 goat anti mouse IgG-Cy3 (Jackson ImmunoResearch 115-166-071, Pennsylvania, USA).

Nucleus was stained with 1:1,000 Hoechst 33258 (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature. The fluorescence signal was observed under the LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany) at the Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University.

5. Adhesion assay of CCA cell lines on PN-coated surface

One μg of recombinant PN (rPN) (Biovendor, Heidelberg, Germany) was coated on 96-well plate surface at 37°C for 2 h. Cells were pre-treated with 1:200 mouse anti-human ITGα5β1 (Chemicon Inc., Temecula, CA) and 1:200 mouse anti-human ITGβ4 (Chemicon Inc., Temecula, CA) at 37°C for 1 h. Cells were centrifuged at 400 g for 5 min once to remove unbound antibodies. Cells with and without antibody blockage were suspended in the serum-free media at a density of 2 x 10^5 cells/ml. The 0.1 ml of cell suspension was then added to each well and incubated at 37°C for 1 h. Unattached cells were removed by rinsing twice with serum-free media. The number of adherent cells was determined by MTS (Promega, Medison, WI) according to the manufacturer's instruction. The percentage of PN-induced cell adhesion was normalized to that of cells attached on 1% BSA-coated wells. Two independent experiments were performed.

6. Neutralization of ITGα5β1 on CCA cells

To ensure role of ITGα5 on PN-induced CCA cell invasion, neutralizing antibody specific to ITGα5β1 heterodimer was employed to block intact ITGα5β1 on the cell membrane of CCA cells. KKU-M213 CCA cells were trysinized and washed with 1X PBS for 2 times. Cell pellet of around 1 x 10^5 cells were incubated with 200 μl of 1:200 anti-human ITGα5β1 (Chemicon Inc., Temecula, CA) diluted in serum-free media at 37°C for 1 h. Cells in antibody solution were centrifuged at 400 g for 5 min to get rid of excess antibody. ITGα5β1-blocked cells were then collected to explore their responses to PN-induced invasion using invasion assay. The numbers of invaded cells induced by PN were compared between with and without anti-ITGα5β1 blocking condition. The experiment was performed in duplicated wells.

7. Invasion assay

To investigate the effect of rPN on the invasion of parental KKU-M213 cells, si*ITGa5*-treated cells, and ITGα5β1-blocked cells, 2 x 10^4 cells of each type were suspended in 100 ng/ml rPN containing complete medium and cultured in the upper chamber of the Matrigel™ invasion chamber (BD Biosciences, San Jose, CA) for 24 h. Invaded cells were fixed with 5% (v/v) glutaraldehyde and then haematoxylin and eosin staining was performed. The number of invaded cells was counted under an inverted microscope by two independent investigators using 100x magnification field. The assays were done in replicate of three independent experiments. Numbers of invaded cells was compared to those without rPN treatment.

8. Western blot analysis of pAKT and pERK

Cells at the mid-exponential phase of the growth curve and having more than 95% cell viability were trypsinized as standard protocol. Cell pellet was collected after centrifugation the cell suspension at 400 g for 5 min in refrigerated centrifuge. The cell pellet was rinsed by cold 1X PBS for 2 times before lysed in 1X sample buffer containing 50 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.05% (w/v) bromophenol blue. Cell lysate was boiled for 10 min and centrifuged to get rid the undissolved proteins and cell debris at 8,000 g for 1 min. The 20 µl of cell lysate was then separated in 8% SDS-PAGE and transferred onto PVDF membrane (Amersham, Buckinghamshire, UK). Membrane was blocked in 5% skim milk containing TBST for 1 h at room temperature. Mouse anti-human pAKT monoclonal antibody (Cell signaling Technology, Inc, Danvers, MA) at the dilution of 1:1,000 and rabbit anti-human pERK1/2 polyclonal antibody (Cell signaling Technology, Inc, Danvers, MA) at the dilution of 1:2,000 were used as primary antibodies by incubating with the membrane for 1 h at room

temperature. The 1:1,000 rabbit anti-mouse IgG-HRP (Zymed, Sanfrancisco, CA) and 1:2,000 goat anti-rabbit IgG-HRP (Abcam, Cambridge, MA) were used as secondary antibodies against their specific first antibodies and incubated for 1 h at room temperature. The immunoreactive signals were visualized by enhance chemiluminescense (ECL). The β-actin protein level was used as an internal control to determine the equal amount of loading proteins.

9. Statistical analysis

Invasion is expressed as mean ± SD. The significance of the different data was determined by the Student's t-test. A *P* value of equal to or less than 0.05 was defined as statistically significant.

Results

1. Integrin expression profile in CCA cell lines

The expression of α-subunits of ITGs including αv, α5, and α6; and β-subunits including β1, β3, β4, and β5 of CCA cells and MMNK1were measured by real time PCR using 50 ng starting cDNA. The result indicates different levels of each subunit in CCA cell lines and MMNK1 cells. Among α-subunit of ITGs, α6 expressed at high level in almost all cell types except KKU-OCA17 (Fig. 1A-B). In addition, α5-subunit showed the highest level among other α-subunits in MMNK1, but moderate expression level was found in KKU-M139, KKU-M213, and KKU-M214. For β-subunits, the results revealed that β1 had highest expression level in KKU-M139, KKU-M156, KKU-M213, KKU-M214, and MMNK1 (Fig. 1A-B). In the other hand, KKU-K100, KKU-M055, and KKU-OCA17 showed ITGβ4 as the highest level of β-subunit expression.

Since certain type of α-subunit ITG can be paired with specific type of β-subunit, the predicted level of intact ITGs were presented based on the minimal level of their counterpart of either α-subunit or β-subunit. The possible levels of ITGs αvβ5, α5β1, and α6β4 were indicated (Fig. 2). The expressions of both α5β1 and α6β4 ITGs were found higher in all cell types than ITGαvβ5. Some cell types had ITGα5β1 as the highest expression level including MMNK1 and KKU-M139. But KKU-M156, KKU-M213, and KKU-M214 showed α6β4 as the highest expressed ITG.

2. Expressions of membrane ITGs α5β1 and α6β4 on CCA cell lines

To confirm the expression of ITGs α5β1 and α6β4 on the membrane of CCA cells, FACS analysis was performed. The result revealed different expression levels of these two ITGs among different types of CCA cell lines. For ITGα5β1, relative fluorescence intensity showed the similar level of expressions in all cell lines of which KKU-M213 showed the highest signal (Fig. 3A). Most of CCA cells originated from well differentiated cancer including KKU-M213 and KKU-M214 expressed high level of ITGα6β4 as similar as KKU-M139 which derived from the moderately differentiation but was squamous cell type (Fig. 3B). In contrast, almost cell lines derived from moderate differentiated type (KKU-M055 and KKU-M156) and poorly differentiated type (KKU-100) had low expression levels of ITGα6β4. In contrast, the expression was found higher in KKU-M139, KKU-M213, and KKU-M214 than other cells. The highest level of ITGα6β4 was detected in KKU-M213 as well as that of ITGα5β1 (Fig. 3A-B). Moreover, using immunocytochemical staining, the result confirmed the abundance of ITGs α5β1 and α6β4 on the membrane of KKU-M213 cells (Fig. 3A-B).

3. Adhesion and invasion of CCA cell lines with PN through ITGs α5β1 and α6β4

To demonstrate the impact of ITGα5β1 and α6β4 in PN-activated tumorigenic function of cells, KKU-M213 cells were used. PN-coated culture plates were utilized to explore the adhesion efficiency of cells. The result showed that CCA cells could bind to PN more than on the plate with BSA coating with statistical significance (Fig. 4A). The binding efficiency was attenuated when cells were blocked the ITGα5β1 by neutralizing antibody (Fig. 4A). Surprisingly, cells bound to surface of plate with increased capability when ITGα6β4 were inhibited. However, using mixture of neutralizing antibodies against α5β1 and α6β4 ITGs, the decreasing of cell binding on PN to that of untreated cells was observed. This result implies that PN may favor to use ITGα5β1 more than ITGα6β4 to activate tumorigenic function of CCA cells. To confirm this, cell invasion induced by recombinant PN was investigated. The result revealed that PN could induce KKU-M213 cell invasion significantly than intrinsic invasive capacity of cells cultured in PN-free medium (Fig. 4B). However, when cells were treated with anti-α5β1 antibody leading to less ITGα5β1 for PN binding, cell invasion stimulated by PN was attenuated.

4. PN-mediated CCA invasion via ITGα5β1 and AKT-dependent signal transduction pathway

To confirm the decreased intact molecule of ITGα5β1 in correspond to the decreasing of cell invasion capacity, KKU-M213 cells were treated with si*ITGα5β1* using lipofectamine. The intact ITGα5β1 receptor on the membrane of CCA cells detected by immunocytochemistry showed that cells were successfully inhibited the expression of ITGα5β1 on the membrane of cancer cells as compared to the normal expression in parental KKU-M213 and mock cells (Fig. 5A). The decreased PN-induced invasive capability of the ITGα51β1-knockdown cells were confirmed (Fig. 5B). The result revealed that cells exposed to si*ITGα5* were dramatically decreased invasive capability after treated with PN (106 ± 18%) compared to no PN treatment condition, whereas significant increased of PN-induced invasion was observed in mock cells (156 ± 18%) as compared to cells without PN induction.

In order to investigate the intracellular signaling pathway activated by PN via ITGα5β1, cells with normal level and ITGα5β1-knockdown were treated with rPN. The result revealed that exogenous PN could induce phosphorylation of AKT in KKU-M213 cells having intrinsic expression of ITGα5β1 significantly at 120-min post-treatment (Fig. 5C). PN could not activate pAKT in cells with transient knockdown of ITGα5β1. However, pERK1/2 did not change as compared between cells with and without ITGα5β1. These results suggested that upon PN-stimulated ITGα5β1, AKT, not ERK, was activated. Hence, pAKT may be an intracellular signal molecule for transducing PN-activation through ITGα5β1 receptor and finally promote CCA invasion.

Discussion and conclusion

Tumor-associated fibroblasts as the major source of growth factors and extracellular matrix involved in tumorigenesis and tumor progression have been reported during a certain decades [26]. Fibroblasts isolated from CCA tissues can produce tumorigenic substances of which play important role in induction of cancer cell proliferation [3]. In support to this finding, the increased expression of tumor-related genes in CCA-derived fibroblasts has been recently explored by our group [4]. Among these genes, PN has been confirmed its high expression in the microenvironment of CCA and relation to short survival time of the patients. In this study, we explored the underlying mechanism that CCA cells respond to PN-driven tumorigenic functions. ITGs as a receptors for PN [15,17,18] were explored the profile in CCA cell lines. Adhesion and invasion assays indicated ITGα5β1 as the receptor for PN in CCA cells. The AKT-mediated was the signaling pathway underlying PN-induced cancer cell invasion.

ITGs consist of various combinations of α- and β-subunits, each with its own binding specificity, signaling properties, and biological function regulation. Their expressions have been explored in primary liver tumors in both biliary epithelial and hepatocytic lineages [22,23]. Using flow cytometry to study the expression of individual subunit of both α- and β-form in CCA cell lines, the results indicated that α1, α3, and β4 are useful markers to distinguish between hepatocellular carcinoma and CCA [23]. In similar way, ITGs α1 and β4 have been confirmed to be of value as markers in the differential diagnosis between these two common liver cancers [22]. Focusing on ITGs in CCA cells, the above mentioned studies indicate that α1 has no expression whereas ITGs α2, α3, α6, β1, and β4 are surely expression in almost CCA cell lines. This is in consistent with our finding that all of CCA cell lines derived from Thai patients used in this study expressed high level of ITG α6.

However, the expression of ITG α5 is not uniformly expressed in CCA [23]. Herein, the result shows the expression of ITG α5 in some CCA cells in particular the cells with high response to PN-induced invasion such as KKU-M213. Though the expression of ITG αv as the most popular reported as PN receptors has no reported in CCA cells, our result revealed that it could be detected in CCA cell lines but in the lower level than ITGs α5 and α6. These results imply that in addition to ITGs α2, α3, and α6, CCA cells can express αv and α5. The ITG α5 can form heterodimer with only ITG β1 [21]. It can be concluded that ITGα5β1 may be abundant and play important impact in caner progression.

The α5β1 is of particular interest because it acts as the receptor for fibronectin during neovascularization [27]. In addition, a fibroblast-expressed protein, Nischarin, has been shown to interact preferentially with the α5β1 and regulate cell migration on fibronectin [28]. As CCA could produce high level of fibronectin [29], the interaction of fibroblast-derived PN on the ITΓα5β1 may regulate the invasiveness of cancer cells within fibronectin-enriched microenvironment. Moreover, the expression of ITΓ αv in CCA cells support the previous finding that ITG αvβ6 is expressed in CCA but not in hepatocellular carcinoma and can be considered as a diagnostic marker for CCA [25].

For β-subunit ITGs, our result showed in the similar way as previously reports [22,23] that ITGs β1 and β4 are expressed in almost CCA cells. In contrast, β4 which seems to be expressed in more differentiated CCA [23] has been found in not only CCA cell lines derived from well differentiated CCA (KKU-M213, KKU-M214, and KKU-OCA17), but also in poorly differentiated, KKU-100. Interestingly, ITG β4 was found higher level in CCA cells as compared to that in MMNK1 immortalized non-tumorigenic biliary cells. Though in the previous report [22], ITG β4 was detected in normal and proliferating biliary epithelial cells

but inconsistent finding was observed in CCA, our study may ensure the presence of ITG β4 in CCA cells. Since ITG β4 can bind only to ITG a6, it is likely to say that ITGα6β4 may express abundantly on CCA membrane. In addition, our study indicated β3 and β5, normally form heterodimers with ITG αv, as the ITGs with low expression in CCA cells. Taken the results above together, it can be suggested that CCA cells express high levels of ITGs α5, α6, β1, and β4. Though the RNA level may not imply the real protein level, it is suggested that heterodimers of ITGs α5β1 and α6β4 may be the major ITG receptors expressed on CCA cell membrane.

The flow cytometry analysis, herein, revealed different levels of ITGs α5β1 and α6β4 expressions in CCA cell lines. The high expression level was found in cell lines derived from well differentiated CCA tissues in particular KKU-M213 and KKU-M214. Immunofluorescence staining confirms the presence of these two ITGs on the membrane of KKU-M213 cells. The favorable binding of PN on ITGs α5β1 rather than ITGα6β4 is indicated by the lower numbers of cells bind onto PN-coated surface after treatment of cells with neutralizing anti-ITGα5β1 antibody. This is supported by our previous report that si*ITGα5*-treated cell have lower PN-induced cell proliferation and invasion compared to the parental cells [4]. Though PN has been demonstrated to activate cells via different ITGs depending on cell type context for example ITGαvβ3 in non-small-cell lung cancer [30], ITGβ4 in pancreatic cancer [17], this is the first report to show the association of PN and ITGα5β1 in tumor promotion effect of CCA cells. In similar to PN-activated signaling pathway reported previously [17,30], PN could activate CCA cell invasion via the stimulation of AKT-dependent, but not ERK, pathway. Activation of AKT-dependent pathway is the upstream signaling molecular needed in the regulation of several biological functions including cell survival, growth and proliferation depending on the specific of ligands [31,32].

PN-stimulated some matrix metalloproteinase expressions are the possible explanation of how PN help tumor cell to invade (data not shown). In addition, the preliminary data in our lab shows that PN can activate cancer cell to migrate (data not shown). which may be the effect of PN-mediated change of cytoskeletal proteins through focal adhesion kinase (FAK) [18]. Finally, the downstream signaling pathway after AKT is of particular interest because the proper inhibitor can be proposed to apply for the attenuation of cancer progression with minimal side effects [33].

In summary, this study provides the evidence for the contribution of CCA-associated fibroblast-derived PN on the activation of tumorigenic properties of CCA cells. PN promotes invasion of cells via ITGα5β-dependent and AKT, but not ERK, signaling pathway. Though the exact downstream signaling pathway after AKT is needed to be explored, the increased pAKT by the autocrine effect of PN in the microenvironment of CCA implies the potential of using AKT inhibitor to attenuate the progression of CCA cells in the patients. In addition, this study highlight the impact of fibroblast-derived substance in the promotion of cancer progression and metastasis. Understanding the mechanisms responsible for fibroblast-associated cancer progression is challenging in the future as the alternative and synergistic cancer-targeted therapy in the CCA patients.

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References

[1] A.E. Sirica, Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy, Hepatology 41 (2005) 5-15.

[2] S. Sriamporn, P. Pisani, V. Pipitgool, K. Suwanrungruang, S. Kamsa-ard, D.M. Parkin, Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand, Trop Med Int Health 9 (2004) 588-594.

[3] C. Chuaysri, P. Thuwajit, A. Paupairoj, S. Chau-In, T. Suthiphongchai, C. Thuwajit, Alpha-smooth muscle actin-positive fibroblasts promote biliary cell proliferation and correlate with poor survival in cholangiocarcinoma, Oncol Rep 21 (2009) 957-969.

[4] K. Utispan, P. Thuwajit, Y. Abiko, K. Charngkaew, A. Paupairoj, S. Chau-in, C. Thuwajit, Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker, Mol Cancer 9 (2010) 13.

[5] T. Terada, K. Makimoto, N. Terayama, Y. Suzuki, Y. Nakanuma, Alpha-smooth muscle actin-positive stromal cells in cholangiocarcinomas, hepatocellular carcinomas and metastatic liver carcinomas, J Hepatol 24 (1996) 706-712.

[6] M. Erkan, J. Kleeff, A. Gorbachevski, C. Reiser, T. Mitkus, I. Esposito, T. Giese, M.W. Buchler, N.A. Giese, H. Friess, Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity, Gastroenterology 132 (2007) 1447-1464.

[7] N. Fukushima, Y. Kikuchi, T. Nishiyama, A. Kudo, M. Fukayama, Periostin deposition in the stroma of invasive and intraductal neoplasms of the pancreas, Mod Pathol 21 (2008) 1044-1053.

[8] J.S. Li, G.W. Sun, X.Y. Wei, W.H. Tang, Expression of periostin and its clinicopathological relevance in gastric cancer, World J Gastroenterol 13 (2007) 5261-5266.

[9] F. Puglisi, C. Puppin, E. Pegolo, C. Andreetta, G. Pascoletti, F. D'Aurizio, M. Pandolfi, G. Fasola, A. Piga, G. Damante, C. Di Loreto, Expression of periostin in human breast cancer, J Clin Pathol 61 (2008) 494-498.

[10] C. Puppin, D. Fabbro, M. Dima, C. Di Loreto, E. Puxeddu, S. Filetti, D. Russo, G. Damante, High periostin expression correlates with aggressiveness in papillary thyroid carcinomas, J Endocrinol 197 (2008) 401-408.

[11] I. Takanami, T. Abiko, S. Koizumi, Expression of periostin in patients with non-small cell lung cancer: correlation with angiogenesis and lymphangiogenesis, Int J Biol Markers 23 (2008) 182-186.

[12] K. Ruan, S. Bao, G. Ouyang, The multifaceted role of periostin in tumorigenesis, Cell Mol Life Sci 66 (2009) 2219-2230.

[13] R.O. Hynes, Integrins: a family of cell surface receptors, Cell 48 (1987) 549-554.

[14] E. Ruoslahti, M.D. Pierschbacher, New perspectives in cell adhesion: RGD and integrins, Science 238 (1987) 491-497.

[15] L. Gillan, D. Matei, D.A. Fishman, C.S. Gerbin, B.Y. Karlan, D.D. Chang, Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility, Cancer Res 62 (2002) 5358-5364.

[16] S. Bao, G. Ouyang, X. Bai, Z. Huang, C. Ma, M. Liu, R. Shao, R.M. Anderson, J.N. Rich, X.F. Wang, Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway, Cancer Cell 5 (2004) 329-339.

[17] P. Baril, R. Gangeswaran, P.C. Mahon, K. Caulee, H.M. Kocher, T. Harada, M. Zhu, H. Kalthoff, T. Crnogorac-Jurcevic, N.R. Lemoine, Periostin promotes invasiveness and resistance of pancreatic cancer cells to hypoxia-induced cell death: role of the beta4 integrin and the PI3k pathway, Oncogene 26 (2007) 2082-2094.

[18] G. Li, R. Jin, R.A. Norris, L. Zhang, S. Yu, F. Wu, R.R. Markwald, A. Nanda, S.J. Conway, S.S. Smyth, D.N. Granger, Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and, alphavbeta5 and focal adhesion kinase (FAK) pathway, Atherosclerosis 208 (2010) 358-365.

[19] R. Shao, S. Bao, X. Bai, C. Blanchette, R.M. Anderson, T. Dang, M.L. Gishizky, J.R. Marks, X.F. Wang, Acquired expression of periostin by human breast cancers promotes tumor

angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression, Mol Cell Biol 24 (2004) 3992-4003.

[20] W. Yan, R. Shao, Transduction of a mesenchyme-specific gene periostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation, J Biol Chem 281 (2006) 19700-19708.

[21] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673-687.

[22] R. Volpes, J.J. van den Oord, V.J. Desmet, Integrins as differential cell lineage markers of primary liver tumors, Am J Pathol 142 (1993) 1483-1492.

[23] M. Enjoji, H. Sakai, M. Nakashima, H. Nawata, Integrins: utility as cell type- and stage-specific markers for hepatocellular carcinoma and cholangiocarcinoma, In Vitro Cell Dev Biol Anim 34 (1998) 25-27.

[24] C. Srisomsap, P. Subhasitanont, P. Sawangareetrakul, D. Chokchaichamnankit, L. Ngiwsara, K. Chiablaem, J. Svasti, Comparison of membrane-associated proteins in human cholangiocarcinoma and hepatocellular carcinoma cell lines, Proteomics Clin Appl 1 (2007) 89-106.

[25] E. Patsenker, L. Wilkens, V. Banz, C.H. Osterreicher, R. Weimann, S. Eisele, A. Keogh, D. Stroka, A. Zimmermann, F. Stickel, The alphavbeta6 integrin is a highly specific immunohistochemical marker for cholangiocarcinoma, J Hepatol 52 (2010) 362-369.

[26] M.J. Bissell, P.A. Kenny, D.C. Radisky, Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes, Cold Spring Harb Symp Quant Biol 70 (2005) 343-356.

[27] S.E. Francis, K.L. Goh, K. Hodivala-Dilke, B.L. Bader, M. Stark, D. Davidson, R.O. Hynes, Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies, Arterioscler Thromb Vasc Biol 22 (2002) 927-933.

[28] S.K. Alahari, J.W. Lee, R.L. Juliano, Nischarin, a novel protein that interacts with the integrin alpha5 subunit and inhibits cell migration, J Cell Biol 151 (2000) 1141-1154.

[29] C.Y. Chen, X.Z. Lin, H.C. Tsao, S.C. Shiesh, The value of biliary fibronectin for diagnosis of cholangiocarcinoma, Hepatogastroenterology 50 (2003) 924-927.

[30] G. Ouyang, M. Liu, K. Ruan, G. Song, Y. Mao, S. Bao, Upregulated expression of periostin by hypoxia in non-small-cell lung cancer cells promotes cell survival via the Akt/PKB pathway, Cancer Lett 281 (2009) 213-219.

[31] J.A. Fresno Vara, E. Casado, J. de Castro, P. Cejas, C. Belda-Iniesta, M. Gonzalez-Baron, PI3K/Akt signalling pathway and cancer, Cancer Treat Rev 30 (2004) 193-204.

[32] D. Sarker, A.H. Reid, T.A. Yap, J.S. de Bono, Targeting the PI3K/AKT pathway for the treatment of prostate cancer, Clin Cancer Res 15 (2009) 4799-4805.

[33] S.S. Castillo, J. Brognard, P.A. Petukhov, C. Zhang, J. Tsurutani, C.A. Granville, M. Li, M. Jung, K.A. West, J.G. Gills, A.P. Kozikowski, P.A. Dennis, Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues, Cancer Res 64 (2004) 2782-2792.

Figure 1 Expression profile of ITGs α- and β-subunits in MMNK1, KKU-K100, KKU-M055, and KKU-M139 (A); in KKU-M156, KKU-M213, KKU-M214, and KKU-OCA17 (B). Bars represent mean ± SD of triplicate experiments.

Figure 2 The possible expressions of different ITGαβ heterodimers are predicted. Bars represent mean ± SD of triplicate experiments.

Figure 3 Flow cytometry analysis of the intact ITGs α5β1 (A) and α6β4 (B) in CCA cell lines. Immunofluorescence staining indicates the expression of both ITGs on the membrane of KKU-M213 cells.

Figure 4 Adhesion assay of untreated KKU-M213 cells compared to those treated with neutralizing anti-ITGs α5β1 and α6β4 antibodies (A). Anti-ITGα5β1 antibody reduces the binding capability of cells on PN-coated surface. Invasion assay of KKU-M213 cells when ITGα5β1 is blocked in comparison to that of the untreated cells (B). Bars represent mean ± SD of triplicate experiments. An asterix (*) means statistical significance compared to control condition.

Figure 5 PN activates cell invasion via ITGα5β1 receptor. Depletion of ITGα5β1 was observed in si*ITGα5*-treated cells by immunofluorescence staining (A). PN-induced invasion of si*ITGα5*-treated cells in comparison to that in mock cells (positive ITGα5) are shown (B). The graph shows percentage of invaded cells exposed to PN compared to that of no PN treatment which assumed to be 100%. Bar represents mean ± SD of duplicate in one experiment. Western blot analysis shows level of pAKT and pERK1/2 in si*ITGα5*-treated cells and mock cells after treated with 100 ng/ml of PN for 30 and 120 min (C). The β-actin level indicates equal amount of loading protein.

Table 1 Characters of primers used in this study

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size (bp)	Accession no.
ITGαv	TGACTGGTCTTCTACCCGC	CTCACAGATGCTCCAAACCA	121	NM_002210
ITGα5	AGTTGCATTTCCGAGTCTGG	CTCTGGGAGCACCAGATACAA	223	NM_002205
ITGα6	GGCCTTATGAAGTTGGTGGA	CTCTGGGAGCACCAGATACAA	144	NM_000210
ITGβ1	TCCCTGAAAGTCCCAAGTGT	TTTCCTGCAGTAAGCATCCA	143	NM_033666
ITGβ3	TGGTCCTGCTCTCAGTGATG	TGAAGGTAGACGTGGCCTCT	180	NM_000212
ITGβ4	TCTCCTACCGCACACAGGA	CTTCACCTGCAGCTCTTTCC	110	NM_001005619
ITGβ5	CTCCACTCTGGGAAACCTGA	AGGACGGTCAGGTTGGACTT	188	NM_002213
β-actin	CACACTGTGCCCATCTACGA	CTCCTTAATGTCACGCACGA	162	X00351

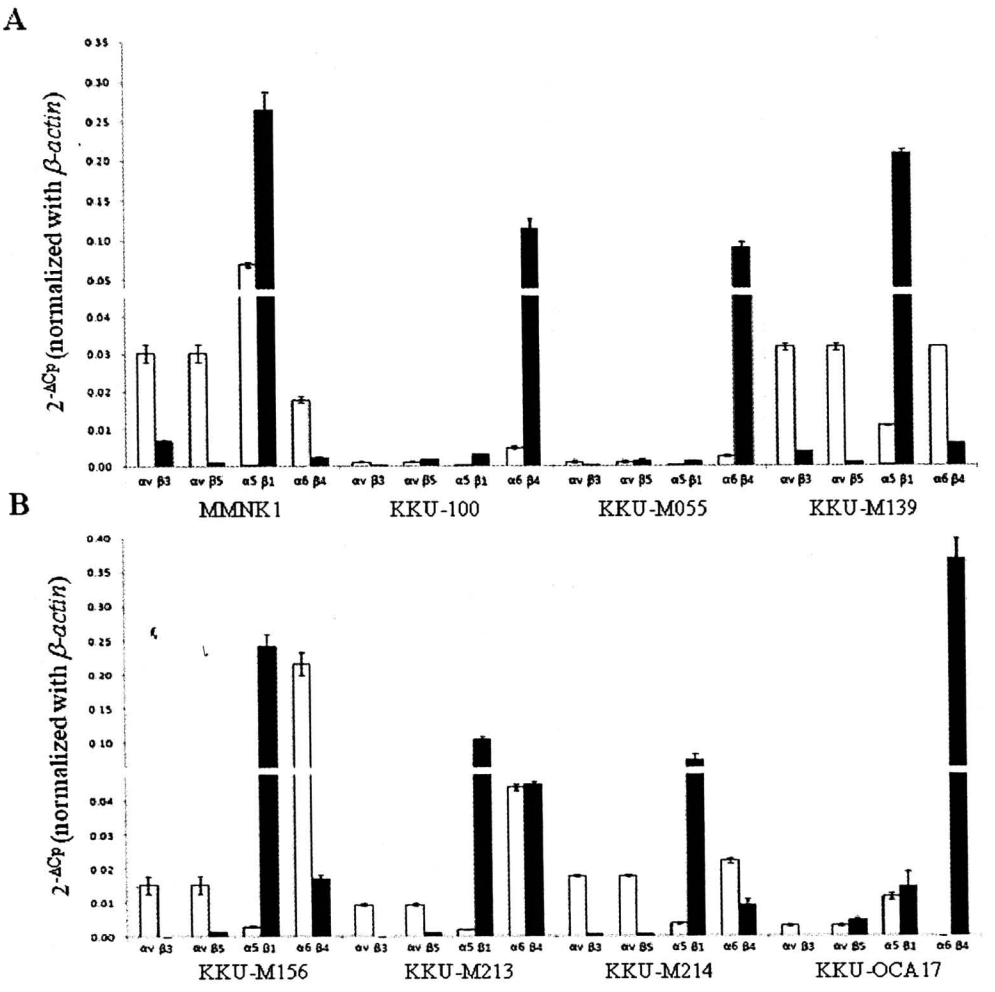
A
2-ΔCp (normalized with β-actin)
αv β3
αv β5
α5 β1
α6 β4
MMNK1
KKU-100
KKU-M055
KKU-M139
B
2-ΔCp (normalized with β-actin)
KKU-M156
KKU-M213
KKU-M214
KKU-OCA17

Figure 1

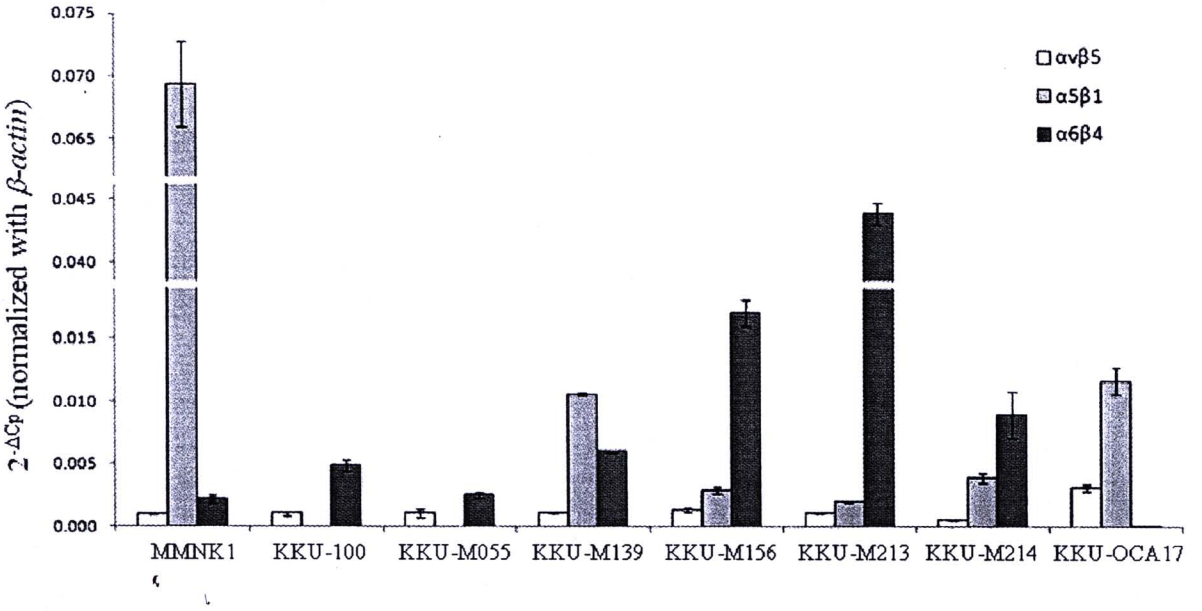
0.075
0.070
0.065
0.045
0.040
0.015
0.010
0.005
0.000
2-ΔCp (normalized with β-actin)
αvβ5
α5β1
α6β4
MMNK1
KKU-100
KKU-M055
KKU-M139
KKU-M156
KKU-M213
KKU-M214
KKU-OCA17

Figure 2

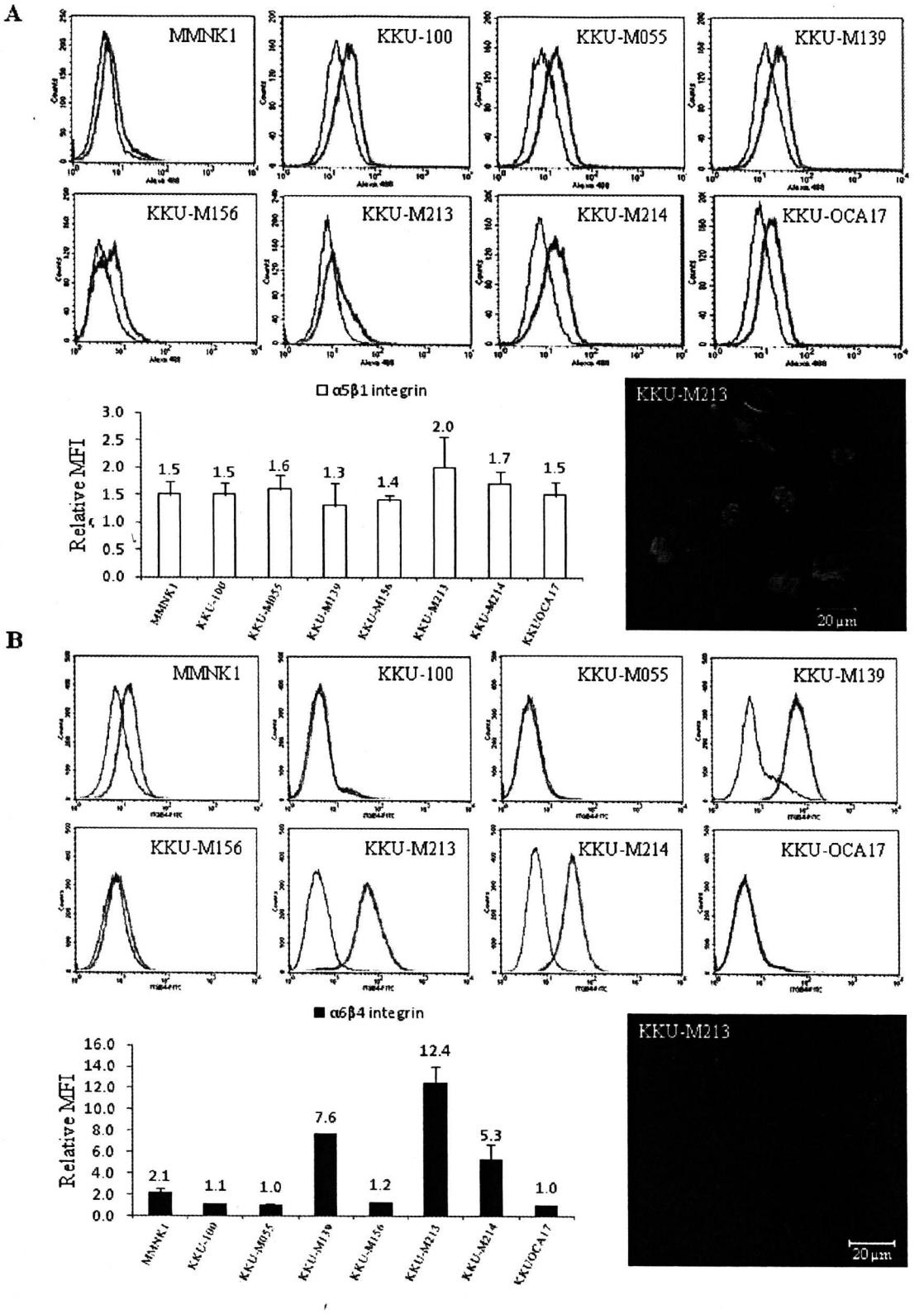
A
MMNK1
KKU-100
KKU-M055
KKU-M139
KKU-M156
KKU-M213
KKU-M214
KKU-OCA17
α5β1 integrin
Relative MFI
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.5
1.5
1.6
1.3
1.4
2.0
1.7
1.5
KKU-M213
20 μm
B
MMNK1
KKU-100
KKU-M055
KKU-M139
KKU-M156
KKU-M213
KKU-M214
KKU-OCA17
α6β4 integrin
Relative MFI
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
2.1
1.1
1.0
7.6
1.2
12.4
5.3
1.0
KKU-M213
20 μm

Figure 3

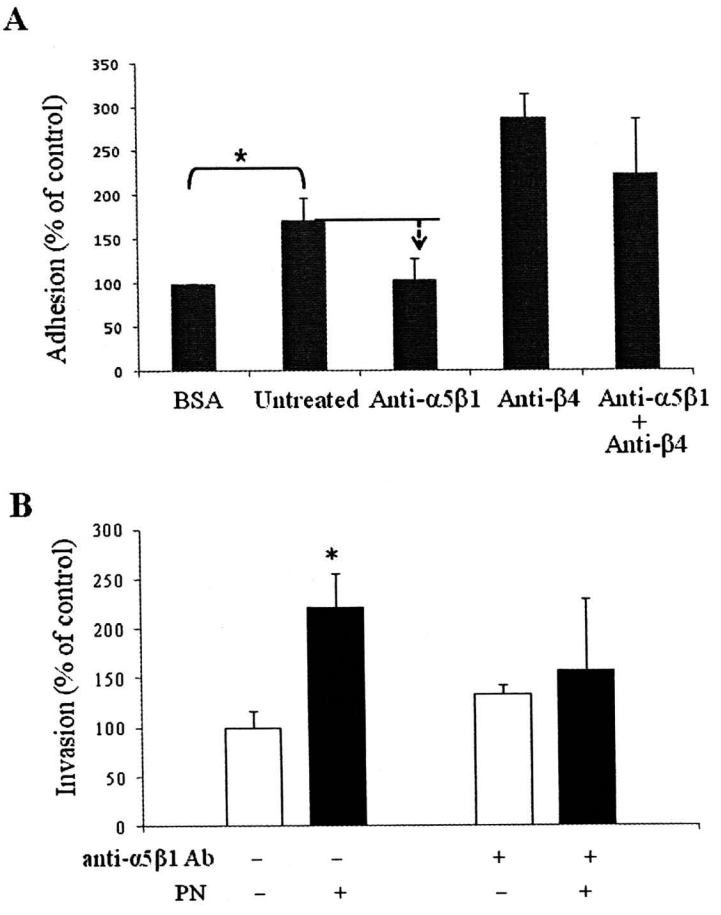
A
Adhesion (% of control)
350
300
250
200
150
100
50
0
*
BSA
Untreated
Anti-α5β1
Anti-β4
Anti-α5β1
+
Anti-β4
B
Invasion (% of control)
300
250
200
150
100
50
0
*
anti-α5β1 Ab – – + +
PN – + – +

Figure 4

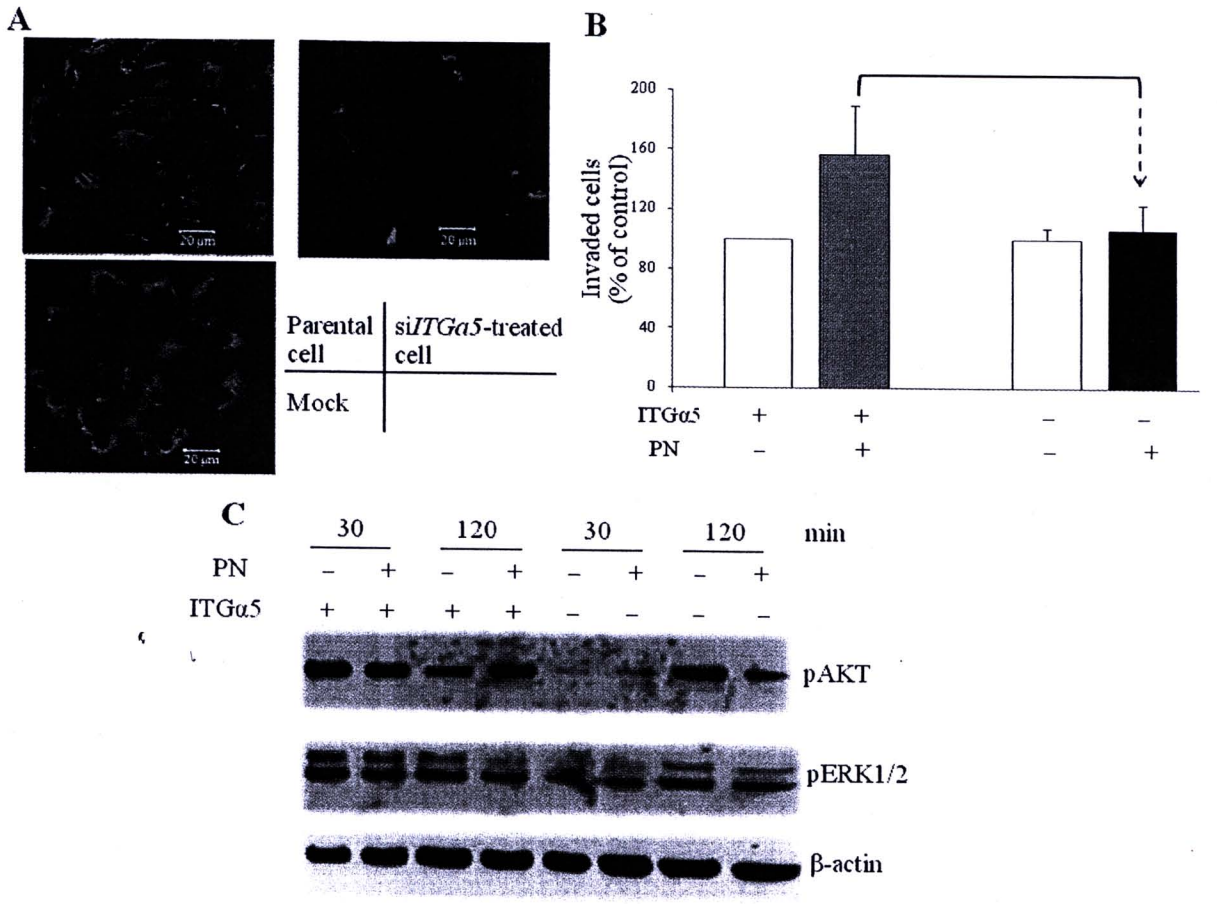
A
20 μm
20 μm
20 μm
Parental cell
siITGα5-treated cell
Mock
B
Invaded cells (% of control)
200
160
120
80
40
0
ITGα5 + + – –
PN – + – +
C
30 120 30 120 min
PN – + – + – + – +
ITGα5 + + + + – – – –
pAKT
pERK1/2
β-actin

Figure 5

Publication ที่ 4

(Tentative title)

Fibroblast-derived angiogenic factors and the prediction value of microvascular density in CCA progression