部癌と膵体尾部癌の両群間で有意な差は認められなかった.

また, 切除された膵癌症例 23 例の癌組織をα4GnT に対する特異抗体で免疫組織学的解析した結果と, 末梢血で検出されたα4GnT mRNA の結果は有意に相関していた.

一方, 慢性膵炎患者群におけるα4GnT mRNA の陽性率は 40.0%であったが, α4GnT mRNA の 発現量は 17.87 ± 6.98 であり, 膵癌に比較して有意に低値であった. また, 健常人群でもα4GnT mRNA は 17.1%に陽性であったが, α4GnT mRNA の発現量は 7.2±0.9 と膵癌に比較して有意に低値であった.

なお, 54 名の膵癌患者については同時に血清中の CEA と CA19-9 を測定し, それぞれ 44.4%, 74.1%の陽性率であったが, α4GnT mRNA の測定結果と CEA や CA19-9 の測定結果とは相関しなかった.

D. 考察

α4GnT mRNA を対象とした real-time RT-PCR 法は胃癌の場合と同様に膵癌の検出に有用であると考えられた. 特に CEA や CA19-9 の測定結果とは相関せず, また検出困難な II 期の膵癌については今回検討し得た症例は3例と少ないものの, その 2 例においてα4GnT mRNA が陽性となったことから, α4GnT mRNA は早期膵癌に対する新たな腫瘍マーカーとして期待できる.

本研究では末梢血中の膵癌細胞を実際に同定するまでには至っていないが, 免疫組織化学的に検討した切除膵癌組織におけるα4GnT 蛋白の発現パターンと, 末梢血におけるα4GnT mRNA の測定結果が有意に相関していることから, 本アッセイ法で検出されたα4GnT mRNA は末梢血中の膵癌細胞由来であることが強く示唆された.

一方, 慢性膵炎患者の 40%と健常人の 17.1%においてもα4GnT mRNA が陽性となったが, その発現量は膵癌に比較し有意に低値であり, またα4GnT mRNA は胃潰瘍や胃生検後等, 胃粘膜障害を生じた場合にも一過性に陽性となることから (Shimizu et al, *Am J Gastroentero*l 95, 3017-3018, 2000), これら非膵癌患者で検出されたα4GnT mRNA は炎症等によって末梢血に流入した胃腺粘液細胞や胃幽門腺化生を示した膵管上皮細胞由来である可能性が考えられる.

今後は本アッセイ法の実用化を目指し, 更に症例数を積み重ねて検討を行う予定である.

E. 結論

α4GnT mRNA を対象とした real-time RT-PCR 法は胃癌の場合と同様に膵癌の検出に有用であると考えられた.

G. 研究発表

1. 論文発表

Kyoshima K, Sakai K, Goto T, Tanabe A, Sato A, Nagashima H, Nakayama J: Gross total surgical removal of malignant glioma from the medulla oblongata: report of two adult cases with reference to surgical anatomy. *J Clin Neurosci* 11, 75-80, 2004.

Hiraoka N, Kawashima H, Petryniak B, Nakayama J, Mitoma J, Marth J D, Lowe J B, Fukuda M: Core 2 branching β1,6-N-acetylglucosaminyltransferase and high endothelial venule-restricted sulfotransferase collaboratively control lymphocyte homing. *J Biol Chem* 279, 3058-3067, 2004.

Nakane T, Satoh T, Inada Y, Nakayama J, Itoh F, Chiba S: Molecular cloning and expression of HRLRRP, a novel heart-restricted leucine-rich repeat protein. *Biochem Biophys Res Commun* 314, 1086-1092, 2004

Yoshizawa A, Ota H, Sakaguchi N, Kanai S, Nakayama J, Matsuzawa K, Tsuzuki S, Takada R, Miyazawa F, Kasahara H, Katsuyama T: Malignant granular cell tumor of the esophagus. (letter) *Virchows Arch* 444, 304-306, 2004.

Kyoshima K, Sakai K, Kanaji M, Oikawa S, Kobayashi S, Sato A, Nakayama J: Symmetric dumbbell ganglioneuromas of bilateral C2 and C3 roots with intradural extension associated with von Recklinghausen's disease: case report. *Surg Neurol* 61, 468-473, 2004.

Kobayashi M, Ito M, Sano K, Honda T, Nakayama J: Pulmonary lymphoepithelioma-like carcinoma: predominant infiltration of tumor-associated cytotoxic T lymphocytes might represent the enhanced tumor immunity. *Intern Med* 43, 323-326, 2004.

Kaneko T, Kawakami R, Fujinaga Y, Oguchi K, Nakayama J, Hongo K, Kadoya M: Degenerative thalamic hamartoma: CT and MR imaging features. *Am J Neuroradiol* 25, 766-768, 2004.

Hatakeyama S, Ohyama C, Minagawa S, Inoue T, Kakinuma H, Kyan A, Arai Y,

Suga T, Nakayama J, Kato T, Habuchi T, Fukuda M N: Functional correlation of trophinin expression with the malignancy of testicular germ cell tumor. *Cancer Res* 64, 4257-4262, 2004.

Isawa M, Takumi Y, Hashimoto S, Nakayama J, Usami S: Polysialic acid and HNK-1 are expressed in the adult rat vestibular endorgans. *Neuroreport* 15, 1575-1578, 2004.

Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, Fukuda M N, Fukuda M, Katsuyama T, Nakayama J: Natural antibiotic function of a human gastric mucin against *Helicobacter pylori* infection. *Science* 305, 1003-1006, 2004.

Sakai K, Peraud A, Mainprize T, Nakayama J, Tsugu A, Hongo K, Kobayashi S, Rutka J T: Inducible expression of p57KIP2 inhibits glioma cell motility and invasion. *J Neurooncol* 68, 217-223, 2004.

Kobayashi M, Mitoma J, Nakamura N, Katsuyama T, Nakayama J, Fukuda M: Induction of peripheral lymph node addressin in human gastric mucosa infected by *Helicobacter pylori*. *Proc Natl Acad Sci USA* 101, 17807-17812, 2004.

2. 学会発表

Nakayama J, Kawakubo M, Ito Y, Kobayashi M, Fukuda N M, Fukuda M, Katsuyama T: A subtype of human gastric mucins expressing alpha1,4-GlcNAc-capped *O*-glycans functions as a natural antibiotic against *Helicobacter pylori* infection. US/Japan Glyco 2004, Honolulu, USA, 2004.

H. 知的財産権の出願・登録状況

1. 特許

U. S. patent (provisional) 60/546.600 (2004 年 2 月 20 日) – "Molecule, functional molecule and process for manufacturing thereof, composition, pharmaceutical, food, beverage, milk, transgenic mammal, process for inhibiting growth of bacteria, process for treating and preventing gastric ulcers, and method for treating gastric cancer".

発明者：中山　淳，川久保雅友，福田穣，勝山　努

厚生労働科学研究費補助金　（第３次対がん総合戦略研究事業）
分担研究報告書

悪性腫瘍において異常を来している血清タンパク質の網羅的解析

分担研究者　近藤　格　国立がんセンター生物学部室長

研究要旨：悪性腫瘍において異常を来す血清タンパク質を調べるための実験系を構築した。液体クロマトグラフィー、蛍光色素、二次元電気泳動法、質量分析機を使い、網羅的かつ定量的に血清タンパク質のプロファイリングを行なうことができるようになった。本方法は悪性腫瘍の早期診断、病態把握のための腫瘍マーカーの開発に有用であると考えられる。

A. 研究目的

本研究の目的は早期診断および病態把握のための血清腫瘍マーカーの開発である。従来ある方法の欠点を克服するため血清タンパク質の新規の発現解析系の構築を試みた。

B. 研究方法

以下のような血清タンパク質発現解析システムの構築を行なった。血清タンパク質のうち比較的量が多いアルブミン、トランスフェリン、抗体などを市販アフィニティーカラムで除去する。その後、イオン交換法で分画をとり、各分画に含まれるタンパク質を蛍光標識する。その際、異なる検体から得られた分画はそれぞれ異なる蛍光色素で標識する。比較したいサンプルどうしを標識後に混ぜたあとで逆相クロマトグラフィーまたは二次元電気泳動で分離・分画する。逆相カラムの各分画はさらに SDS-PAGE 電気泳動によって分離される。ゲル中のタンパク質は蛍光シグナルとしてレーザースキャナーで観察され、血清タンパク質の発現量はシグナルの強度として定量される。タンパク質の同定は質量分析で行なわれる。

（倫理面への配慮）

今回使用した検体はすでに倫理委員会で承認を受けている。今後新たに膵がんと肺がんの血清を使用する計画であり、そのため国立がんセンターの倫理審査委員会に審査を請求している。

C. 研究結果

10 数種類のイオン交換カラムおよび逆相カラムをスクリーニングし、もっとも分離・保持がよいものを選択した。さらに蛍光標識、泳動、分画の諸条件を検討した。最終的に、数１００の血清タンパク質を感度よく分離・定量することができる実験系を構築するこ

とができた。一部はゲルからタンパク質を回収して質量分析にかけて、タンパク質の同定ができることを確認した。

D. 考察

血清タンパク質はダイナミックレンジが広く種類が多いため全長タンパク質の発現解析は一般に困難であると言われている。そのため血清タンパク質についてはタンパク質分解酵素でペプチドに消化してから解析が行なわれるのが一般的である。しかし、この方法では検体ごとの翻訳後修飾（切断や糖鎖修飾など）による違いが分からないことが多い。一方で悪性腫瘍においては翻訳後修飾の異常が報告されていることから、その解析は重要である。市販されているほとんどのカラムは何１０００種類もの全長のタンパク質の混合サンプル（血清）を解析用に分離するようには設計されていないので、各種のカラムをスクリーニングするところから始め、血清タンパク質の分離に最適と思われる組み合わせを決めた。また、電気泳動による誤差を押さえるために、異なるサンプルを異なる蛍光色素で標識して一枚のゲルで電気泳動を行なうようにした。さらに、分離の最終段階に二次元電気泳動法を使っているので、翻訳後修飾の異常を検出することも可能である。液体クロマトグラフィー、（二次元）電気泳動、蛍光色素を組み合わせたこのような発現解析系は世界でも稀であることから、他ではできない発見をこれから期待している。また、本システムは実験結果を画像としてデータベース化することが可能である。多検体を用いた実験や各種腫瘍の縦断的な研究にはデータベースが有用であると考えられる。データベース化に向けた方法の開発が今後の課題である。問題点としては質量分析によるタンパク質の同定が挙げられる。蛍光色素によるタンパク質の検出感度は計算上は質量分析の同定感度を上回るため、検出できても同定できないタンパク質が出現する可能性がある。そのためのアプリケーションも考えていく必要がある。また、タンパク質の同定の過程で、本システムでは検出ができないタンパク質が明らかになっていくと推測される。その場合は分離モードの若干の修正が必要になるだろう。

E. 結論

本年度は血清タンパク質解析システムを構築した。実用レベルに仕上がっており、実際の臨床検体の解析を来年度に行なう。

E.　健康危険情報

なし

G.　研究発表

1. 論文発表

1. Yokoo H, Kondo T, Fujii K, Yamada T, Todo S, Hirohashi S, Proteomics signature corresponding to alpha

fetoprotein expression in liver cancer cells, Hepatology, 40, 609-617, 2004.

2. Seike M, Kondo T, Fujii K, Yamada T, Gemma A, Kudoh S, Hirohashi S, Proteomic signature of human cancer cells, Proteomics, 4, 2776-2788, 2004.
3. 近藤格、二次元電気泳動を用いた臨床マーカーの開発 -必要とされる技術開発と臨床研究への応用-、J Electrophoresis 、48、1-3、2004.

2. 学会発表

1. 第５４回日本電気泳動学会基調講演「プロテオミクスの時代における電気泳動と質量分析」
2. 第７７回日本生化学学会シンポジウム「腫瘍マーカー開発のためのがんのプロテオーム研究」
3. 第６３回日本癌学会学術総会シンポジウム「肝がんのプロテオーム解析～肝がんの早期再発に関わるタンパク質の探索～」

H. 知的財産権の出願・登録状況

特記事項なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
「がん検診に有用な新しい腫瘍マーカーの開発」班
分担研究報告書

膵癌のペプチド性腫瘍マーカーの開発に関する研究

分担研究者　佐々木　一樹
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研究要旨
膵癌培養細胞株から選択的に分泌される２種類のペプチドについて有効性を評価する為の血中での測定系を構築した。

A.　研究の目的
代表的な難治がんである膵癌について現在の腫瘍マーカーの問題点を補完しうる新しいマーカーを開発する。

B.　研究方法
膵がん培養細胞の上清より見いだされた候補ペプチドの血中での検出、測定可能性を検討する為にELISAおよびRIAを用いる測定系を構築する。
（倫理面への配慮）
共同研究先で包括同意を得た検体を使用した。

C.　研究結果
前年度までに同定したDMBT1のＣ端ペプチドの ELISA 系で、健常者(24 例)に対し、慢性膵炎（25 例）膵癌（34 例）の血清中で高値になる傾向を認めた。また、ある分泌蛋白質のプロペプチド部分に対する RIA 系を構築し、CA19-9 陰性の進行膵癌３例で健常者に比して有意に高値を呈することが判明した。

D.　考察
２つのマーカーペプチドそれぞれ単独では、健常者と慢性膵炎、膵癌の区別は不可能であった。昨年、遺伝子発現レベルからの研究でこれらのペプチドをコードする遺伝子が膵癌で高発現することが報告され、培養細胞を用いる手法の妥当性が間接的に支持された。

E.　結論
膵癌培養細胞から同定されたペプチドが血中で測定可能であることを示したが、現状では、健常者と慢性膵炎、膵癌を区別しうるマーカーは見いだされなかった。

F. 健康危険情報

なし

G. 研究発表

５．　論文発表

なし

６．学会発表

なし

H. 知的財産権の出願・登録状況（予定を含む。）

特許収得

公開特許 WO2003/027138

「新規なスクリーニング法によるがんマーカーの探索」

別紙５

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujii K, Kondo T, Yokoo H, Yamada T, et al.	Proteomic study of human hepatocellular carcinoma using two-dimensional difference gel electrophoresis with saturation cysteine dye.	Proteomics			in press
Naishiro Y, Yamada T, et al.	Morphological and transcriptional responses of untransformed intestinal epithelial cells to an oncogenic beta-catenin protein.	Oncogene			in press
Hara T, Honda K, Ono M, Naito K, Hirohashi S, Yamada T.	Identification of two serum biomarkers of renal cell carcinoma by surface-enhanced laser desorption/ionization mass spectrometry.	J. Urology			in press
Idogawa M, Yamada T, et al.	Poly(ADP-ribose) polymerase-1 (PARP-1) is a component of the oncogenic T-cell factor-4 (TCF-4)/β-catenin complex.	Gastroenterology			in press
Nitori N, Ino Y, Nakanishi Y, Yamada T, et al.	Prognostic significance of Tissue Factor in Pancreatic Ductal Adenocarcinoma	Clinical Cancer Research			in press
Seike T, Kondo T, Fujii K, Okano T, Yamada T, et al.	Proteomic signatures for histological types of lung cancer	Proteomics			in press
Honda K, Yamada T, et al.	Actinin-4 increases cell motility and promotes lymph node metastasis of colorectal cancer.	Gastroenterology	128(1)	51-62	2005
Seike M, Kondo T, Fujii K, Yamada T, et al.	Proteomic signature of human cancer cells.	Proteomics	4(9)	2776-2788	2004
Yokoo H, Kondo T, Fujii K, Yamada T, et al.	Proteomic signature corresponding to alpha fetoprotein expression in liver cancer cells.	Hepatology	40(3)	609-617	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Honda K, Yamada T, et al.	Alternative splice variant of actinin-4 in small cell lung cancer.	Oncogene	23(30)	5257-5262	2004
Liu QY, Lei JX, LeBlanc J, Sodja C, Ly D, Charlebois C, Walker PR, Yamada T, et al.	Regulation of DNaseY activity by actinin-alpha4 during apoptosis.	Cell Death Differ	11(6)	645-654	2004
山田哲司	プロテオミクスの現状と将来	Modern Media (モダンメディア)	50巻10号	227-232	2004
山田哲司、他	MDR1はβ-cateninとTCF4転写複合体の標的遺伝子である	分子消化器病	1巻4号	336-341	2004
本田一文、山田哲司	難治がんの早期診断マーカーの探索	検査と技術	33巻2号	172-174	2005
Maekawa M, Taniguchi T, et al.	Pilot study of arbitrarily primed PCR-single stranded DNA conformation polymorphism analysis for screening genetic polymorphisms related to specific phenotypes.	Clin Chim Acta			in press
Maekawa M, Taniguchi T, Ishikawa J, Toyoda S, Takahata N.	Problem with Detection of an Insertion-Type Mutation in the BCHE Gene in a Patient with Butyrylcholinesterase Deficiency.	Clin Chem	50	2410-2411	2004
Shinmura K, Tao H, Goto M, Igarashi H, Taniguchi T, Maekawa M, et al.	Inactivating mutations of the human base excision repair gene NEIL1 in gastric cancer.	Carcinogenesis	25	2311-2317	2004
Ishikawa J, Taniguchi T, Higashi H, Miura K, Suzuki K, Takeshita A, Maekawa M.	High lactate dehydrogenase isoenzyme 1 in a patient with malignant germ cell tumor is attributable to aberrant methylation of the LDHA gene.	Clin Chem	50	1826-1828	2004
Maekawa M, Taniguchi T, Higashi H, Sugimura H, Sugano K, Kanno T	Methylation of mitochondrial DNA is not a useful marker for cancer detection.	Clin Chem	50	1480-1481	2004
Maekawa M, Nagaoka T, Taniguchi T, Higashi H, et al.	Three-dimensional microarray compared with PCR-sngle-strand conformation polymorphism analysis/DNA sequencing for mutation analysis of K-ras codons 12 and 13.	Clin Chem	50	1322-1327	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Miyakura Y, Sugano K, Akasu T, Yoshida T, Maekawa M, Saitoh S, et al.	Extensive but hemiallelic methylation of the hMLH1 promoter region in early-onset sporadic colon cancers with microsatellite instability.	Clin Gastroenterol Hepatol	2	147-156	2004
Ishikawa J, Fujita K, Kanno T, Maekawa M.	Lactate dehydrogenase (LD) extra isoenzyme electrophoretic band between LD1 and LD2 caused by a complex with alpha1-lipoprotein.	A case report. Clin Chem Lab Med	42	102-104	2004
前川真人	膵癌の腫瘍マーカー	膵臓			in press
前川真人	原発不明癌	日本分子腫瘍マーカー研究会誌			in press
Kyoshima K, Sakai K, Goto T, Tanabe A, Sato A, Nagashima H, Nakayama J	Gross total surgical removal of malignant glioma from the medulla oblongata: report of two adult cases with reference to surgical anatomy.	J Clin Neurosci	11	75-80	2004
Hiraoka N, Kawashima H, Petryniak B, Nakayama J, Mitoma J, Marth J D, Lowe J B, Fukuda M	Core 2 branching β1,6-N-acetylglucosaminyltransferase and high endothelial venule-restricted sulfotransferase collaboratively control lymphocyte homing.	J Biol Chem	279	3058-3067	2004
Nakane T, Satoh T, Inada Y, Nakayama J, Itoh F, Chiba S	Molecular cloning and expression of HRLRRP, a novel heart-restricted leucine-rich repeat protein.	Biochem Biophys Res Commun	314	1086-1092	2004
Yoshizawa A, Ota H, Sakaguchi N, Kanai S, Nakayama J, et al.	Malignant granular cell tumor of the esophagus.(letter)	Virchows Arch	444	304-306	2004
Kyoshima K, Sakai K, Kanaji M, Oikawa S, Kobayashi S, Sato A, Nakayama J	Symmetric dumbbell ganglioneuromas of bilateral C2 and C3 roots with intradural extension associated with von Recklinghausen's disease: case report.	Surg Neurol	61	468-473	2004
Kobayashi M, Ito M, Sano K, Honda T, Nakayama J	Pulmonary lymphoepithelioma-like carcinoma: predominant infiltration of tumor-associated cytotoxic T lymphocytes might represent the enhanced tumor immunity.	Intern Med.	43	323-326	2004
Kaneko T, Kawakami R, Fujinaga Y, Oguchi K, Nakayama J, et al.	Degenerative thalamic hamartoma: CT and MR imaging features.	Am J Neuroradiol	25	766-768	2004
Hatakeyama S, Ohyama C, Minagawa S, Inoue T, Kakinuma H, Kyan A, Arai Y, Suga T, Nakayama J, et al.	Functional correlation of trophinin expression with the malignancy of testicular germ cell tumor.	Cancer Res	64	4257-4262	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Isawa M, Takumi Y, Hashimoto S, Nakayama J, Usami S	Polysialic acid and HNK-1 are expressed in the adult rat vestibular endorgans.	Neuroreport	15	1575-1578	2004
Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, Fukuda M N, Fukuda M, Katsuyama T, Nakayama J	Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection.	Science	305	1003-1006	2004
Sakai K, Peraud A, Mainprize T, Nakayama J, et al.	Inducible expression of p57KIP2 inhibits glioma cell motility and invasion.	J Neurooncol	68	217-223	2004
Kobayashi M, Mitoma J, Nakamura N, Katsuyama T, Nakayama J, Fukuda M	Induction of peripheral lymph node addressin in human gastric mucosa infected by Helicobacter pylori.	Proc Natl Acad Sci USA	101	17807-17812	2004
近藤格	二次元電気泳動を用いた臨床マーカーの開発 -必要とされる技術開発と臨床研究への応用-	J Electrophoresis	48	1-3	2004

Actinin-4 Increases Cell Motility and Promotes Lymph Node Metastasis of Colorectal Cancer

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Background & Aims: Enhanced motility of cancer cells by remodeling of the actin cytoskeleton seems crucial in the process of cancer invasion and metastasis. We previously identified an actin-binding protein, actinin-4, as a new biomarker of cancer invasion and an indicator of prognosis for patients with breast cancer. However, its involvement in the mechanisms of cancer invasion and metastasis remains undetermined. The current study tested the role of actinin-4 in the motility and metastatic potential of colorectal cancer cells. *Methods & Results:* Quantitative immunofluorescence histochemistry showed that the expression level of the actinin-4 protein was increased in 73.1% (19/26) of the cases of colorectal cancer over the corresponding normal intestinal epithelium. The increased expression of actinin-4 was most significant in dedifferentiated cancer cells at the invasive front. A colorectal cancer cell clone capable of inducing actinin-4 using the tetracycline-regulatory system (designated DLD1 Tet-off ACTN-4) was established. Upon the induction of actinin-4, DLD1 Tet-off ACTN-4 cells spread filopodia and significantly increased their motility ($P = .00027$); actinin-4 protein was concentrated at the leading edges of these actin-rich podia. When injected into the mesocecum of severe combined immunodeficient mice, DLD1 Tet-off ACTN4 cells, but not the control cells, metastasized into regional mesenteric lymph nodes, resembling the behavior of clinical cancers. The expression of actinin-4 in focally dedifferentiated cancer cells at the invasive front was significantly correlated with the frequency of lymph node metastasis of colorectal cancer ($P = .038$). *Conclusions:* Actinin-4 actively increases cell motility and promotes lymph node metastasis of colorectal cancer.

Metastasis is one of the major obstacles to the treatment of colorectal cancer. The surgical management of colorectal cancer without lymph node and organ metastasis is relatively uncomplicated, but the treatment of metastasized disease remains challenging.[1,2] Although the entire molecular and cellular mechanisms of cancer invasion and metastasis remain largely unexplored, the acquisition of enhanced motility by cancer cells seems to be a requirement for these processes.[3–6]

Various molecular components of the cytoskeletal, cell adhesion, and signaling systems seem to be involved in the regulation of cell motility.[7–10] In this study, we focused on a molecule that regulates the actin cytoskeleton. During the process of cell movement, the actin cytoskeleton is dynamically remodeled, and characteristic actin-rich protrusive structures, known as filopodia and lamellipodia, are created at the leading edge to generate mechanical forces.[10,11] The remodeling of the actin cytoskeleton and formation of filopodia and lamellipodia is believed to be initiated by the activation of small guanosine triphosphate–binding proteins, Cdc42 and Rac.[11] WASP family proteins, and subsequently the Arp2/3 complex, are activated in the cascades evoked by Cdc42 and Rac.[12,13] However, effector molecule(s) that directly regulate actin dynamics have not yet been identified.

Effectors regulating the actin cytoskeleton are believed to include various classes of actin-binding proteins. α-Actinin is a family of actin-binding proteins. So far, 4 isoforms of human α-actinin have been identified, including 2 nonmuscle types, actinin-1 and actinin-4.[14–16] Actinin-1 is a structural protein localized at points of focal contacts and adherens junctions.[17,18] Actinin-4 was identified by our laboratory as another actinin molecule with a distinct subcellular localization.[16] In contrast to actinin-1, the actinin-4 protein was highly concentrated at the leading edge of motile cells and in cytoplasmic regions with sharp cell extensions.[16] Actinin-4 was preferentially localized to moving structures, such as the dorsal ruffles of mouse macrophages.[19] The expression level of actinin-4 was significantly increased in cells exhibiting enhanced motility.[16] The cytoplasmic localization of actinin-4 was closely associated with the invasive phenotype of breast cancer and was a predictor of the

Abbreviations used in this paper: Dox, doxycycline; HA, hemagglutinin; SCID, severe combined immunodeficient.

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prognosis of patients with breast cancer.[16] Yamagata et al reached the conclusion that actinin-4 was a significant prognostic predictor of non–small cell lung cancer using a large-scale complementary DNA microarray analysis.[20] In another complementary DNA microarray analysis, actinin-4 was identified as one of the genes up-regulated by Rac1 and Cdc42 but not by RhoA.[21] All of these results indirectly support the notion that actinin-4 is involved in cell movement, cancer invasion, and metastasis. However, whether actinin-4 is actively involved in the molecular mechanisms of these processes remains undetermined.

In this study, we constructed a colorectal cancer cell clone capable of inducing actinin-4 under the strict control of the tetracycline-regulatory system[22] to avoid unrelated factors and to pinpoint the precise biological significance of actinin-4. We obtained direct evidence of the involvement of actinin-4 in the modulation of the actin cytoskeleton and the enhancement of colorectal cancer cell motility. The increased expression of actinin-4 seems to be involved in the infiltrative growth and lymphatic spread of colorectal cancer.

Materials and Methods

Antibodies

Anti-hemagglutinin (HA) rat monoclonal antibody (clone 3F10) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anti-actin mouse monoclonal antibody (clone AC-15) was purchased from Abcam Inc (Cambridge, England). Anti–E-cadherin mouse monoclonal HECD1 and anti–actinin-4 rabbit polyclonal (Ab-1) antibodies were produced in our laboratory, as described previously.[16,23] Another anti–actinin-4 polyclonal (Ab-2) antibody was newly raised against the synthetic peptide NQSYQYGPSSAGNGAGC and affinity purified. These 2 anti–actinin-4 antibodies yielded essentially the same results.

Immunohistochemistry

Thin sections (5 μm) of formalin-fixed and paraffin-embedded specimens of 26 colorectal cancer specimens were used for the immunofluorescence histochemistry study (Figures 1 and 2). After incubation with anti–actinin-4 rabbit polyclonal (Ab-1) and anti–E-cadherin mouse antibodies at 4°C overnight, each protein was detected with Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-mouse antibodies (Molecular Probes, Inc, Eugene, OR), respectively. Specimens were examined using a confocal microscope (2000MP; Bio-Rad Laboratories, Hercules, CA). Fluorescence intensity was evaluated using the surface plotting and line profiling functions of the LaserPix image analysis software (Bio-Rad Laboratories). We measured the fluorescence intensity of 3 randomly selected normal colonic crypts and 3 cancerous glands in each case.

Specimens of another larger series of colorectal cancer (52 cases) were examined using conventional immunoperoxidase staining with anti-actinin polyclonal antibody (Ab-2) (Table 1).[16]

Immunofluorescence Cytochemistry

Cells grown on glass coverslips (Asahi Technoglass, Tokyo, Japan) were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After blocking with 10% normal swine serum (Vector Laboratories, Inc, Burlingame, CA) for 30 minutes at room temperature, the cells were incubated with anti-HA rat monoclonal antibody at 4°C overnight. After incubation with Alexa Fluor 488 anti-rat immunoglobulin (IgG) G and Alexa Fluor 594 phalloidin (Molecular Probes, Inc), the specimens were examined as previously described above.

Immunoblot Analyses

Cells were extracted with lysis buffer (10 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% NP-40, 1 mg/mL NaN_3) containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) on ice for 30 minutes before centrifugation (12,000*g* for 30 minutes). Cell lysates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After incubation with primary antibodies at 4°C overnight, the blots were detected with horseradish peroxidase–conjugated anti-mouse IgG, anti-rat IgG, or anti-rabbit IgG antibodies and enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Corp, Piscataway, NJ), as instructed by the supplier. The blot intensity was quantified using an LAS-3000 image analyzer and Multi Gauze software (Fuji Film, Tokyo, Japan).

Scanning Electron Microscopy

Cells grown on cover glasses were washed twice with phosphate-buffered saline and fixed with 2.5% glutaraldehyde at 4°C for 16 hours. The cells were then washed 3 times with phosphate-buffered saline, postfixed with 2% osmium tetroxide at 4°C for 30 minutes, and dehydrated in an ascending ethanol series. After critical point drying using liquid CO_2, the specimens were coated with gold-palladium and examined under a scanning electron microscope (S-900; Hitachi, Hitachi, Japan).[24]

Establishment of DLD1 Tet-off ACTN4

A human colorectal cancer cell line, DLD1, was obtained from American Type Culture Collection (Rockville, MD) and cultivated in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). DLD1 was originally established from a primary adenocarcinoma of the sigmoid colon.[25]

DLD1 was engineered to expresses the N-terminally HA-tagged actinin-4 protein under control of the tetracycline-regulatory promoter system (BD Biosciences, San Jose, CA), as instructed by the supplier. A fragment (encoding amino acids

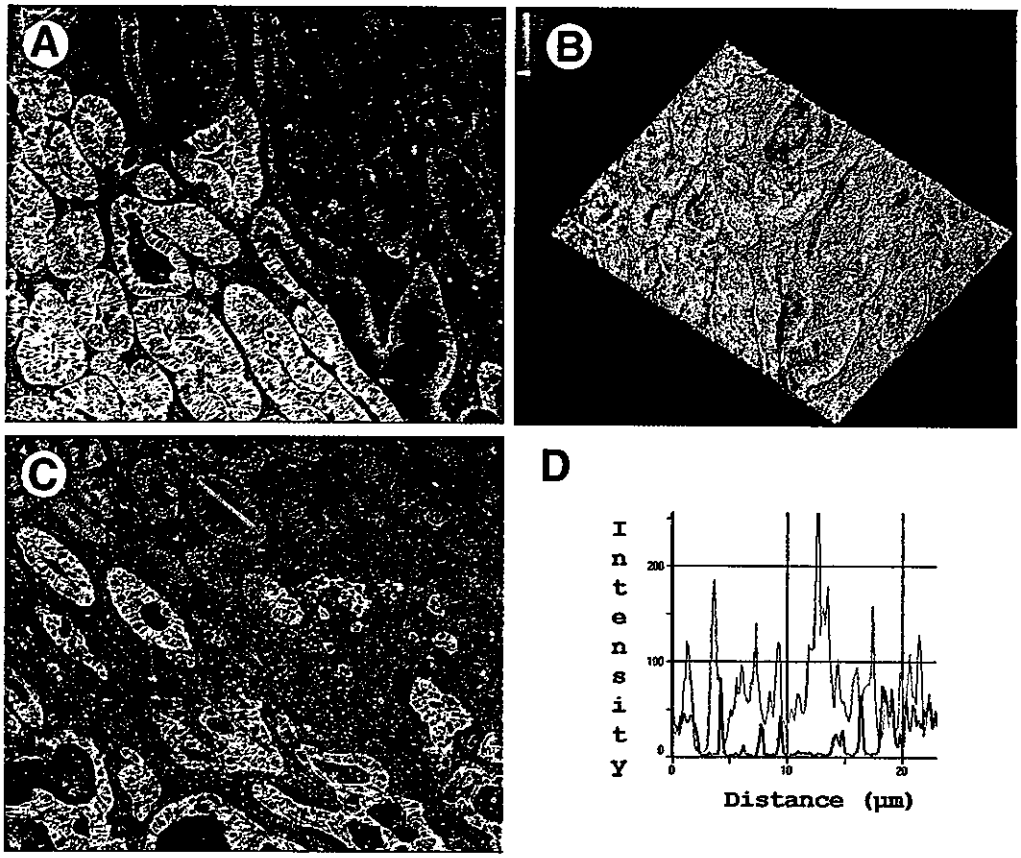

Figure 1. (*A* and *C*) Conventional immunofluorescence histochemical images of human colorectal cancer (2 cases) with anti–actinin-4 antibody (Ab-1). Cancer is seen on the lower left and normal glands on the upper right in *A* and *C*. (*B*) Three-dimensional pseudo-color visualization of the spatial distribution and expression level of the actinin-4 protein in *A*. The fluorescence intensity of the areas was measured using the Radiance 2000MP confocal microscopy (Bio-Rad). The difference in the fluorescence intensity is visualized in a color gradient, as indicated on the upper left (from low to high, blue/green/yellow/red) using the surface plotting function of the Laserpix image analysis software (Bio-Rad). (*D*) Histogram of the fluorescence intensity of colorectal cancer (*red line*) and normal intestinal epithelium (*blue line*) is visualized using the line profiling function of the previously mentioned software. The position of the lines used for the measurements is shown in *C*. A red line in *C* is placed on a randomly selected cancer nest and a blue line on a normal gland.

29–911; NP-004915) of the actinin-4 complementary DNA was amplified by polymerase chain reaction using a pair of primers (5′-cttacgcgtaccatggactacccatacgacgtcccagactacgctggcgactacatggccc-3′ and 5′-cggcatcgattcacaggtcgctctcgccataca-3′), digested with restriction enzymes (*Mlu*I and *Cla*I), and subcloned into pTRE2-pur (pTRE2-ACTN4). The composition of the construct was confirmed by sequencing. Cells were transfected using Lipofectamine 2000 (Invitrogen) reagent and cloned by limiting dilution. To suppress the tetracycline-regulatory promoter, doxycycline (Dox) (Sigma-Aldrich) was added to the culture medium at concentrations of .01–.1 μg/mL.

Cell Motility Assays

Cell motility was evaluated using the conventional wound-healing assay[16] and the ArrayScan II system (Beckton Coulter, Inc, Fullerton, CA).

Confluent cell monolayers were gently scratched with plastic pipette tips. After incubation for 16 hours, cells infiltrating the "wound" areas were examined using a phase-contrast microscope (Nikon Instruments Inc, Tokyo, Japan).

Cell motility was quantified using the Cell Motility HitKit (Cellomics, Inc, Pittsburgh, PA). Briefly, 125 cells/well were seeded into 96-well plates, which had been coated with type I collagen and blue fluorescent beads, and incubated in the presence of 10% fetal bovine serum for 20 hours. Cells were fixed with 5.5% formaldehyde and stained with rhodamine-labeled phalloidin. Areas of fluorescent beads denuded by cell movement were measured using the ArrayScan II system. The movement of at least 50 cells was measured in each experiment. The details of the ArrayScan system and the Cell Motility HitKit are available at http://www.cellomics.com/content/menu/Cell_Motility_Kit/.

Animal Experiments

Severe combined immunodeficient (SCID) female mice were obtained from Clea Japan (Tokyo, Japan) and maintained in a specific pathogen-free environment. A laparotomy was performed under general anesthesia, and 2×10^5 DLD1 Tet-Off ACTN4 or control cells were injected into the subserosal region of the spleen or 5×10^5 cells were injected into the mesocecum of the animals.[26] Primary

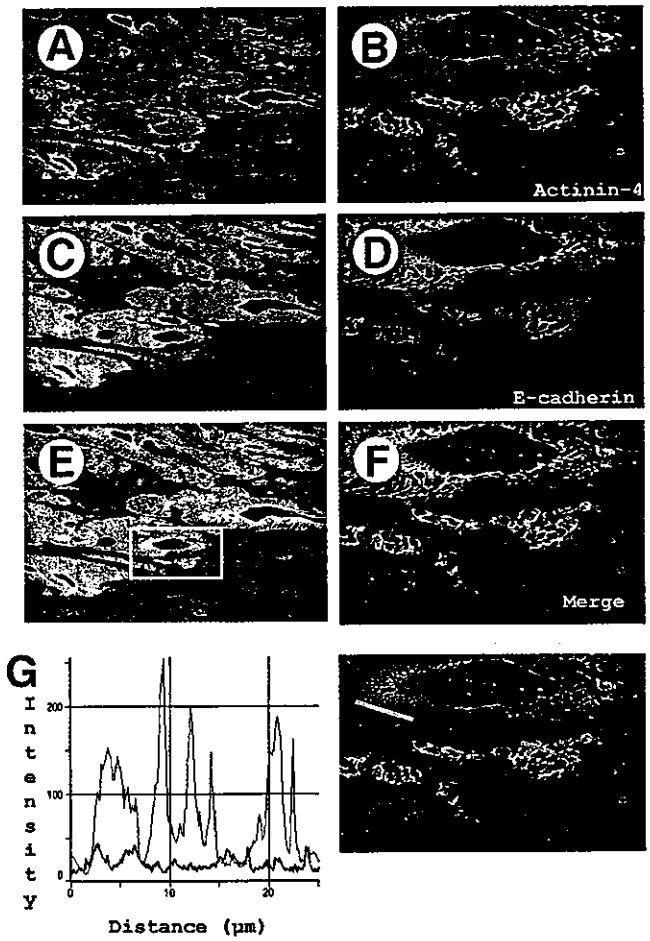

Figure 2. (*A–F*) Immunofluorescence histochemical analysis of the invasive front of human colorectal cancer using anti–actinin-4 rabbit polyclonal (Ab-1) (red, *A*, *B*, *E*, and *F*) and anti–E-cadherin mouse monoclonal (green, *C–F*) antibodies. The areas of *A*, *C*, and *E* corresponding to the area in the square in *E* were enlarged and are shown in *B*, *D*, and *F*, respectively. (*G*) Line profile of the fluorescence intensity of cancer cells infiltrating the stroma and cancer cells forming a glandular structure in a representative case. The right panel is a black-and-white image of *B* that shows the lines used for the measurements. A representative histogram of the relative fluorescence intensity along a line placed on a dedifferentiated cancer nest (*red*) and a randomly selected line placed on a cancerous gland that did not exhibit a dedifferentiation histology (*blue*) is shown in the left panel.

or metastatic tumor formation was evaluated 5 weeks later under a dissecting microscope (Nikon Instruments Inc). Autopsied tissues were processed for histologic examination and immunohistochemical analyses with anti-HA antibody.[27] All animal experiments were reviewed and approved by the ethical committee of the National Cancer Center Research Institute (Tokyo, Japan).

Statistical Analysis

The Student t test was used for the comparison of continuous variables and Fisher exact test for the comparison of categorical variables. The difference was considered significant when the P value was $<.05$.

Results

Increased Expression of Actinin-4 Protein in Colorectal Cancer

The expression of actinin-4 protein was examined in 26 cases of colorectal carcinoma using immunofluorescence histochemistry (Figure 1). The expression level of actinin-4 was increased in the cancer cells when compared with normal intestinal epithelial cells in the same specimens (Figure 1*A* and *B*). We measured the fluorescence intensity along lines of the same length placed on 3 randomly selected normal colonic crypts and 3 cancerous glands in each case and found that the expression of actinin-4 was higher in the cancer cells than in the normal tissues in 73.1% (19/26) of the cases. Images of a representative case are shown in Figure 1*C* and *D*.

The increased expression of actinin-4 was most significant in cancer cells that had infiltrated with the fibrous stroma (red; Figure 2*A*, *B*, and *E–G*), where cancer cells lost their polarity and glandular structure and the expression of E-cadherin was diminished (green; Figure 2*C–F*).

We previously defined the status of colorectal cancer cells that were dissociated from the polarized glandular structure with infiltration in a single or solitary trabecular nest as "focal dedifferentiation."[28] The clinical significance of the increased actinin-4 expression associated with focal dedifferentiation is described below (Table 1).

Establishment of DLD1 Tet-off ACTN4 and DLD1 Tet-off Control

A colorectal cancer cell line, DLD1, was engineered to express the N-terminally HA-tagged actinin-4 protein under control of the tetracycline-regulatory promoter system. Parental DLD1 cells expressed a small amount of endogenous actinin-4 protein (data not shown). We first established a stable clone, designated as DLD1 Tet-off, by introducing a tetracycline-responsive transactivator tTA into native DLD1 cells. DLD1 Tet-off was then transfected with pTRE2-ACTN4 or empty pTRE2-pur, and stable clones, designated DLD1 Tet-off ACTN4 and DLD1 Tet-off control, respectively, were isolated.

Although a small amount of leakage expression (detected by anti-HA antibody) was observed even in the presence of Dox (ACTN4, Dox (+); Figure 3*A*), the removal of Dox from the culture medium increased the expression level of the actinin-4 protein by about 5-fold in DLD1 Tet-off ACTN4 (ACTN4, Dox (−); Figure 3*A* and *B*). The removal of Dox had no effect on the expression of endogenous actinin-4 in the DLD1 Tet-off control (Control, Dox (+), and Dox (−); Figure 3*A* and *B*).

Table 1. Correlation Between the Expression of Actinin-4 in Focal Dedifferentiated Cells and Clinicopathologic Variables of Colorectal Cancer

Variables	Actinin-4 expression in focal dedifferentiation		
	Increased (n = 28)	Not increased (n = 13)	P[a]
Age (*y*, mean ± SD)	62.21 ± 5.89	65.08 ± 10.05	.354[b]
Sex			
Male	16	7	
Female	12	6	1.000
Maximum diameter of the tumor (*cm*, mean ± SD)	6.11 ± 2.43	5.25 ± 2.04	.243[b]
Primary tumor[c] (depth of invasion)			
T3	26	13	
T4	2	0	1.000
Regional lymph node metastasis[c]			
N0	7	8	
N1 or N2	21	5[d]	.038[e]
Distant metastasis[e]			
M0	19	8	
M1	9	5	.734
TNM stage[c]			
II	5	8 [*]	*P = .001[f]
III	14	0 [**]	**P = .010[g]
IV	9	5	
Histopathologic grade[e]			
G1	18	5	
G2	8	7 [***]	***P = .179[h]
G3	2	1	
Liver metastasis			
Negative	19	8	
Positive	9	5	.734
Lung metastasis			
Negative	25	12	
Positive	3	1	1.000

[a]Analyzed using the Fisher exact test, except for age and maximum diameter of the tumor.
[b]Analyzed using the Student *t* test.
[c]Classified according to the TNM classification of malignant tumors of the colon and rectum (UICC).[29]
[d]Note that all 5 cases had distant organ (liver) metastasis.
[e]Significant difference between N0 and N1–2.
[f]Significant difference between II and III.
[g]Significant difference between II and III–IV.
[h]No significant difference between G1 and G2–3.

Increased Expression of Actinin-4 Induces Cell Scattering and Podia Formation

DLD1 Tet-off ACTN4 cells dramatically altered their morphology (Figures 3 and 4) after the removal of Dox from the culture medium. When cultured without Dox for 72–96 hours, the cell clusters of DLD1 Tet-off ACTN4 became dispersed (ACTN4, Dox (−); Figure 3*D*), and individual cells extended lengthy filopodia (ACTN4, Dox (−); Figure 3*E*). Scanning electron microscopy studies revealed irregular membrane ruffling and the formation of protrusions at the leading edge of these podia as well as formation of microvilli on the dorsal surface (ACTN4, Dox (−); Figure 4*B*, *D*, and *E*). These morphologic changes were never observed in the DLD1 Tet-off control cells (Control, Dox (+), and Dox (−); Figure 3*F* and *G* and Figure 4*F* and *G*).

Confocal immunofluorescence microscopy (Figure 5) revealed that actinin-4 protein was highly concentrated in the membrane ruffles and protrusions at the tips of the podia (Figure 5*A* and *B*; green, Figure 5*E–H*) and colocalized with filamentous actin (Figure 5*C* and *D*; red, Figure 5*E–H*).

Expression of Actinin-4 Increases Cell Motility

Based on the above morphologic observations, we speculated that the induction of actinin-4 might increase cell motility. Wound-healing assays in our previous study showed that the actinin-4 protein was significantly up-regulated in cells forced to be motile. DLD1 Tet-off ACTN4 cells became highly motile (Figures 5 and 6) merely by the removal of Dox from the culture medium.

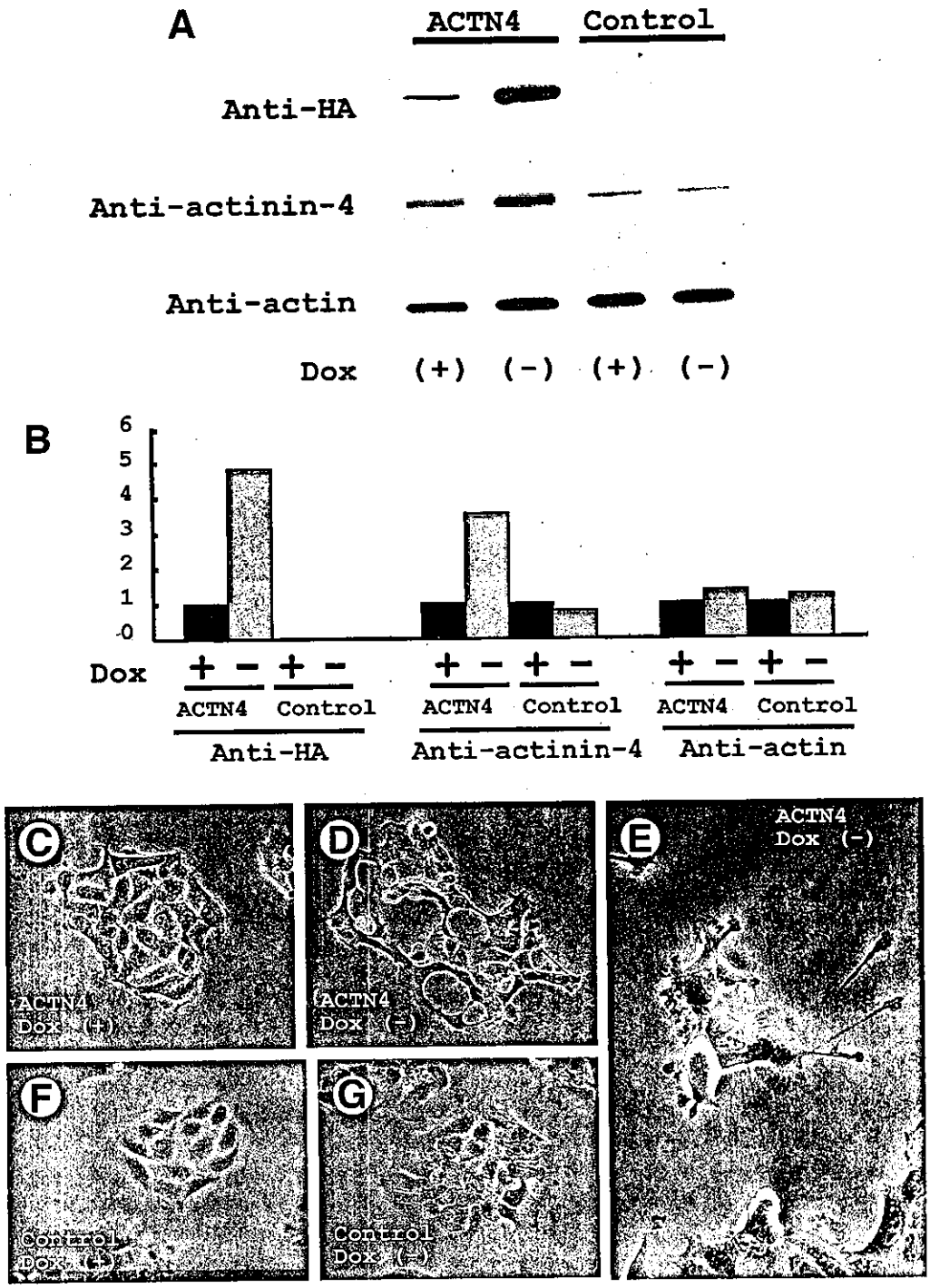

Figure 3. (*A*) Western blot analysis of DLD1 Tet-off ACTN4 (ACTN4) and control (Control) cells cultured in the presence (+) or absence (−) of .1 μg/mL Dox for 72 hours with anti-HA, anti-actinin-4 (Ab-1), and anti-actin antibodies. (*B*) Relative intensity of the blots in *A*. Values of Dox (−) were normalized to those of Dox (+), which were defined as equal to one. (*C–G*) Phase-contrast microscopy images showing (*C–E*) DLD1 Tet-off ACTN4 and (*F* and *G*) control cells cultured in the (*C* and *F*) presence or (*D*, *E*, and *G*) absence of .1 μg/mL Dox for 72 hours.

In the presence of Dox (without the induction of actinin-4; Figure 6*A*), DLD1 Tet-off ACTN4 cells rarely migrated into the wounds; however, a large number of cells migrated into the wound in the absence of Dox (with the induction of actinin-4; Figure 6*B*). These cells were often sharply elongated, with podia extending toward the wound area (Figure 6*B*). DLD1 Tet-off control cells rarely migrated into the wound, either in the presence or the absence of Dox (data not shown).

To quantify cell motility, we measured the areas (circumscribed by the blue lines in Figure 7*A* and *B*) denuded by the movement of single cells (circumscribed by red lines). DLD1 Tet-off ACTN4 cells removed significantly larger areas in the absence of Dox than in the presence of Dox ($*P = .00027$; Student t test) (Figure 7*C*). The areas denuded by DLD1 Tet-off control cells were not altered either in the presence or absence of Dox (Figure 7*C*).

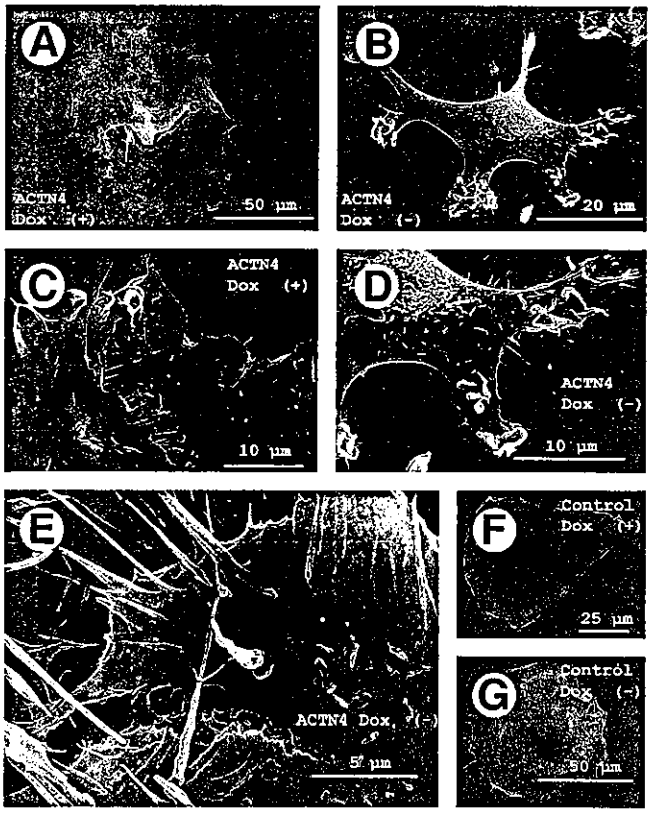

Figure 4. Scanning electron microscopy images showing (*A–E*) DLD1 Tet-off ACTN4 and (*F* and *G*) DLD1 Tet-off control cells cultured in the (*A*, *C*, and *F*) presence or (*B*, *D*, *E*, and *G*) absence of Dox.

Actinin-4 Promotes Lymph Node Metastasis

Enhanced cell movement may promote cancer metastasis. We examined the metastatic activity of DLD1 Tet-off ACTN4 cells in SCID mice. Because the addition of Dox into drinking water was not sufficient to suppress tetracycline-regulated gene expression (data not shown), we compared the metastatic ability of DLD1 Tet-off ACTN4 and control cells without the administration of Dox in vivo (Figures 8–10). Because these clones were derived from the same DLD1 Tet-off clone (see above), the biological differences between DLD1 Tet-off ACTN4 and the control were unlikely to have been caused by clone-to-clone variations.

Five weeks after the injection of DLD1 Tet-off ACTN4 cells into the spleen of severe SCID mice, a significantly higher number of metastases was formed in the lymph nodes surrounding the spleen, stomach, duodenum, and pancreas than in the DLD1 Tet-off control cells ($P = .013$) (Figure 8). In animals injected with DLD1 Tet-off control cells, tumor formation was limited to the vicinity of the splenic hilum. Histologic examinations revealed no liver metastases in either the DLD1 Tet-off ACTN4 or the DLD1 Tet-off control (data not shown).

Histologic examination (H&E staining [Figure 9*A* and *C*] and immunostaining with anti-HA antibody [Figure 9*B* and *D*]) of xenografted DLD1 Tet-off ACTN4 cells revealed infiltrative growth (Figure 9*A* and *B*) and lymphatic permeation (Figure *C* and *D*). The scattering of small tumor clusters in massive fibrous stroma (Figure 9*A* and *B*) resembled the focal dedifferentiation seen in clinical colorectal cancer. In contrast, DLD1 Tet-off control cells formed rather large and solid tumor nests with expansive growth (Figure 9*E* and *F*). Focal dedifferentiation was not observed in the control group.

To evaluate the metastatic ability of cancer cells under conditions closer to the actual clinical situation, we performed so-called orthotopic implantations (Figure 10).

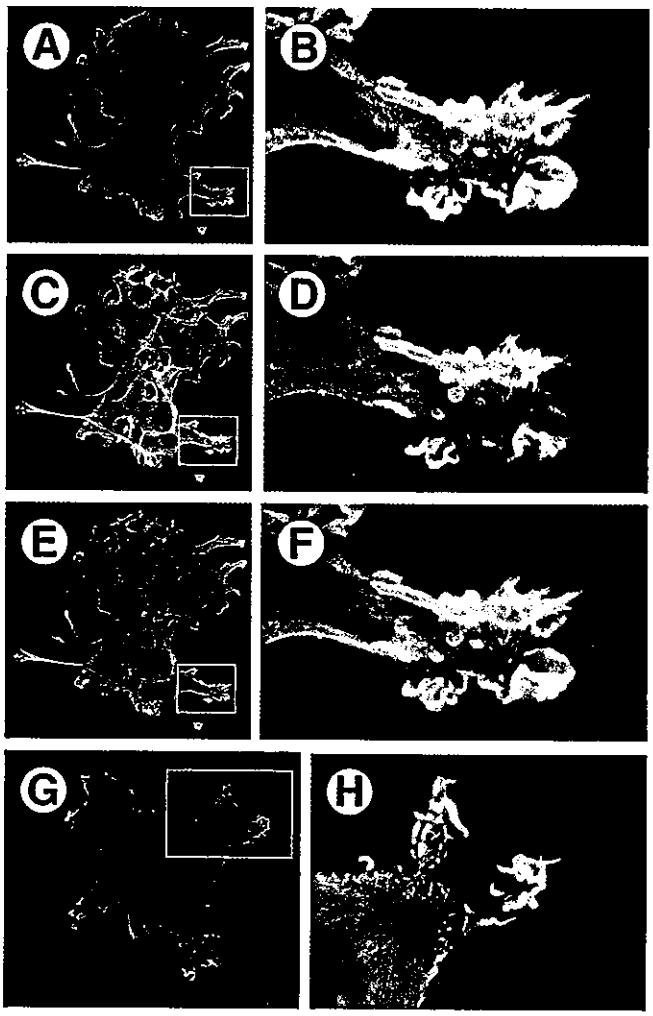

Figure 5. (*A–H*) Confocal immunofluorescence microscopy images showing the localization of exogenously expressed actinin-4 protein and the actin cytoskeleton in DLD1 Tet-off ACTN4 cell clusters cultured without Dox. Actinin-4 protein was detected using anti-HA antibody (*A* and *B*; green, *E–H*), and filamentous actin polymers were detected by phalloidin (*C* and *D*; red, *E–H*).

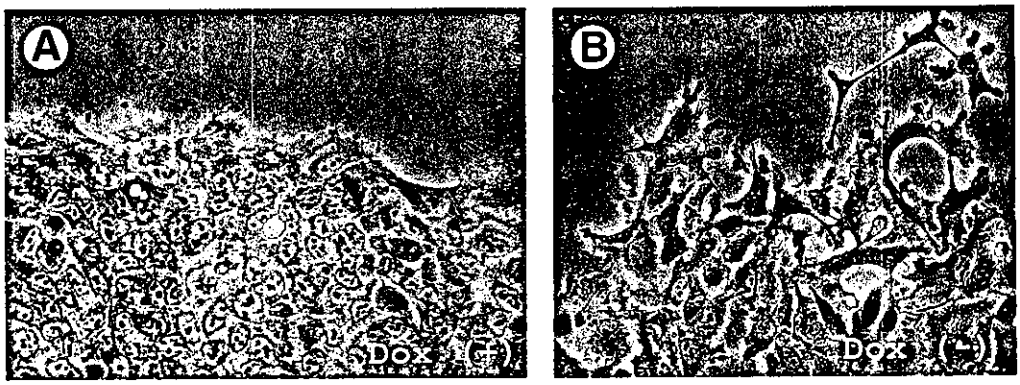

Figure 6. Wound-healing assay. Artificial linear defects were introduced into confluent monolayers of DLD1 Tet-off ACTN4 cells. Cells were cultured with (*A*) .1 μg/mL Dox or (*B*) without Dox for 16 hours.

DLD1 Tet-off ACTN4 (n = 7) and control (n = 5) cells were injected into the mesocecum of SCID mice. DLD1 Tet-off ACTN4 metastasized to the regional mesenteric lymph nodes (7/7) (blue arrows, Figure 10*A* and *B*), resembling the behavior of clinical colorectal cancer. The DLD1 Tet-off control cells formed "primary" tumors of the same size as the DLD1 Tet-off ACTN4 cells in the mesocecum, but lymph node metastasis was not observed (0/5) (data not shown). Histologic examination (Figure 10*C* and *D*) showed infiltrative growth and lymphatic permeation (arrows, Figure 10*D*) of the DLD1 Tet-off ACTN4 cells associated with massive stromal fibrosis in the affected lymph nodes. Again, no liver metastases were found in either the DLD1 Tet-off ACTN4 or the DLD1 Tet-off control (data not shown).

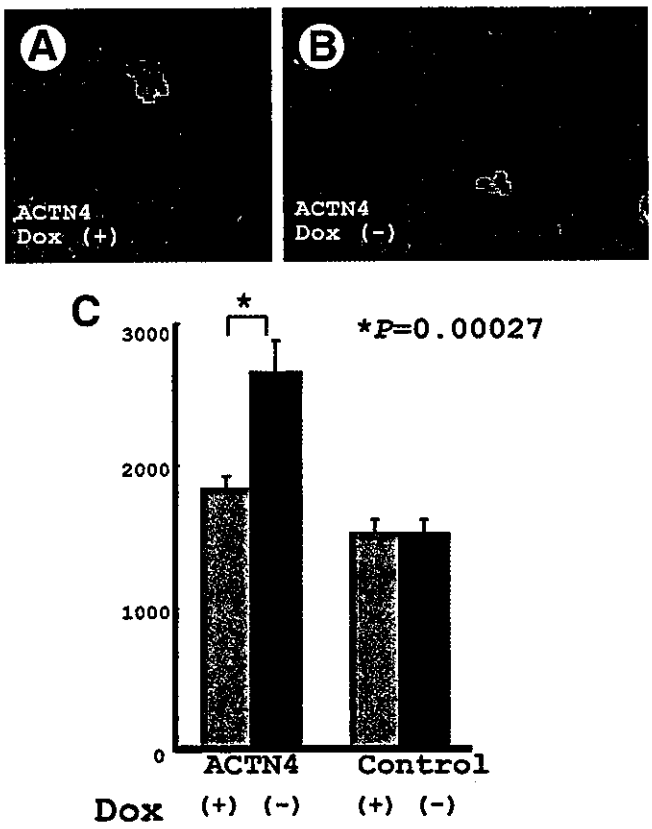

Figure 7. Cell motility quantification. (*A* and *B*) DLD1 Tet-off ACTN4 cells were seeded into 96-well plates that had been precoated with type I collagen and blue fluorescent beads and then incubated (*A*) with or (*B*) without .1 μg/mL Dox for 20 hours. The areas denuded by the movement of DLD1 Tet-off ACTN4 cells, during the period of 20 hours in the presence or absence of Dox, are circumscribed by the *blue lines*. The *red lines* circumscribe single cells of DLD1 Tet-off ACTN4. (*C*) The areas of fluorescent beads denuded by the migration of single cells of DLD1 Tet-off ACTN4 (ACTN4) and control (Control) during the period of 20 hours in the presence (+) or absence (−) of Dox were measured using the ArrayScan II system. The movement of at least 50 cells was measured in each experiment. *Error bars* represent the SEM.

Increased Expression of Actinin-4 in Focally Dedifferentiated Colorectal Cancer Cells Was Correlated With Lymph Node Metastasis

Finally, we immunohistochemically examined a larger series of clinical colorectal cancer specimens (52 cases) (Table 1) to clarify the clinical significance of the expression of actinin-4. Focal dedifferentiation was observed in 41 of the 52 cases (78.8%). Elevated expression of actinin-4 in the focally dedifferentiated cancer cells (Figure 2 and data not shown) was observed in 28 of these 41 cases (68.3%) and was compared with various clinical parameters, including those described in the TNM classification of malignant tumors of the colon and rectum (UICC)[29] (Table 1). Elevated expression of actinin-4 in the focally dedifferentiated cancer cells was significantly correlated with lymph node metastasis (7/15 in N0 vs 21/26 in N1–2; $P = .038$) (N0, no regional lymph node metastasis; N1, metastasis in 1–3 regional lymph nodes; N2, metastasis in 4 or more regional lymph nodes[29]) but not with other clinical features, such as liver metastasis, age, sex, depth of invasion, or overall tumor differentiation (Table 1). The correlation with lymph node metastasis was more significant among the 27 cases without distant organ metastasis (5/13 in stage II [T3–4, N0, M0] vs 14/14 in stage III [any T, N1–2, M0];