表3. 大腸癌術後サーベイランスに関するメタアナリシス

報告者(年)	症例数*	再発率*	再発時期	再発巣治癒切除率*	予後
Rosen ら[9] 米国 (1998)	2,005(963：1,042)	31%：27% (NS)	—	26%：9%	5年生存率* 62%：48% ($p = 0.003$)
Renehan ら[10] 英国 (2002)	1,342(666：676)	32%：33% (NS)	intensive 群が 8.5ヵ月早い ($p < 0.001$)	—	intensive 群で予後がよい risk ratio：081 ($p = 0.007$)
Jeffery ら[11] ニュージーランド (2002)	1,342(666：676)	32%：33% (NS)	—	24%：9%	intensive 群で予後がよい risk ratio：0.73 ($p = 0.007$)
Figueredo ら[12] カナダ (2003)	1,679(858：821)	(NS)	—	—	intensive 群で予後がよい risk ratio：0.80 ($p = 0.0008$)

*intensive 群：対照群

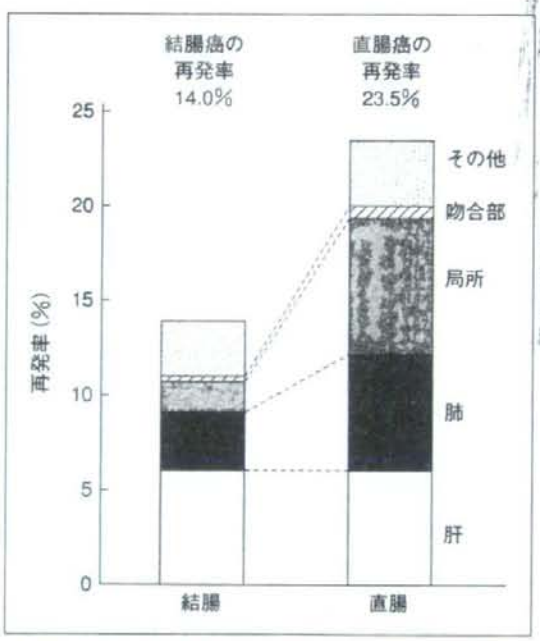

図3. 結腸癌・直腸癌における初回再発部位別再発率の比較(Rsは結腸として集計)

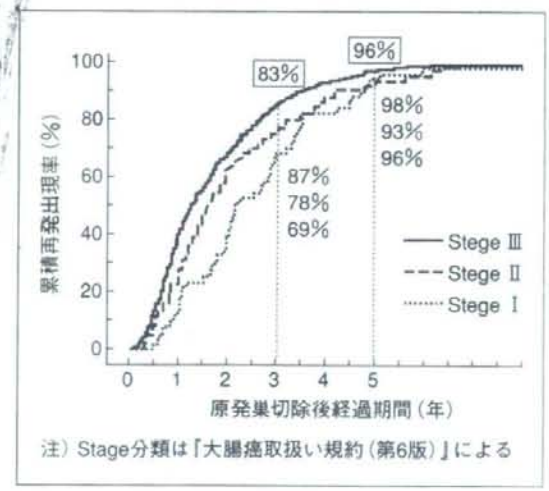

図4. Stage別累積再発出現率

表4. 欧米のガイドラインにおける大腸癌術後サーベイランスの変化

	ASCO		ESMO	
	2000年[13] →	2005年[15]	2001年(結腸癌)[14] →	2007年(結腸癌)[16]
診　察	術後3年間は3～6ヵ月ごと 以降は1年ごと	術後3年間は3～6ヵ月ごと 術後4～5年は6ヵ月ごと	術後2年間は6ヵ月ごと	術後2年間は6ヵ月ごと
腫瘍マーカー(CEA)	術後2年間以上は2～3ヵ月ごと (Stage II・III症例)	術後3年以上は3ヵ月ごと (Stage II・III症例)	再発を疑う症状があるとき	術後3年間は3～6ヵ月ごと 術後4～5年は6～12ヵ月ごと
胸部X線検査	CEA上昇時および再発を疑う症状があるとき	—	再発を疑う症状があるとき	術後5年間は1年ごと
腹部超音波検査	—	—	術後3年間は1年ごと	術後3年間は6ヵ月ごと 術後4～5年は1年ごと
CT	—	高リスク群：術後3年間は年1回の胸部・腹部CT 骨盤CT：放射線未照射の直腸癌術後	再発を疑う症状があるとき	高リスク群：術後3年間の胸部・腹部CTを考慮
大腸内視鏡検査	3～5年ごと	術後3年目 正常ならその後5年ごと すべての大腸癌患者は術前にクリーンコロンであることを確認すべきである	5年ごと	術後1年目 その後3年ごと

ASCO：American Society of Clinical Oncology，ESMO：European Society of Medical Oncology

ンスを推奨するように変化した[15,16](表4).

III. 適切な術後サーベイランスとは

では，再発を切除可能な状態で発見するために，どのような間隔で，どれだけの期間，サーベイランスを行うべきであろうか？

術後サーベイランスを行ううえで，再発の特徴(再発の起こりやすい時期，起こりやすい臓器)を認識しておくことはたいへん重要である．前述のプロジェクト研究では，集積した5,317例のうち906例の再発例を詳細に検討し，その結果が『大腸癌治療ガイドライン―医師用(2005年版)』[1,2]に記された．

初回再発臓器のうち，もっとも多いのは肝再発(373例，7.0%)であり，次いで肺再発(251例，4.7%)，局所再発(206例，3.9%)の順であった．直腸癌では結腸癌に比し，有意に再発率が高率であった(23.5%，14.0%，$p<0.001$)．再発臓器では，結腸癌では初回再発の約半数(186例，6.8%)が肝再発であるのに比し，直腸癌では肝再発(96例，7.3%)，肺再発(89例，6.7%)，局所再発(100例，7.6%)がほぼ同数であった(図3)．これより，直腸癌の術後は，結腸癌に比し肺再発・局所再発にも留意すべきである．

また，再発の80%以上が術後3年以内に，95%以上が術後5年以内に診断されていた(図4)．術後5年を超えて診断された再発は，全5,317例のわずか0.6%(33例)のみであった．これより，術後3年までのサーベイランスはintensiveに行い，また少なくとも術後5年間は再発の可能性を念頭におき，サーベイランスを継続すべきであると考える．

おわりに

再発大腸癌を治癒せしめるには，再発を治癒切除可能な状態で発見することが重要であり，術後サーベイランスは，再発大腸癌の診断・治療において，その結果を左右する重要なポイントであるとともに，本邦の良好な治療成績の一端を担っているといえる．

しかし，至適サーベイランス間隔，検査法，費用対効果比など，いまだ解決すべき問題はある．前述したプロジェクト研究の症例集積期間は1991～1996年であり，その後のヘリカルCTの急速な普及に伴い，現在の術後サーベイランスの主流がCTへと変化しているなど，現在とは実情がやや異なっているのも事実である．常に，より新しいデータを集積・検討し，臨床にフィードバックしていくことが望まれる．

症例の集積・検討に多大なご尽力をいただいた「大腸癌術後再発に関するフォローアップに関する研究プロジェクト」参加各施設の先生方に深謝する．

◆◆◆ 文献 ◆◆◆

1) 大腸癌研究会(編)：大腸癌治療ガイドライン—医師用，2005年版，金原出版，東京，p20，40-45，2005
2) Kobayashi H, Mochizuki H, Sugihara K et al : Characteristics of recurrence and surveillance tools after curative resection for colorectal cancer ; a multicenter study. Surgery **141** : 67-75, 2007
3) Makela J, Laitinen SO, Kairaluoma MI et al : Five-year follow-up after radical surgery for colorectal cancer ; results of a prospective randomized trial. Arch Surg **130** : 1062-1067, 1995
4) Ohlsson B, Breland U, Ekberg H et al : Follow-up after curative surgery for colorectal carcinoma ; randomized comparison with no follow-up. Dis Colon Rectum **38** : 619-626, 1995
5) Kjeldsen B, Kronborg O, Fenger C et al : A prospective randomized study of follow-up after radical surgery for colorectal cancer. Br J Surg **84** : 666-669, 1997
6) Pietra N, Sarli L, Costi R et al : Role of follow-up in management of local recurrences of colorectal cancer. Dis Colon Rectum **41** : 1127-1133, 1998
7) Schoemaker D, Black R, Giles L et al : Yearly colonoscopy, liver CT, and chest radiography do not influence 5-year survival of colorectal cancer patients. Gastroenterology **114** : 7-14, 1998
8) Secco GB, Fardelli R, Gianquinto D et al : Efficacy and cost of risk-adapted follow-up in patients after colorectal cancer surgery ; a prospective randomized and controlled trial. Eur J Surg Oncol **28** : 418-423, 2002
9) Rosen M, Chan L, Beart R et al : Follow-up of colorectal cancer ; a meta-analysis. Dis Colon Rectum **41** : 1116-1126, 1998
10) Renehan AG, Egger M, Saunders M et al : Mechanisms of improved survival from intensive follow up in colorectal cancer ; a hypothesis. Br J Cancer **92** : 430-433, 2005
11) Jeffery GM, Hickey BE, Hider P : Follow-up strategies for patients treated for non-metastatic colorectal cancer. Cochrane Database Syst Rev **1** : CD002200, 2002
12) Figueredo A, Rumble RB, Maroun J et al : Follow-up of patients with curatively resected colorectal cancer ; a practice guideline. BMC Cancer **3** : 26, 2003
13) Besson AB, Desch CE, Flynn PJ et al : 2000 Update of American Society of Clinical Oncology Colorectal Cancer Surveillance Guidelines. J Clin Oncol **18** : 3586-3588, 2000
14) ESMO : ESMO minimum clinical recommendations for diagnosis, adjuvant treatment and follow-up of colon cancer. Ann Oncol **12** : 1053-1054, 2001

15) Desch CE, Besson AB, Somerfield MR et al : Colorectal cancer surveillance ; 2005 update of an American Society of Clinical Oncology practice guideline. J Clin Oncol 23 : 8512-8519, 2005

16) ESMO Guidelines Working Group, Van Cutsem EJ : Colon cancer ; ESMO clinical recommendations for diagnosis, adjuvant treatment and follow-up. Ann Oncol 18 [Suppl 2] : ii21-22, 2007

*　　*　　*

お知らせ

◆真菌症フォーラム第10回学術集会

会　　期：2009年2月21日(土)　11：00～18：40(受付：10：00開始)
　　終了後，情報交換会があります．
会　　場：ヒルトン名古屋5階「扇の間」
会　　長：木内哲也(名古屋大学移植外科)
参 加 費：3,000円(抄録集・情報交換会費含む．事前登録はありません)
共　　催：真菌症フォーラム/ファイザー(株)
テ ー マ：「Compromised hostに学ぶ」
プログラム
　招待講演「Compromised hostにおける深在性真菌症―一般臨床への教訓」
　ランチョンセミナー「ハイ・リスクグループにおける深在性真菌症とその対策」
　シンポジウム「Compromised hostにおける深在性真菌症―予防から標的治療まで」
　①Keynote lecture―感染免疫からみた深在性真菌症の病態と対策，②臓器移植領域，③血液領域，④HIV領域，⑤総合討論
演題募集：深在性真菌症全般について，ふるってご応募ください．皮膚科領域の真菌症は対象外とします．
　要望演題(口演発表)：免疫不全下の深在性真菌症の病態・予防・診断・治療に関する演題を募集します．
　一般演題(ポスター発表)：基礎/検査領域，内科系領域，外科系/救急領域
登録期間：2008年8月20日(水)正午～9月25日(木)正午(厳守)
問い合わせ先：〒105-0004 東京都港区新橋2-20 新橋駅前ビル1号館5階
　(株)協和企画コンベンション事業局内
　真菌症フォーラム第10回学術集会運営事務局
　TEL：03-3573-2060/FAX：03-3573-2064
　E-mail：mycoses_10@kk-kyowa.co.jp

大腸癌化学療法における緊急対応と手術
――有害事象と外科的処置
Urgent treatment in the chemotherapy for colorectal cancer

植竹宏之(写真)　杉原健一
Hiroyuki UETAKE[1] and Kenichi SUGIHARA[2]
東京医科歯科大学大学院医歯学総合研究科応用腫瘍学講座[1]，同腫瘍外科学分野[2]

◎抗癌剤治療中は腫瘍出血，腸管穿孔など，外科的処置が必要な有害事象が起こりうる．とくにBevacizumabを用いると，創傷治癒遅延も考慮しなければならない．周術期は化学療法施行中の患者の全身状態を十分に把握し，使用された薬剤の有害事象をよく理解して治療にあたる必要がある

Key word：腫瘍出血，消化管閉塞，消化管穿孔，血栓・塞栓症，Bevacizumab

近年，新規薬剤や多剤併用療法の開発，支持療法の進歩によって，癌化学療法は長足の発展を遂げ，奏効率の上昇と生存期間の改善が得られている．一方，癌化学療法における有害事象の発現率は，軽微なものまで含めると100%に近い．重篤な副作用の兆候を見逃したり対処法を誤ったりすれば患者のQOLを著しく悪化させるばかりか，ときに患者の生命を左右することになりかねない．現在の強力な癌化学療法における有害事象対策においては，外科医の知識や手技が必要となる場合が増加している．さらに，緊急処置や手術を要する有害事象が近年とくに注目される理由は，大腸癌に対するBevacizumab〔アバスチン®，血管内皮細胞増殖因子(vascular endothelial growth factor：VEGF)に対する遺伝子組換え抗ヒトモノクローナル抗体〕療法の有効性が示され，わが国においても2007年6月保険収載されたことが大きい．Bevacizumabは他の抗体治療薬と同様，それまでの経験からは予測が困難な副作用を惹起することが臨床試験において報告された．Bevacizumabの有害事象のなかには，腫瘍出血，消化管穿孔といった外科的処置が必要となるものが含まれている(表1)．

本稿においては癌化学療法時の腫瘍出血，消化管閉塞，消化管穿孔，血栓・塞栓症，創傷治癒遅延について概説する．

腫瘍関連出血

切除不能大腸癌に対するBevacizumab療法において，重篤な腫瘍関連出血の頻度がみられるのは約2%である[1,2]．原発性肺癌，とくに非小細胞癌では腫瘍関連出血の頻度が高く，“生命をおびやかす肺出血”も9%の症例に起こったので，肺転移を有する大腸癌患者に対してBevacizumab療法を行う場合には十分な注意を要する．脳転移を有する患者に対しては，原則Bevacizumabの投与は禁忌となっている．消化管出血は吐血や下血をきたすことが多いので，診断は困難ではない．しかし，出血部位の同定や治療はかならずしも容易ではない．治療は内視鏡検査や血管造影で出血部位(原発巣，消化管への転移巣)の同定を試み，まず内視鏡下あるいはinterventional radiologyの手法を用いた止血を行う．止血不能と判断したときには手術(出血部位の切除)を行う．腫瘍出血のリスクは，投与開始5カ月以降，投与回数は10回以上，累積投与量5,000 mgと報告されている．術中，術後は創傷治癒遅延に留意した術式を選択し術後管理を行う．

表 1　Bevacizumab療法の特徴的有害事象と発生頻度

		発生頻度(%)		
		すべての grade	grade 3, 4	BRiTE study*
1	高血圧症	6～32	0～25	12
2	蛋白尿	19～38	0.8～1	—
3	動脈血栓症	8.6～13	1.2～9	1.5
4	創傷治癒遅延	2～5	—	1.4
5	腫瘍関連出血	29～69	0～15.6	2.2
6	消化管穿孔	＊＊	1.0～4.2	1.7

＊：BRiTE study における“serious adverse event”の頻度[1]．
＊＊：腸管穿孔は grade 3 以上．

消化管閉塞(イレウス)

消化器癌化学療法中の患者の多くは腹部手術の既往があり，腸管癒着がある．画像診断でとらえられない腹膜再発をきたしている場合もある．また，薬物有害反応として便秘を起こしやすい薬剤には植物アルカロイド，タキサン系薬剤，イリノテカンなどがある．このように，腸管の正常な蠕動および内容物の通過を阻害する因子のある患者に対して化学療法が行われ，さらに有害事象として腸管蠕動の抑制や粘膜障害による腸管内容の貯留がイレウスの誘引となりうる．診断には腹部単純 X 線検査のほか，造影 CT を行い，閉塞部位または責任病変の同定に努める．腹膜刺激症状がなく絞扼性イレウスではないと判断されれば，イレウス管を挿入して腸管の減圧・浮腫の軽減をめざすとともに閉塞部位の診断を確実にする．イレウス管を挿入しても腸管の通過障害が改善しない場合は，患者の全身状態や骨髄機能を考慮して適切な手術時期を決定し，イレウス解除術を行う必要がある．

サイドメモ　イレウス管挿入

腹膜刺激症状がなく絞扼性イレウスではないと判断されたイレウス患者に対して，わが国ではイレウス管を挿入して腸管の減圧・浮腫の軽減をめざすとともに，閉塞部位の診断を確実にする処置・治療が一般的に行われている．海外では“経鼻胃管挿入により 24 時間以内に改善しない腸閉塞症は手術適応”とすることが一般的で，イレウス管挿入は推奨されていない．

表 2　Bevacizumab療法における消化管穿孔のリスク

- 急性憩室炎
- 腹腔内膿瘍
- 腸管閉塞
- 腫瘍の存在(腫瘍の消化管穿孔)
- 癌性腹膜炎
- 腹部または骨盤部の放射線照射の既往

消化管穿孔

抗癌剤治療中に起こる腸管穿孔の機序は，①高度の粘膜障害，腸管壁の萎縮による穿孔，②抗腫瘍効果が発揮されたことによる腫瘍の穿孔，である[3]．腸管悪性リンパ腫に対して化学療法を施行中に腸管穿孔を合併した，としばしば報告される[4]．腸管穿孔は緊急手術の適応であり，穿孔部腸管の切除あるいは空置が必要である．術式は，穿孔部口側をストマとする，穿孔部を体外に誘導しループ状にストマとする，穿孔部腸管を切除吻合して口側に一時的なストマをおく，などがある．

消化管穿孔は Bevacizumab 療法のもっとも重大な有害事象であり，手術など適切な治療が施されなければ致死的である．発生頻度は約 2%である[1,5]．発生時期は Bevacizumab 投与後 1 週間から 1 年以上後まで多岐にわたるが，約 70%は 60 日以内に発生する．Kozloff らが報告した消化管穿孔のリスクを表 2 に示した[5]．消化管穿孔をきたした症例のうち，これらのリスクファクターのいずれかを満たす症例は 54.5%であるが，いずれも満たさない症例が 31.8%である．加えて，異時性再発症例や二次治療症例の腹膜播種の有無は診断が容易ではない．したがって，リスクファクターを有さない症例に対しても，消化管穿孔に対して

十分注意が必要である．Hurwitz らによれば，IFL（イリノテカン＋5-FU/LV）または 5-FU/LV＋Bevacizumab 群における消化管穿孔（9 例）の発生時期は 8～383 日（中央値 127 日），穿孔部位は胃 1 例，小腸 2 例，大腸 4 例（不明 2 例）であった[1,6]．消化管穿孔は緊急手術の適応であり，腫瘍の穿孔では穿孔部の切除が基本である．創傷治癒遅延の可能性を考慮すると，腸管吻合する場合は口側に一時的なストマをおく方法が（とくに結腸では）安全と思われる．

血栓，塞栓症

動脈血栓症の頻度は抗癌剤に Bevacizumab を付加することにより上昇し，**表 1** に示すように Grade 3 以上は 1.2～9％に合併する（抗癌剤のみの場合は 0.8～1.7）．動脈血栓症の内訳は，心筋梗塞，脳梗塞，脳出血などで，リスクファクターは年齢（65 歳以上）および動脈血栓症の既往，である．治療については，ワーファリンによる“full-dose anticoagulation”を行う（それに伴う出血のリスクは上昇しなかったと報告されている[1]）が，心筋梗塞や急性動脈閉塞では緊急手術となる場合がある．

欧米の報告では，静脈血栓の頻度は抗癌剤に Bevacizumab を付加しても増加しないとされてきたが，①静脈血栓症の頻度がもともと低い，②直腸癌手術で側方郭清がなされていると，腸骨静脈領域の血流はスムーズではないと想像される，③術前放射線療法は欧米に比べわが国では普及していない，などの欧米と異なる要素がある．Bevacizumab 療法による静脈血栓症の合併頻度は，わが国では今後の検討が必要と思われる．

創傷治癒遅延

緊急手術の際，Bevacizumab 療法における創傷治癒遅延を考慮しなければならない．Hurwitz らの報告によると，創傷治癒遅延の発生頻度は IFL＋プラセボ群で 0.5％（396 例中 1 例）であったのに対し，Bevacizumab 併用群では 1％（392 例中 4 例）であった[6]．Bevacizumab 投与中に手術を行うと，10％の頻度で創傷治癒遅延または出血をきたす（プラセボ群 0％）．Bevacizumab の半減期は約 20 日であり[7-9]，Bevacizumab 投与終了後 28～60 日経過すれば創傷治癒遅延は 2％となる．

おわりに

以上，化学療法時の腫瘍関連出血，消化管閉塞，消化管穿孔，血栓・塞栓症と，それらに対する緊急対応について概説した．わが国の消化器癌化学療法の多くは外科医により行われているのが現状であるが，今後は腫瘍内科医が行う比率が増すと思われる．Bevacizumab 療法がわが国へ導入されたことや，患者の生存期間が延長したことによって，外科医が癌化学療法にかかわるあらたな局面が出現した．その代表的なものが化学療法施行中の緊急の外科的処置・手術である．化学療法中の患者の全身状態を十分把握し，使用された薬剤の有害事象をよく理解して緊急処置，手術，術後管理にあたる必要がある．

文献

1) Hurwitz, H. et al.：Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.*, **350**：2335-2342, 2004.
2) Kabbinavar, F. F. et al.：Combined analysis of efficacy：The addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. *J. Clin. Oncol.*, **23**：3706-3712, 2005.
3) 大矢雅敏・他：消化器症状（2）外科の立場から．コンセンサス癌治療，**5**：182-183，2006.
4) 神谷和則・他：塩酸イリノテカンによる化学療法中に結腸穿孔をきたした 1 例．日本消化器外科学会雑誌，**38**：249-255，2005.
5) Kozloff, M. et al.：Efficacy of bevacizumab plus chemotherapy as first-line treatment of patients with metastatic colorectal cancer：Updated results from a large observational registry in the US（BRiTE）. *J. Clin. Oncol.*, **24**（18S）：3537, 2006.
6) Hurwitz, H. et al.：Bevacizumab in combination with fluorouracil and leucovorin：An active regimen for first-line metastatic colorectal cancer. *J. Clin. Oncol.*, **23**：3502-3508, 2005.
7) Kerr, D. J.：Targeting angiogenesis in cancer：Clinical development of bevacizumab. *Nat. Clin. Prac. Oncol.*, **1**（1）：39-43, 2004.
8) Margolin, K. et al.：Phase I b trial of intravenous recombinant humanized monoclonal antibody to vascular endothelial growth factor in combination with chemotherapy in patients with advanced cancer：Pharmacologic and long-term Ssfety data. *J. Clin. Oncol.*, **19**：851-856, 2001.
9) Gordon, M. S. et al.：Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J. Clin. Oncol.*, **19**：843-850, 2001.

Reduced expression of the *claudin-7* gene correlates with venous invasion and liver metastasis in colorectal cancer

TAKASHI OSHIMA[1], CHIKARA KUNISAKI[1], KAZUE YOSHIHARA[1], ROPPEI YAMADA[1], NAOTO YAMAMOTO[1], TSUTOMU SATO[1], HIROCHIKA MAKINO[1], SHIGERU YAMAGISHI[1], YASUHIKO NAGANO[1], SHOICH FUJII[1], MANABU SHIOZAWA[2], MAKOTO AKAIKE[2], NOBUYUKI WADA[3], YASUSHI RINO[3], MUNETAKA MASUDA[3], KATSUAKI TANAKA[1] and TOSHIO IMADA[4]

[1]Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama-shi, Kanagawa-ken 232-0024; [2]Department of Surgery, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama-shi, Kanagawa-ken 241-0815; [3]Department of first Surgery, Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama-shi; [4]Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa-ken 236-0004, Japan

Received October 15, 2007; Accepted December 5, 2007

Abstract. Claudins, members of a large family of adherent junction proteins, regulate the integrity and function of tight junctions and influence tumorigenesis. Studies have suggested that altered levels of different claudins are related to carcinoma-cell invasion and disease progression. This study examined the relationship between the relative expression of claudin genes and clinicopathological factors, especially invasion and metastasis, in patients with colorectal cancer. We studied surgical specimens of cancer tissue and adjacent normal mucosa from 205 patients with untreated colorectal carcinoma. The relative expression levels of *claudin-1*, *-3*, *-4* and *-7* mRNA in cancer and in normal adjacent mucosa were measured by quantitative real-time, reverse-transcription polymerase chain reaction. The relative expression levels of the *claudin-1*, *-3* and *-4* genes were higher in cancer than in normal adjacent mucosa, whereas the relative expression of the *claudin-7* gene was similar. An analysis of the relationship between the clinicopathological features and gene expression showed that reduced expression of *claudin-7* correlated with venous invasion and liver metastasis. There was also a correlation between *claudin-3* and *-4* gene expression. Our results suggested that a reduced expression of the *claudin-7* gene might lead to venous invasion and liver metastasis in colorectal cancer. Reduced expression of the *claudin-7* gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer.

Correspondence to: Dr Takashi Oshima, Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama-shi, Kanagawa-ken 232-0024, Japan
E-mail: ohshimatakashi@yahoo.co.jp

Key words: claudin-1, claudin-3, claudin-4, claudin-7, colorectal cancer

Introduction

In simple epithelium, tight junctions are positioned at the boundaries of apical and basolateral plasma membranes. These junctions are thought to play an important role in the paracellular barrier and cell polarity (1-4). Several lines of evidence indicate that the granular cell layer of stratified epithelium of the skin possesses tight junctions that are crucial for barrier function (1,2,5,6). The tight junctions consist of membrane and peripheral proteins. Claudins are membrane proteins composed of four transmembrane domains and two extracellular loops, through which they bind to corresponding claudins in cell-to-cell contact. Claudin-1, -3, -4 and -7 are four representative members of the 24-claudin multigene family (4), associated with cancer. An enhanced expression of claudin-1 has been reported in colorectal cancer (7). Ovarian epithelial cells that express claudin-3 and -4 show increased invasiveness *in vitro* (8). Claudin-4 is a potent inhibitor of the invasiveness and phenotype of pancreatic cancer cells (9). The loss of claudin-7 expression has been observed in ductal carcinoma of the breast and squamous cell carcinoma of the head and neck (10,11). Usami *et al* (12) reported that a reduced expression of claudin-7 correlates with tumor invasion and metastasis in squamous cell carcinoma of the esophagus. However, whether the expression of claudin-1, -3, -4 and -7 is associated with the malignant potential of colorectal cancer remains to be clarified.

In this study, we measured the expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes in 205 pairs of cancer tissue and adjacent normal mucosa obtained from patients with colorectal cancer. To evaluate the clinical significance of the claudins, we examined the correlation between the relative expression of these genes and the clinicopathological features.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients

Table I. PCR primers and conditions.

Gene	Primer	Temperature (C)	Product size (bp)
Claudin-1	5'-CCAGTTAGAAGAGGTAGTGTG-3' 5'-GAGAGGAAGGCAGTGAATC-3'	60	168
Claudin-3	5'-ACCACCACCACCACCAAC-3' 5'-GGGCTTCCTGGCTTCTGG-3'	65	113
Claudin-4	5'-TGCCTTGCTCACCGAAACCC-3' 5'-CCTCTAAACCCGTCCATCCACTC-3'	64.5	95
Claudin-7	5'-GGAGACGACAAAGTGAAGAAG-3' 5'-GCCATACCAGGAGCAAGC-3'	60	99
β-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60	171

with untreated colorectal carcinoma. The patients underwent surgery at the Yokohama City Medical Center, Gastroenterological Center and at the Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the Ethics Committees of the Yokohama City Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study. Each tissue sample was embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and immediately stored at -80˚C until use. No patient had any other malignancies. The histopathological features of specimens stained with hematoxylin and eosin were examined and sections that consisted of >80% carcinoma cells were used to prepare total RNA.

Quantitative real-time, reverse-transcription polymerase chain reaction (PCR). Total RNA isolated from colorectal cancer and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD). Complemetary DNA (cDNA) was synthesized from 2 μg of total RNA with an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with an iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl containing cDNA derived from 75 ng of RNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP and dTTP at a concentration of 400 μM each and 50 units/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C, followed by 50 cycles of denaturation of the cDNA for 30 sec at 94˚C, annealing for 30 sec at an appropriate temperature (Table I) and a primer extension for 1 min at 72˚C followed by 72˚C for 10 min. The PCR primer sequences of MMP2, MMP9, MT-MMP, RECK and ß-actin, used as an internal control, are shown in Table I.

Statistical analysis. Gene expression levels of colorectal cancer were compared with those of normal adjacent mucosa with the use of the Wilcoxon test. The relationship between gene expression and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion and liver metastasis, were evaluated with the χ^2 test. Associations between variables were assessed using the Mann-Whitney U test. Correlation coefficients between the different variables were calculated by simple regression analysis. Each statistical analysis was performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and a difference was considered significant at P-value <0.05.

Results

Comparison of claudin-1, -3, -4 and -7 mRNA expression between colorectal cancer tissue and adjacent normal mucosa. Claudin-1, -3 and *-4* gene expression levels were higher in cancer than in normal adjacent mucosa (P<0.001, P=0.001 and P<0.001) (Fig. 1A, B and C). The *claudin-7* gene expression level of cancer did not differ significantly from that of normal adjacent mucosa (P=0.524) (Fig. 1D).

Relationship of claudin-1, -3, -4 and -7 gene expression levels to clinicopathological features. Expression levels of the *claudin-1, -3, -4* and *-7* genes were categorized as low or high according to their median values. The relationship between the expression of these genes and clinicopathological features was then examined. The expression levels of the *claudin-1, -3, -4* and *-7* genes were unrelated to age, gender, tumor size, lymph node metastasis and lymphatic invasion. There were correlations between *claudin-1* expression and histological type (P=0.047) and between *claudin-4* expression and tumor location (P=0.039). Moreover, a reduced expression of the *claudin-7* gene correlated with venous invasion (P=0.029) and liver metastasis (P=0.022) (Table II).

Associations of claudin-1, -3, -4 and -7 gene expression with lymph node metastasis in patients with colorectal cancer. There was no significant association between the expression level of any gene and the presence or absence of lymph node metastasis (Fig. 2).

Associations of claudin-1, -3, -4 and -7 gene expression with venous invasion in patients with colorectal cancer. Claudin-3 and *claudin-7* gene expression levels were higher in the absence than in the presence of venous invasion (P=0.043, P=0.001) (Fig. 3).

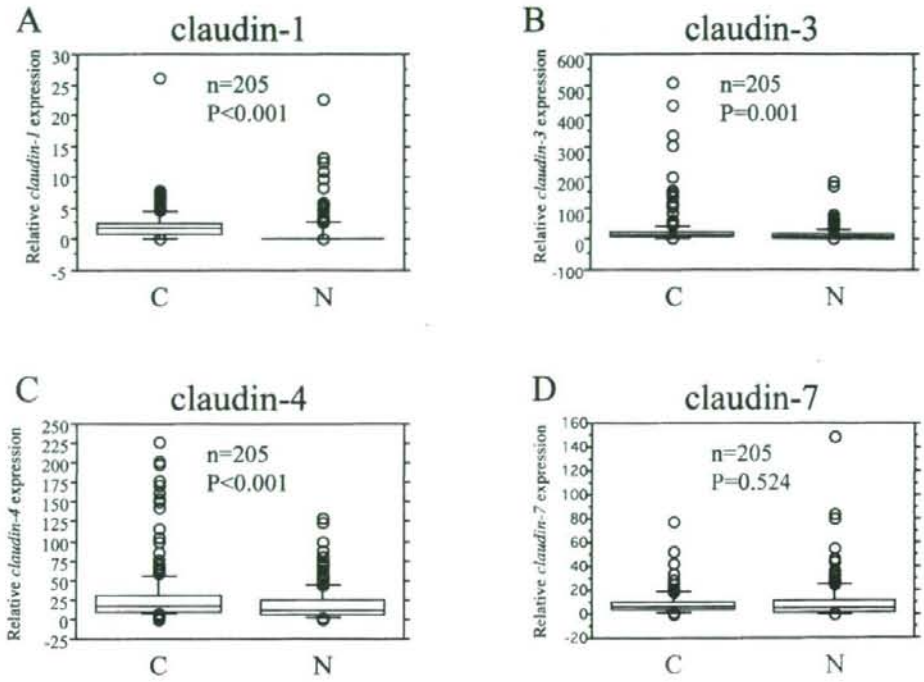

Figure 1. A comparison of *claudin-1*, *-3*, *-4* and *-7* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. The *claudin-1*, *-3* and *-4* gene expression levels were higher in cancer than in normal adjacent mucosa (P<0.001, P=0.001, P<0.001). *Claudin-7* gene expression levels did not differ significantly between cancer and normal adjacent mucosa.

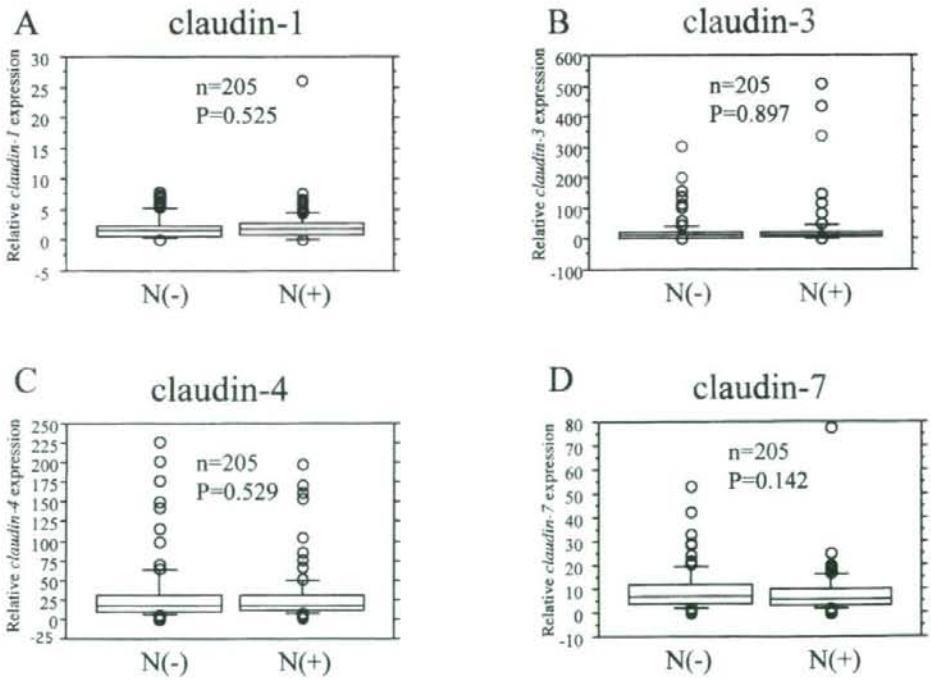

Figure 2. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. The expression level of none of the genes examined correlated with the presence or absence of lymph node metastasis.

Table II. Relationship between the expression of claudin-1, -3, -4 or -7 genes and clinicopathological features.

Variables/categories	claudin-1 expression		P-value	claudin-3 expression		P-value	claudin-4 expression		P-value	claudin-7 expression		P-value
	low (n=102)	high (n=103)		low (n=102)	high (n=103)		low (n=102)	high (n=103)		low (n=102)	high (n=103)	
Age	65.6±11.3	66.0±10.3	0.775	65.6±11.1	66.0±10.5	0.805	65.7±11.2	65.8±10.4	0.917	65.1±11.0	66.5±10.6	0.344
Gender												
Male	58	54	0.524	51	61	0.160	50	62	0.108	50	62	0.108
Female	44	49		51	42		52	41		52	41	
Size												
≤5 cm	58	57	0.826	57	58	0.951	56	59	0.731	54	61	0.365
>5 cm	44	46		45	45		46	44		48	42	
Histological type												
Well differentiated	28	33	0.047	26	35	0.362	29	32	0.809	28	33	0.762
Moderately differentiated	54	62		60	56		60	56		60	56	
Poorly differentiated	20	8		16	12		13	15		14	14	
Depth of invasion												
T1	10	9	0.846	9	10	0.294	7	12	0.320	10	9	0.085
T2	44	50		41	53		52	42		38	56	
T3	41	39		44	36		36	44		46	34	
T4	7	5		8	4		7	5		8	4	
Lymph node metastasis												
Absent	50	45	0.930	46	49	0.722	51	44	0.296	42	53	0.140
Present	52	58		56	54		51	59		60	50	
Location												
Colon	61	51	0.139	62	50	0.784	66	46	0.039	56	56	0.940
Rectum	41	52		40	53		36	57		46	47	
Lymphatic invasion												
Absent	66	68	0.843	67	67	0.924	75	59	0.145	70	64	0.829
Present	36	35		35	36		27	44		32	39	
Venous invasion												
Absent	40	37	0.237	40	37	0.626	35	42	0.340	28	49	0.029
Present	62	66		62	66		67	61		74	54	
Liver metastasis												
Absent	70	69	0.802	69	70	0.802	72	67	0.396	59	80	0.022
Present	32	34		34	32		30	36		43	23	

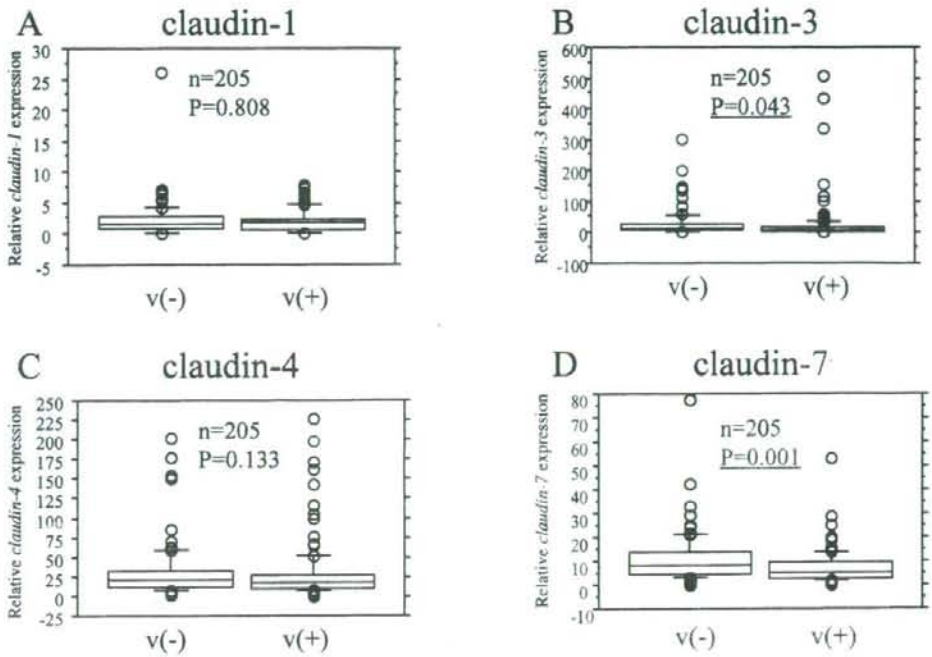

Figure 3. Associations of *claudin-1*, *-3*, *-4* and *-7* gene expression levels with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. P-values were calculated by the Mann-Whitney U test. *Claudin-3* and *-7* gene expression levels were higher in the absence than in the presence of venous invasion (P=0.043, P=0.001).

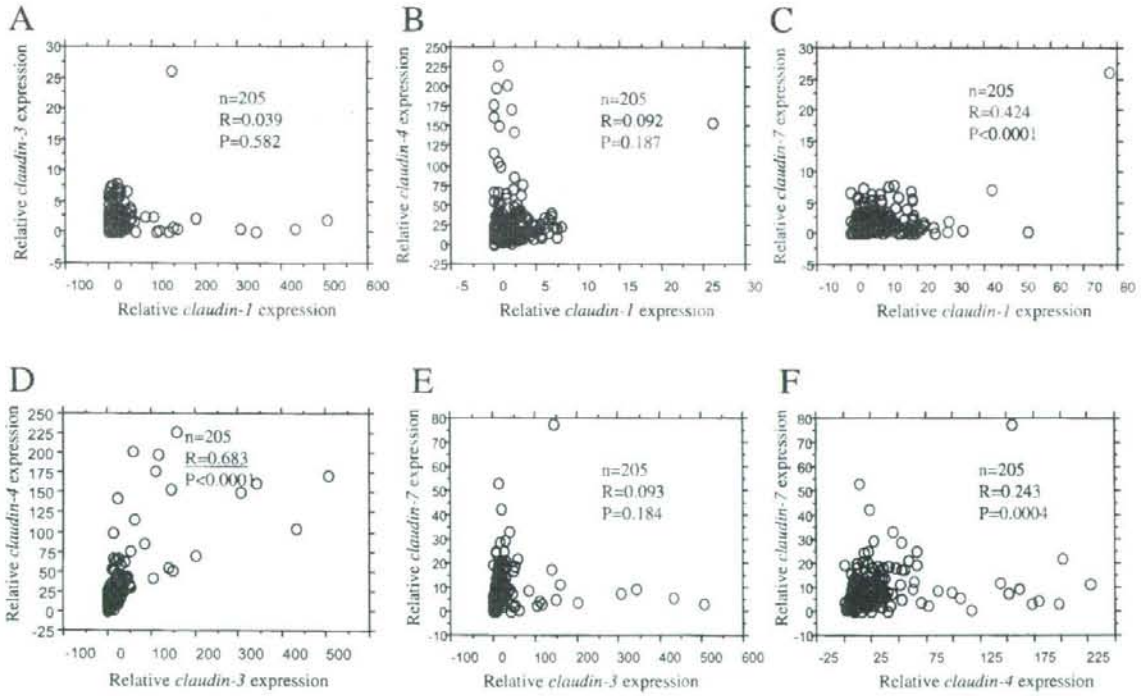

Figure 4. Correlation among *claudin-1*, *-3*, *-4* and *-7* gene expression levels in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. The expression of the *claudin-3* gene correlated with that of the *claudin-4* gene (R=0.683).

Correlations among claudin-1, -3, -4 and -7 gene expression. Correlations between gene expression levels are shown in Fig. 4. The expression of the *claudin-3* gene correlated with that of the *claudin-4* gene (R=0.683).

Discussion

Cell-to-cell adhesiveness is generally reduced in various human cancers. The dissociation of cancer cells from primary cancer nests is a crucial step in metastasis. The suppression of cell-to-cell adhesiveness may trigger the release of cancer cells from primary cancer nests and increase tumor invasiveness (13). In this study, we examined the expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes in colorectal cancer and the relationship of such levels to clinicopathological variables.

We compared the mRNA expression of each *claudin* gene between colorectal cancer tissue and adjacent normal mucosa. Dhawan *et al* (14) reported that the expression of claudin 1 is higher in human primary colon carcinoma and metastasis than in normal colorectal tissue. Pan *et al* (15) found that the expression of claudin-3 and -4 is significantly higher in human endometrial carcinoma than in normal endometrial tissue at the protein and mRNA levels. As for claudin-7, Kominsky *et al* (10) reported that this gene is down-regulated in breast cancers as compared with normal breast tissue. However, Sobel *et al* (16) found no significant difference in the expression of claudin-7 between human invasive cervical carcinoma and normal cervical tissue. In our study, expression levels of the *claudin-1*, *-3* and *-4* genes were higher in cancer than in normal adjacent mucosa, whereas the expression level of the *claudin-7* gene cancer did not differ significantly between cancer and normal adjacent mucosa.

We then examined the relationship between claudin gene expression levels and clinicopathological features. Sheehan *et al* (17) reported that a decreased expression of claudin-1 correlates with high tumor grade and biochemical disease recurrence in prostate carcinomas. Resnick *et al* (18) showed that a low expression level of claudin-1 is associated with a higher tumor grade and recurrence in patients with colorectal cancer. In our study, claudin-1 expression was associated with the histological type. As for claudin-3 and -4, Sheehan *et al* (17) reported that the expression of claudin-3 correlates with advanced-stage tumors and recurrence, whereas the expression of claudin-4 correlates with only advanced-stage tumors. Pan *et al* (15) found a slight though insignificant trend towards positive associations of claudin-3 and -4 levels with tumor grade and disease stage in patients with endometrial carcinoma. Our study found no significant relationship between the expression level of the *claudin-3* gene and any clinicopathological feature. The expression of the *claudin-4* gene correlated with only tumor location. As for claudin-7, Kominsky *et al* (10) reported that the loss of claudin-7 expression is associated with nodal metastasis in primary breast carcinomas. Sauer *et al* (19) found that a reduced expression of claudin-7 correlates with metastatic disease in breast carcinoma. Usami *et al* (12) demonstrated that a reduced expression of claudin-7 correlates with metastasis in squamous cell carcinoma of the esophagus. In our study, a reduced expression of the *claudin-7* gene correlated with venous invasion and liver metastasis in colorectal cancer.

When expression levels of the *claudin-1*, *-3*, *-4* and *-7* genes were contrasted with the presence or absence of lymph node metastasis, no correlation was noted for any gene. We also examined potential correlations of gene expression levels with the presence or absence of venous invasion. Sauer *et al* (19) reported that a reduced expression of claudin-7 correlates with metastatic disease. Usami *et al* (12) found that a reduced expression of claudin-7 correlates with tumor invasion in squamous cell carcinoma of the esophagus. In our study, *claudin-3* and *-7* gene expression levels were higher in the absence than in the presence of venous invasion. This finding suggested that reduced *claudin-3* or *-7* gene expression levels might contribute to venous invasion in colorectal cancer.

We then examined correlations among *claudin-1*, *-3*, *-4* and *-7* gene expression in colorectal cancers. Expression of the *claudin-3* gene was found to correlate with that of the *claudin-4* gene.

In conclusion, our results show that a reduced expression of the *claudin-7* gene correlates with venous invasion and liver metastasis in colorectal cancer. Reduced levels or the absence of claudin-7 expression may thus be a novel marker or predictor of metastasis.

References

1. Tsukita S and Furuse M: Claudin-based barrier in simple and stratified cellular sheets. Curr Opin Cell Biol 14: 531-536, 2002.
2. Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A and Tsukita S: Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156: 1099-111, 2002.
3. Eaton S and Simons K: Apical, basal, and lateral cues for epithelial polarization. Cell 82: 5-8, 1995.
4. Tsukita S, Furuse M and Itoh M: Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2: 285-293, 2001.
5. Morita K, Tsukita S and Miyachi Y: Tight junction-associated proteins (occludin, ZO-1, claudin-1, claudin-4) in squamous cell carcinoma and Bowen's disease. Br J Dermatol 151: 328-334, 2004.
6. Morita K, Furuse M, Fujimoto K and Tsukita S: Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96: 511-516, 1999.
7. Miwa N, Furuse M, Tsukita S, Niikawa N, Nakamura Y and Furukawa Y: Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. Oncol Res 12: 469-476, 2001.
8. Agarwal R, D'Souza T and Morin PJ: Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. Cancer Res 65: 7378-7385, 2005.
9. Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Löhr M, Leder G, Iwamura T, Adler G and Gress TM: Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 63: 6265-6271, 2003.
10. Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallioniemi OP and Sukumar S: Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. Oncogene 22: 2021-2033, 2003.
11. Al Moustafa AE, Alaoui-Jamali MA, Batist G, Hernandez-Perez M, Serruya C, Alpert L, Black MJ, Sladek R and Foulkes WD: Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. Oncogene 21: 2634-2640, 2002.
12. Usami Y, Chiba H, Nakayama F, Ueda J, Matsuda Y, Sawada N, Komori T, Ito A and Yokozaki H: Reduced expression of claudin-7 correlates with invasion and metastasis in squamous cell carcinoma of the esophagus. Hum Pathol 37: 569-577, 2006.

13. Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol 153: 333-339, 1998.
14. Dhawan P, Singh AB, Deane NG, No Y, Shiou SR, Schmidt C, Neff J, Washington MK and Beauchamp RD: Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 115: 1765-1776, 2005.
15. Pan XY, Wang B, Che YC, Weng ZP, Dai HY and Peng W: Expression of claudin-3 and claudin-4 in normal, hyperplastic, and malignant endometrial tissue. Int J Gynecol Cancer 17: 233-241, 2007.
16. Sobel G, Páska C, Szabó I, Kiss A, Kádár A and Schaff Z: Increased expression of claudins in cervical squamous intraepithelial neoplasia and invasive carcinoma. Hum Pathol 36: 162-169, 2005.
17. Sheehan GM, Kallakury BV, Sheehan CE, Fisher HA, Kaufman RP Jr and Ross JS: Loss of claudins-1 and -7 and expression of claudins-3 and -4 correlate with prognostic variables in prostatic adenocarcinomas. Hum Pathol 38: 564-569, 2007.
18. Resnick MB, Gavilanez M, Newton E, Konkin T, Bhattacharya B, Britt DE, Sabo E and Moss SF: Claudin expression in gastric adenocarcinomas: a tissue microarray study with prognostic correlation. Hum Pathol 36: 886-892, 2005.
19. Sauer T, Pedersen MK, Ebeltoft K and Naess O: Reduced expression of claudin-7 in fine needle aspirates from breast carcinomas correlate with grading and metastatic disease. Cytopathology 16: 193-198, 2005.

Clinicopathological significance of the gene expression of matrix metalloproteinases and reversion-inducing cysteine-rich protein with Kazal motifs in patients with colorectal cancer: *MMP-2* gene expression is a useful predictor of liver metastasis from colorectal cancer

TAKASHI OSHIMA[1], CHIKARA KUNISAKI[1], KAZUE YOSHIHARA[1], ROPPEI YAMADA[1], NAOTO YAMAMOTO[1], TSUTOMU SATO[1], HIROCHIKA MAKINO[1], SHIGERU YAMAGISHI[1], YASUHIKO NAGANO[1], SHOICH FUJII[1], MANABU SHIOZAWA[2], MAKOTO AKAIKE[2], NOBUYUKI WADA[3], YASUSHI RINO[3], MUNETAKA MASUDA[3], KATSUAKI TANAKA[1] and TOSHIO IMADA[4]

[1]Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama-shi, Kanagawa-ken 232-0024; [2]Department of Surgery, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama-shi, Kanagawa-ken 241-0815; [3]Department of First Surgery; [4]Yokohama City University, 3-9 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa-ken 236-0004, Japan

Received October 15, 2007; Accepted January 29, 2008

Abstract. Matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and membrane-type matrix metalloproteinase 1 (MT1-MMP) are involved in colorectal cancer invasion and metastasis. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) inhibits MMP-2, MMP-9 and MT1-MMP. We examined the clinicopathological significance of the relative expression of these genes in patients with colorectal cancer, especially with regard to metastasis. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal carcinoma. *MMP-2*, *MMP-9*, *MT1-MMP*, *RECK* and *β-actin* mRNA of cancer tissue and adjacent normal mucosa were measured by quantitative real-time reverse-transcriptase polymerase chain reaction. *MT1-MMP* gene expression was higher in cancer tissue than in adjacent normal mucosa. In contrast, *MMP-2*, *MMP-9* and *RECK* gene expression levels were lower in cancer tissue than in adjacent normal mucosa. As for the relationship between the gene expression and clinicopathological factors, *MMP-2* expression correlated with the depth of invasion, venous invasion and liver metastasis; *MMP-9* and *RECK* expression correlated with venous invasion. There were positive correlations among the gene expression levels of *MMP-2*, *MMP-9* and *RECK*. *MMP-2* gene expression was considered a useful predictor of liver metastasis from colorectal cancer.

Correspondence to: Dr Takashi Oshima, Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune-cho, Minami-ku, Yokohama-shi, Kanagawa-ken 232-0024, Japan
E-mail: ohshimatakashi@yahoo.co.jp

Key words: matrix metalloproteinase-2, matrix metalloproteinase-9, membrane-type matrix metalloproteinase 1, Kazal motifs, colorectal cancer

Introduction

Colorectal cancer, one of the most prevalent cancers worldwide (1), is the second leading cause of cancer-related mortality in developed countries (2). Tumor cell invasion and metastasis involve multiple steps, including proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM), altered cell adhesion and the physical movement of tumor cells. Among the many steps of tumor invasion and metastasis, excessive degradation of the matrix is one of the hallmarks of this process (3).

Matrix metalloproteinases (MMPs) are a key family of proteolytic enzymes involved in extracellular matrix degradation. In colorectal cancer, several MMPs have been found to be associated with tumor stage, prognosis, or both (4). Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) have been implicated in the progression, invasion and metastasis of colorectal cancer in animal models and patients (5). MMP-2 and MMP-9 can degrade denatured collagen and type IV, V, VII, IX and X collagens. Type IV collagen is particularly abundant in basement membranes. These gelatinases are now also thought to be involved in cell differentiation, apoptosis, angiogenesis, immune response and cancer cell growth (6). The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene was originally

Table I. PCR primers and conditions.

Gene	Primer	Temperature (C)	Product size (bp)
MMP-2	5'-CCCTCCCTTCAACCATTCCC-3' 5'-TTCCAGCAGACACCATCACC-3'	55.6	186
MMP-9	5'-TGGTCCTGGTGCTCCTGGTG-3' 5'-GCTGCCTGTCGGTGAGATTGG-3'	61.2	111
MT1-MMP	5'-AAGAGGAGAAGAGCAAACAG-3' 5'-CGGTAGGCACTGAACTTG-3'	55.1	91
RECK	5'-ACTGCCGAGAATACTGTCAAGCC-3' 5'-ACTATCCGTTGGGTTCCTCATTGG-3'	64.9	161
ß-actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

discovered in an expression cloning screen designed to isolate the transformation of suppressor genes against activated ras oncogenes (5,7,8). The RECK gene encodes a membrane-anchored glycoprotein and is down-regulated during the malignant conversion of cells (9). Although RECK is widely expressed in normal tissues and non-neoplastic cell lines, its expression is strongly suppressed in oncogene-transformed fibroblasts and several tumor-derived cell lines (9,10). RECK inhibits MMP-2, MMP-9 and membrane-type matrix metalloproteinase 1 (MT1-MMP) secretion and activity, suggesting that it participates in the regulation of MMPs and tumor invasiveness (11). RECK is also vital to developmental vasculogenesis and its down-regulation has been implicated in tumor angiogenesis and progression (9,11,12).

In this study, we examined the clinicopathlogical significance of the relative expression of these genes in patients with colorectal cancer, especially with regard to metastasis.

Materials and methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 205 patients with untreated colorectal carcinoma. The patients underwent surgery at the Yokohama City Medical Center, Gastroenterological Center and Kanagawa Cancer Center between 2002 and 2006. Informed consent was obtained from each patient and the Yokohama City Medical Center Committee and Kanagawa Cancer Center Committee approved the study. Each sample was embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and stored at -80˚C, immediately before use. The patients had no other form of malignancy. After examining the histopathological features of specimens stained with hematoxylin and eosin, sections including >80% carcinoma cells were used for total RNA preparation.

Quantitative real-time reverse-transcriptase polymerase chain reaction (PCR). Total RNA from colorectal cancer tissue and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD). cDNA was synthesized from 2 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20˚C until use. Quantitative real-time PCR was performed with iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl, containing cDNA derived from 75 ng of RNA, 0.27 μM of each primer, 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP and dTTP at a concentration of 400 μM each and 50 U/ml of iTag DNA polymerase. The PCR consisted of 10 min at 94˚C followed by 50 cycles of denaturation of the cDNA for 30 sec at 94˚C, annealing for 30 sec at an appropriate temperature according to Table I and a primer extension for 1 min at 72˚C, followed by 72˚C for 10 min. The PCR primer sequences of MMP-2, MMP-9, MT1-MMP, RECK and ß-actin, used as an internal control are shown in Table I.

Statistical analysis. Associations of the gene expression levels of colorectal cancer with those of adjacent normal mucosa were evaluated by the Wilcoxon test. The relationship between the gene expression levels and potential explanatory variables, including age, gender, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion and liver metastasis, were assessed with the χ^2 test. Associations among variables were evaluated with the Mann-Whitney U test. Correlation coefficients between different variables were determined by a simple regression analysis. Statistical analyses were performed using Statview J 5.0 software (Abacus, CA). Two-sided P-values were calculated and P-values <0.05 were considered to indicate statistical significance.

Results

Comparison of MMP-2, MMP-9, MT1-MMP and RECK mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were lower in cancer tissue than in adjacent normal mucosa (P=0.004, 0.001 and 0.006; Fig. 1A, B and D). In contrast, *MT1-MMP* gene expression in cancer tissue was higher than that in adjacent normal mucosa (P=0.038; Fig. 1C).

Relationship between clinicopathological features to MMP-2, MMP-9, MT1-MMP and RECK gene expression levels. After

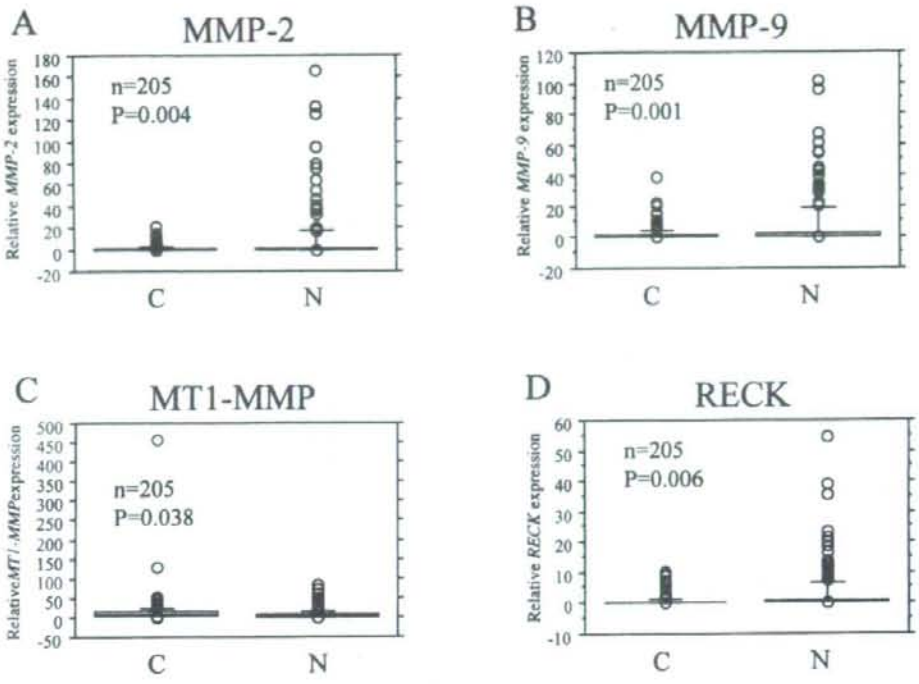

Figure 1. Comparison of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression between colorectal cancer tissue and adjacent normal mucosa. *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue (P=0.0462, 0.0488 and 0.0491). However, the *MT1-MMP* gene expression level did not differ significantly between cancer tissue and adjacent normal mucosa.

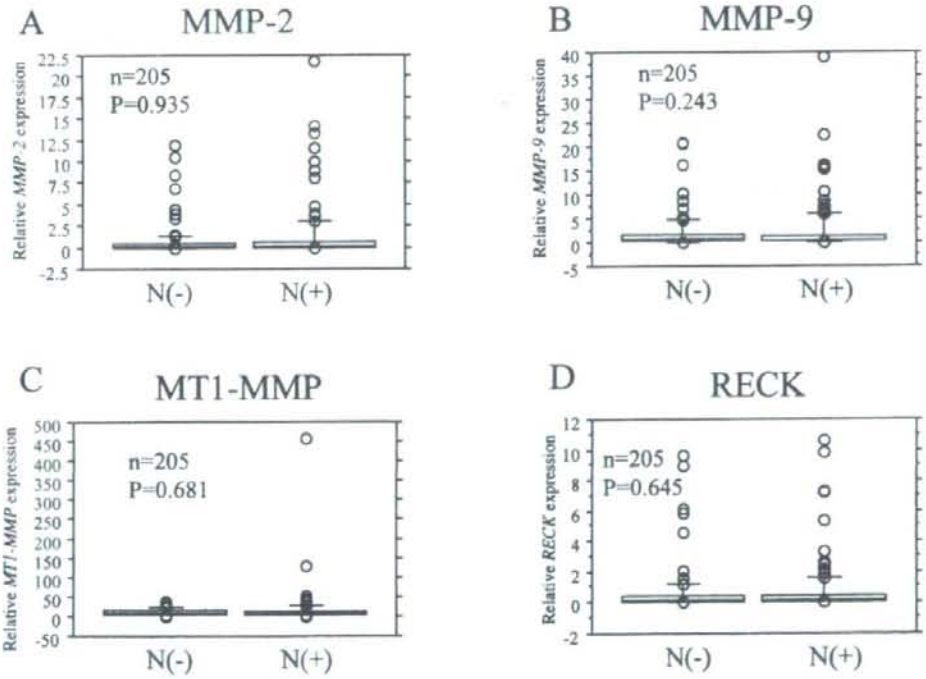

Figure 2. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with lymph node metastasis in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed by the Mann-Whitney U test. The presence or absence of lymph node metastasis was unrelated to the expression level of any gene.

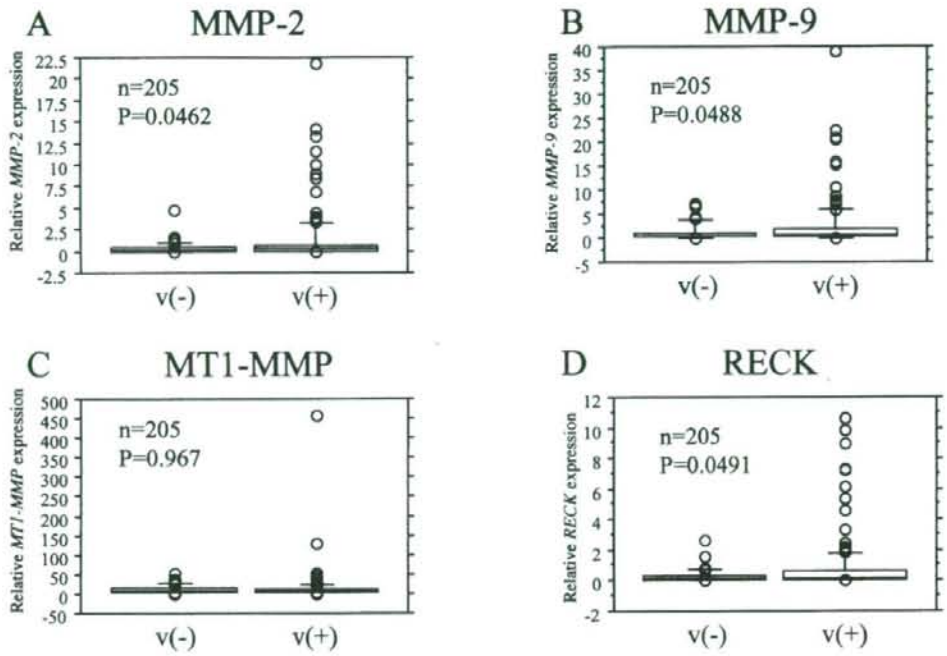

Figure 3. Association of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression with venous invasion in 205 patients with colorectal cancer. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, the median. P-values were assessed by the Mann-Whitney U test. The presence and absence of venous invasion was significantly related to the gene expression levels of *MMP-2*, *MMP-9* and *RECK*.

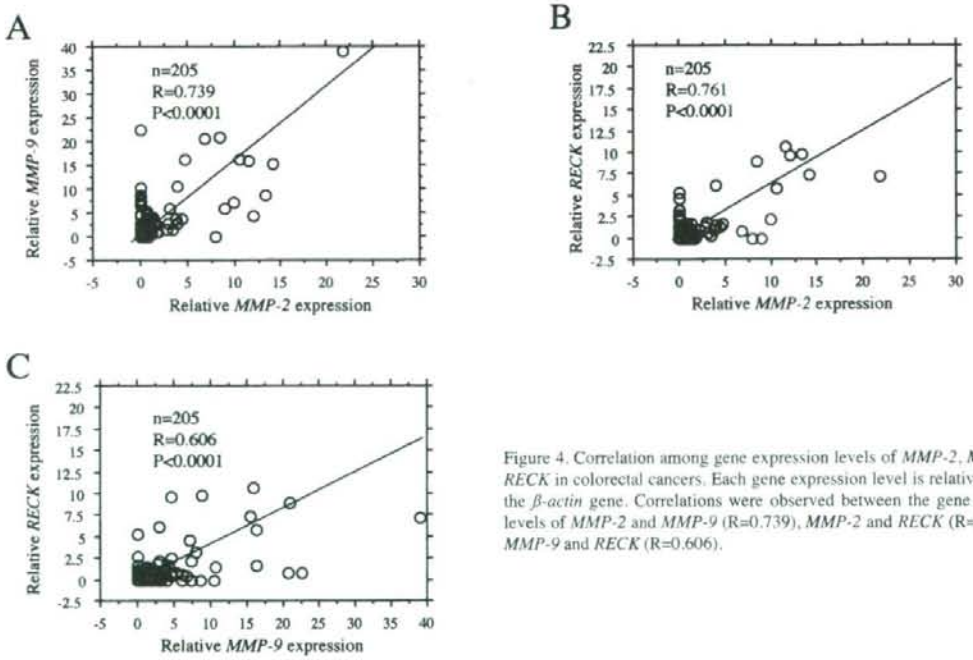

Figure 4. Correlation among gene expression levels of *MMP-2*, *MMP-9* and *RECK* in colorectal cancers. Each gene expression level is relative to that of the *β-actin* gene. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and *MMP-9* and *RECK* (R=0.606).

categorizing expression levels of *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* genes as low or high according to their respective median values, we examined the relationship between the expression levels of each gene and clinicopathological features. *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression levels were unrelated to age, gender, tumor size, histological type, lymph node metastasis, tumor location and lymphatic invasion. *MMP-2* expression was significantly

Table II. The relationship between the expression of MMP-2, MMP-9, MT-MMP or RECK genes and clinicopathological features.

Variables/categories	MMP-2 expression			MMP-9 expression			MT1-MMP expression			RECK-7 expression		
	low (n=103)	high (n=102)	P-value	low (n=103)	high (n=102)	P-value	low (n=102)	high (n=103)	P-value	low (n=103)	high (n=102)	P-value
Age	66.6±10.2	65.0±11.3	0.294	66.2±10.6	65.4±10.9	0.586	65.9±11.3	65.2±10.2	0.929	64.9±11.9	66.7±9.5	0.229
Gender												
Male	52	60	0.231	57	55	0.838	53	59	0.444	54	58	0.523
Female	51	42		46	47		49	44		49	44	
Size												
≤5 cm	59	56	0.731	60	55	0.532	61	54	0.287	60	55	0.532
>5cm	44	46		43	47		41	49		43	47	
Histological type												
Well differentiated	32	31	0.995	31	32	0.395	31	31	0.495	28	33	0.492
Moderately differentiated	57	57		61	53		59	55		62	53	
Poorly differentiated	14	14		11	17		11	17		13	16	
Depth of invasion												
T1	16	3	0.018	11	8	0.272	12	7	0.455	10	9	0.337
T2	46	48		50	44		49	45		53	41	
T3	36	44		39	41		36	44		34	46	
T4	5	7		3	9		5	7		6	6	
Lymph node metastasis												
Absent	51	44	0.360	43	52	0.185	47	48	0.940	49	46	0.722
Present	52	58		60	50		55	55		54	56	
Location												
Colon	61	51	0.185	58	54	0.628	59	53	0.401	60	52	0.296
Rectum	42	51		45	48		44	50		43	50	
Lymphatic invasion												
Absent	70	64	0.490	70	64	0.490	72	63	0.155	67	68	0.807
Present	33	37		33	37		30	40		36	34	
Venous invasion												
Absent	48	30	0.011	47	31	0.025	43	35	0.228	47	31	0.025
Present	55	72		56	71		56	68		56	71	
Liver metastasis												
Absent	77	62	0.032	69	70	0.802	72	67	0.396	70	69	0.962
Present	26	40		34	32		30	36		33	33	

related to the depth of invasion (P=0.018). *MMP-2*, *MMP-9*, and *RECK* gene expression levels were significantly related to venous invasion (P=0.011, 0.025 and 0.035). *MMP-2* expression was also significantly related to liver metastasis (P=0.032) (Table II).

Comparison of MMP-2, MMP-9, MT1-MMP and RECK gene expression levels according to the presence and absence of lymph node metastasis. There were no significant differences in *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* gene expression levels according to the presence or absence of lymph node metastasis (Fig. 2).

Comparison of MMP-2, MMP-9, MT1-MMP and RECK gene expression levels according to the presence or absence of venous invasion. MMP-2, *MMP-9* and *RECK* gene expression levels differ significantly according to the presence or absence of venous invasion (P=0.0462, 0.0488 and 0.0491) (Fig. 3).

Correlation among MMP-2, MMP-9 and RECK expression. The results of a correlation analysis are shown in Fig. 4. Correlations were observed between the gene expression levels of *MMP-2* and *MMP-9* (R=0.739), *MMP-2* and *RECK* (R=0.761) and *MMP-9* and *RECK* (R=0.606) (Fig. 4).

Discussion

MMP-2 and MMP-9 play key roles in the development and progression of human malignancies (13-15). These matrix metalloproteinases mediate the destruction of extracellular matrix and are considered an important early step in tumor invasion and metastasis. MMP-2 and MMP-9 also have angiogenic activity and participate in early tumorigenesis and tumor growth, including metastasis (16,17). The over-expression of MT1-MMP in tumor cells promotes growth (18). The RECK gene is believed to regulate multiple MMP family members, such as MMP-2, MMP-9 and MT1-MMP (12).

Several previous studies have compared *MMP-2*, *MMP-9*, *MT1-MMP* and *RECK* mRNA expression levels between colorectal cancer tissue and adjacent normal mucosa. Kim *et al* (19) reported that *MMP-2* and *MMP-9* gene expression levels (n=24) are higher in colorectal cancer than in adjacent normal mucosa. Lubbe *et al* (20) found that the *MMP-9* gene expression level in colorectal cancer (n=28) is higher than that in adjacent normal mucosa. However, in our study (n=205), *MMP-2*, *MMP-9* gene expression levels were higher in adjacent normal mucosa than in cancer tissue. We believe that this result was related to the higher expression of MMP-2 and MMP-9 in interstitial tissues than in cancer cells. Atkinson *et al* (21) showed that the *MT1-MMP* gene expression level is higher in cancer tissue than in adjacent normal mucosa, while Takeuchi *et al* (22) reported that the *RECK* gene expression level is higher in adjacent normal mucosa than in colorectal cancer. In our study, *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue. Conversely, the *MT1-MMP* gene expression level was higher in cancer tissue than in adjacent normal mucosa.

Zheng *et al* (23) studied the relationship between the clinicopathological features and gene expression levels of MMPs. The expression levels of MMP-2 and MMP-9 were found to be closely linked to venous and lymph node invasion. Ogata *et al* (24) reported that MMP-9 expression is related to lymph node metastasis and severe venous invasion. Takeuchi *et al* (22) reported that RECK expression is significantly associated with lymph node metastasis, Dukes' stage and venous invasion. In our study, *MMP-2*, *MMP-9* and *RECK* expression levels were significantly related to venous invasion. *MMP-2* expression was also significantly related to tumor depth and liver metastasis. MT1-MMP has been reported to specifically activate MMP-2 (25). The association of *MMP-2* expression with tumor depth, venous invasion and liver metastasis may be related to the finding that the *MT1-MMP* gene expression level was higher in cancer tissue than in adjacent normal mucosa in our study.

In a study examining interrelations among RECK, MMP-2, and MMP-9, van der Jagt *et al* found that RECK expression levels strongly correlate with the inhibition of MMP-2 enzyme activity, though not with the inhibition of MMP-9 activity (26). Masui *et al* reported a significant negative correlation between RECK activation and MMP-2 activation (27). In our study, correlations were observed between gene expression levels of *RECK* and *MMP-2*, *RECK* and *MMP-9* and *MMP-2* and *MMP-9*. These results demonstrated a positive correlation between the expression of *RECK* and *MMP-2* at the mRNA level, although RECK inhibited MMP-2 activity at the enzyme level.

In conclusion, our study showed that *MMP-2*, *MMP-9* and *RECK* gene expression levels were higher in adjacent normal mucosa than in cancer tissue and correlated with each other. Expression levels of these genes were significantly related to venous invasion. *MMP-2* gene expression is considered a useful predictor of liver metastasis from colorectal cancer.

References

1. Jemal A, Murray T, Ward E, *et al*: Cancer statistics. CA Cancer J Clin 55: 10-30, 2005.
2. Greenwald P: Colon cancer overview. Cancer 70: 1206-1215, 1992.
3. Liotta LA and Stetler-Stevenson WG: Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res 51: 5054-5059, 1991.
4. Wagenaar-Miller RA, Gorden L and Matrisian LM: Matrix metalloproteinases in colorectal cancer: is it worth talking about? Cancer Metastasis Rev 23: 119-135, 2004.
5. Noda M, Kitayama H, Matsuzaki T, *et al*: Detection of genes with a potential for suppressing the transformed phenotype associated with activated ras genes. Proc Natl Acad Sci USA 86: 162-166, 1989.
6. Mook OR, Frederiks WM, Van C and Noorden J: The role of gelatinases in colorectal cancer progression and metastasis. Biochim Biophys Acta 1705: 69-89, 2004.
7. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y and Noda M: A ras-related gene with transformation suppressor activity. Cell 56: 77-84, 1989.
8. Takahashi C, Akiyama N, Matsuzaki T, Takai S, Kitayama H and Noda M: Characterization of a human MSX-2 cDNA and its fragment isolated as a transformation suppressor gene against v-Ki-ras oncogene. Oncogene 12: 2137-2146, 1996.
9. Takahashi C, Sheng Z, Horan TP, *et al*: Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Natl Acad Sci USA 95: 13221-13226, 1998.
10. Sasahara RM, Takahashi C and Noda M: Involvement of the Sp1 site in ras-mediated downregulation of the RECK metastasis suppressor gene. Biochem Biophys Res Commun 264: 668-675, 1999.
11. Oh J, Takahashi R, Kondo S, *et al*: The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. Cell 107: 789-800, 2001.