18. Aarnio M, Mecklin JP, Aaltonen LA, Nystrom-Lahti M, Jarvinen HL. Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Int J Cancer 1995;64:430–3.
19. Kawakami K, Yasutomoi M, Baba S. Analysis of the HNPCC registries data reported at the 43rd Japanese Society for Cancer of the Colon and Rectum (JSCCR) meeting. In: Baba S, ed. New strategies for treatment of hereditary colorectal cancer. Tokyo: Churchill Livingstone, 1996:229–33.
20. Utsunomiya J, Miyaki M. Studies of hereditary non-polyposis colorectal cancer in Japan. Int J Clin Oncol 1998;3:53–74.
21. Watson P, Vasen HF, Mecklin JP, Jarvinen H, Lynch HT. The risk of endometrial cancer in hereditary non-polyposis colorectal cancer. Am J Med 1994;96:516–20.

特集　最新　直腸癌手術

仙骨合併骨盤内臓全摘術

上原圭介*　山本聖一郎*　赤須孝之*
藤田　伸*　森谷宜皓**

はじめに

直腸癌の局所再発に対しては，化学療法，放射線療法，外科療法などが単独あるいは集学的治療として行われているが，満足のいく治療法はいまだ確立されていない。放置すれば長期にわたり出血・疼痛・腸閉塞・会陰潰瘍などの合併症で悲惨な経過をたどるケースも少なくなく，当院では1983年以来，再発直腸癌に対して積極的に外科的完全切除を行ってきた。

仙骨合併骨盤内臓全摘術（total pelvic exenteration with distal sacretomy；TPES）は骨盤壁に浸潤の及ぶ再発直腸癌（fixed recurrent tumor；FRT）に対して，仙骨を含む隣接臓器とともに腫瘍を *en bloc* に摘出し，surgical margin を free とできる根治的治療であり，FRT に対する基本術式と考えている。

本稿では TPES の手術手技を中心として，適応，合併症，遠隔成績につき述べる。

I. 手術適応

TPES はきわめて大きな侵襲を伴う術式のため，利点・欠点を正確に評価し，手術適応を十分に考慮しなくてはならない。原則的に切除可能な1～2個の肝転移を除き，遠隔転移がなく，骨盤内に限局した局所再発癌のみを適応としている。仙骨切断レベルの決定には術前のMRIが欠かすことのできない検査である（図1）。仙骨切断レベルは歩行障害や脊髄液瘻を来すことのないS2下縁までとしており，これより高位での再発巣に対しては仙骨前面の表層切除あるいは Waldeyer 筋膜のみの切除に留めている。より高位での仙骨切断は仙骨原発腫瘍に対しては行われることもあるが，再発直腸癌に対しては行うべきではない。仙骨のS2以下の切断術において合併切除可能な非骨性骨盤壁は仙棘靱帯，仙結節靱帯，尾骨筋，内閉鎖筋の一部，梨状筋，骨盤隔膜およびS3以下の仙骨神

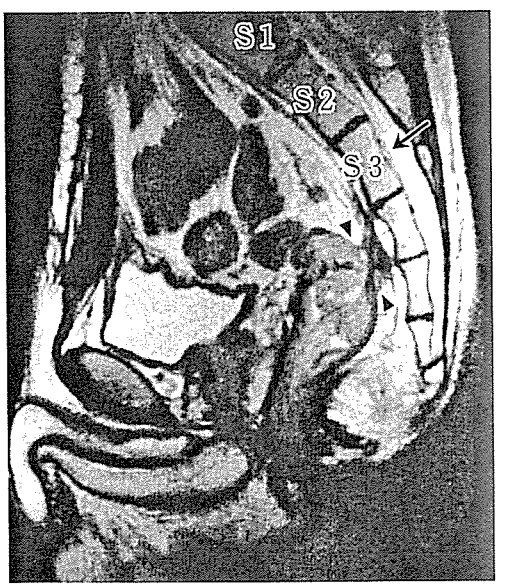

図1　矢頭は RFT，矢印は仙骨切断レベル

* Keisuke UEHARA et al.　国立がんセンター中央病院大腸外科
** Yoshihiro MORIYA　同手術部長

経となる。

また，次のような症例は適応外としている。下肢の浮腫，坐骨神経痛などを来す症例は癌の神経，リンパ組織への広範な浸潤が考えられる。また，初回手術時に側方郭清を行っている症例や側方リンパ節転移を認める症例も適応外である。

II. 手術手技

手術は表1に示す手順で行う。

1. 腹腔操作

体位は砕石位とし，正中切開で開腹する。肝転移，腹膜播腫，大動脈周囲リンパ節転移などの遠隔転移がなく，再発巣が骨盤内に限局していることを確認し，手術遂行の是非を決定する。

まず，膀胱前腔（Retzius腔）の展開を行う。外腸骨血管に沿った後腹膜切開を骨盤内に延長し，骨盤壁近くで精管（女性では子宮円索）を結紮・切離する。膀胱前面を恥骨後面に沿って疎な組織の剝離を進めると，前立腺の前側方に恥骨前立腺靱帯と内骨盤筋膜（endopelvic fascia）を確認できる。前立腺の両側でendopelvic fasciaを電気メスで切開し肛門挙筋を露出する。続いて，前立腺表面を走行する深陰茎背静脈の深枝であるdorsal vein complex（以下，DVC）の処理を行う。鳶巣式bunching鉗子を用い，左右のendopelvic fascia切離断から前立腺前面に沿って組織を収束させると，この中にDVCが含まれることになる（bunching method）。鉗子の前後で2-0タイクロン糸を用い二重結紮・切離する。良好な視野が得られれば，ここで尿道の切離を行ってもよいが，会陰操作時に行ってもよい（図2）。

腹部大動脈分岐部に戻り，郭清しつつ岬角を露出する。仙骨骨膜を露出する層で仙骨切断予定部位まで剝離を進める。この際，仙骨前面には瘢痕組織が覆っており，仙骨前面静脈層の確認が困難なため出血を伴うが，出血部位をしっかりと確認し，吸引しながら電気凝固を行えば止血可能である。それでも止血できない場合

表1 手術の手順

1. 腹腔操作
① 前方（Retzius腔）の展開とDVC（dorsal vein complex）の処理，② 仙骨前面の展開，③ 側方郭清および内腸骨血管の処理と仙骨切断レベルの決定
2. 会陰操作
① 肛門挙筋の切離，② 尿道の切離
3. 体位変換，仙骨部操作
① 大殿筋の仙骨からの切離，② 椎弓切除，仙骨神経の確認，③ 仙骨切断
4. 体位変換，再建操作
① 回腸導管，人工肛門の作製，② ドレーン挿入と閉腹

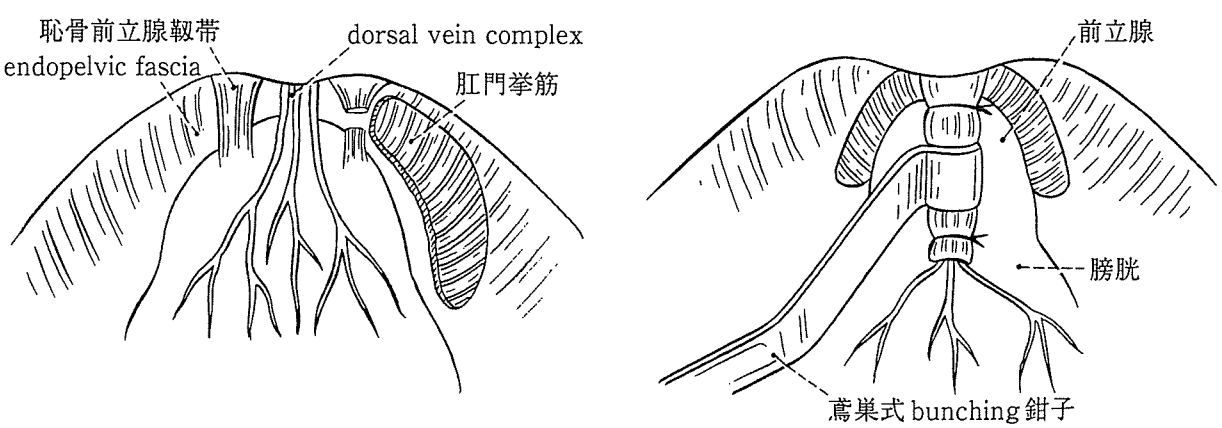

図2 Dorsal vein complexの処理

は，綿球で圧迫し，術野の展開を十分に行ったのち，再度止血を試みるべきである。

ついで内腸骨血管系の処理を行う。左右総腸骨動脈から内・外腸骨動脈分岐部を露出し，外腸骨血管に沿い内閉鎖筋を露出しながら内下方に剝離操作を進める。閉鎖動・静脈は切離し，閉鎖神経は温存する。この時点で腰仙骨神経，第1・第2仙骨神経を同定し，温存すべき第2仙骨神経にはテーピングを行うと，のちに行う仙骨操作での神経誤認を回避できる（図3）。内腸骨動脈は第一次分枝である上殿動脈が分岐したあとで，二重結紮・切離する。上殿動脈の温存は必須ではないが，会陰創の血流が悪くなるため，温存するよう努めている。内腸骨静脈本幹の切離に先立ち，骨盤壁を貫通する下殿動・静脈，陰部動・静脈などの末梢分枝をていねいに結紮・切離し，最後に内腸骨静脈本幹を結紮・切離する。内腸骨静脈の切離を先行すれば，静脈系のうっ血を招き，のちの操作で思わぬ大出血につながる可能性がある。骨盤内静脈系からの出血コントロールがきわめて大切な本術式において，重要なポイントの一つである。

尿管は周囲の結合織と栄養血管を可能なかぎり温存し，左右総腸骨血管との交叉部よりできるかぎり膀胱側で切離する。一側または両側に7 Fr.のシングルJカテーテルをスプリントカテーテルとして挿入し，術中の尿量モニタリングを行う。

2．会陰操作

会陰の皮膚切開は男性では直腸切断術に準じ，女性では女性器を含めた切離線で腹部創につなげる。肛門挙筋を付着部で切離し，腹腔側の剝離層につなげる。この操作での骨盤底筋群からの出血を減らすため，できれば内腸骨静脈本幹の結紮前に会陰操作を行っておくとよい。

3．仙骨操作

仙骨切断操作には整形外科医の協力が必要である。腹部および会陰創の仮閉鎖を行い，体位を砕石位から腹臥位へ変更する。注意点は腹圧の上昇を避けることで，腹圧が上昇すると静脈還流が悪くなり，椎骨静脈叢の圧が上昇し，出血量が増加する。我々は椎弓切除用の4点支持架台を使用し腹圧上昇を防止している。

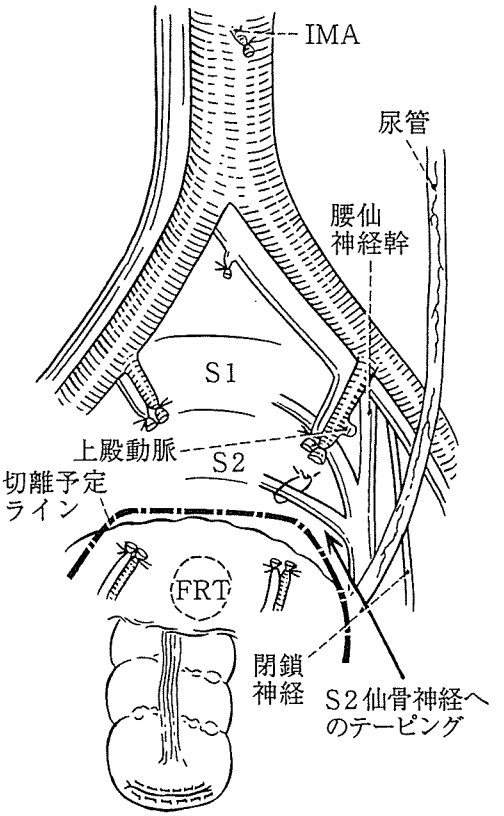

図3　内腸骨血管の処理および仙骨神経のテーピング

会陰創背側端から仙骨切断予定部位より約10 cm頭側までの正中切開をおく。大殿筋を仙骨に沿って剝がし，仙骨背側面を十分に露出する。次に仙骨を固定する靱帯・筋肉の切離を行う。まず，坐骨結節を確認後，仙結節靱帯を切離する。続いて会陰より示指を挿入し，仙棘靱帯の走行を確認後，これを坐骨棘近傍で切離する。これらの靱帯を切離したのち，梨状筋の切離を行うが，梨状筋の腹側には坐骨神経・仙骨神経が走行しており，注意が必要である。この際，腹腔側で行ったテーピングが非常に有用となる。切離が進んだら示指で仙骨前面の剝離層を確認し，仙骨切断レベルの最終確認を行う。

仙骨切断は，まず正中仙骨稜を削り，仙骨管を開放する。硬膜の尾側端はS2下縁あたりとされており，S2下縁以下での仙骨切除では通

常，硬膜の結紮処理は不要である。左右の示指を挿入し，腹腔側剝離層との交通を再度確認し，ノミと鎚を用いて迅速に仙骨切断を行う。腫瘍，仙骨を一塊として摘出後，視野は良好となり，電気メス・骨蠟ですばやく止血する（図4）。

男性では尿道断端を3-0バイクリル糸で確実に閉鎖する。怠れば経尿道的に骨盤死腔炎を起こす原因となる。十分に止血を確認後，大殿筋起始部・皮下を3-0バイクリル糸で，皮膚を2-0ナイロン糸でwater-tightに3層で縫合閉鎖する。

4. 再建と閉腹

体位を仰臥位とし，洗浄・止血を行ったのち，尿路再建および人工肛門造設を行う。尿路再建には回腸導管を用いる。回腸末端より約20 cm口側で，2本の栄養血管を含む約10～15 cmの回腸を使用する。長すぎる導管は尿の再吸収を来すため好ましくない。導管内を生食洗浄後，尿管回腸吻合を5-0バイクリル糸で行い，7 Fr.のシングルJカテーテルをスプリントカテーテルとする（図5）。高さのあるurostomaを作るため，上行結腸を肝彎曲まで十分に授動し，右側結腸全体を頭側へよける。この操作によって回腸導管間膜の過度の緊張を回避できる。低いurostomaは患者のQOLを悪化させるため避けなければならない。

回腸回腸吻合はリニアカッターを用い機能的端端吻合を行っている。吻合部が骨盤底に落ち込むと，骨盤死腔炎から二次的に縫合不全を来

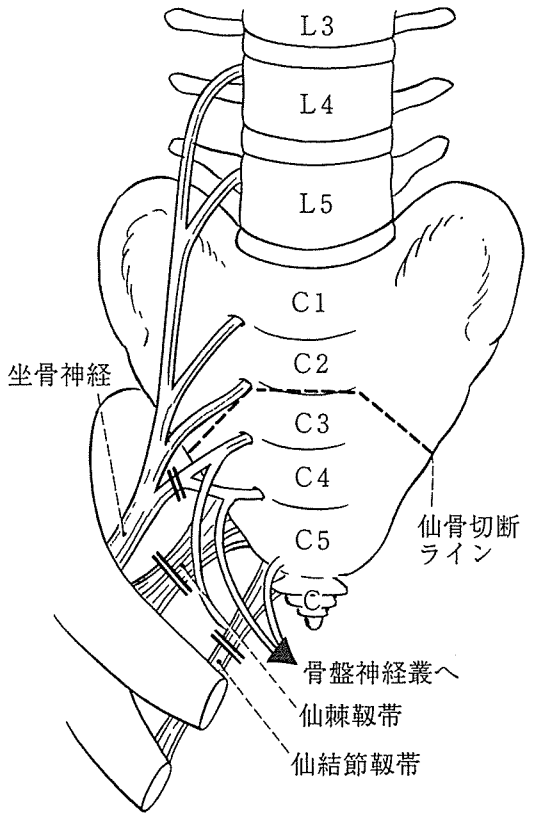

図 4　仙骨の切断ライン

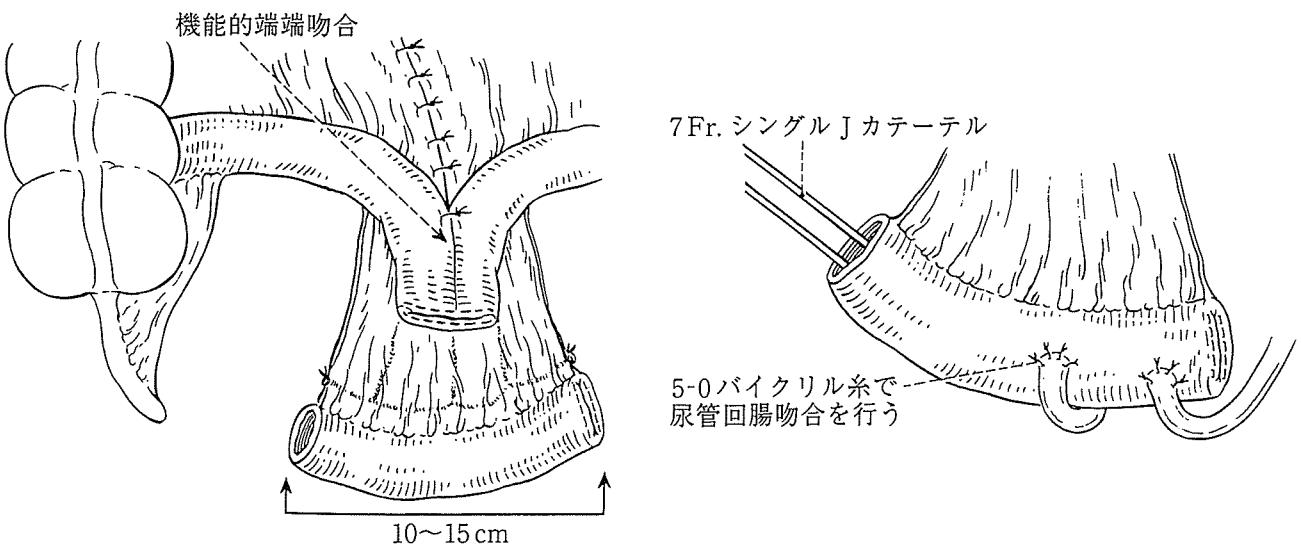

図 5　回腸導管による尿路再建

し，小腸会陰瘻を形成する可能性がある。小腸会陰瘻は患者のQOLをいちじるしく損なうため，吻合部が骨盤底に落ち込まないように，腸間膜を固定するとよい。この操作は術前放射線照射例では必須である。

骨盤死腔炎の防止のためには術中汚染の防止や完全な止血が必要なことはいうまでもないが，血流の良好な大網の充塡も感染防止に有用である。骨盤底に10 mmプリーツドレーンを留置し，術後は10 mm H_2Oで持続吸引を行っている。また，手術侵襲の大きさから術後腸管運動の回復が遅れるため，胃瘻を造設している。

III. 手術成績

1983～2001年の19年間に57例のTPESを行い，48例（84%）にmargin freeとなるR0手術が可能であったが，平均手術時間は741分，平均出血量は3,650 mlとある程度の手術侵襲は避けられない。しかしながら前期症例（1983～1992年）と後期症例（1993～2001年）を比較すると平均出血量は4,229 mlから2,500 mlへ有意（p=0.002）に減少し，良好なlearning curveを示していると考えられる。在院死も前期に2例認めるものの，後期では経験していない。

仙骨切断レベルはS3上縁が23例（40%）ともっとも多く，ついでS4下縁，S2下縁の順であった。

合併症は全体の58%で認め，仙骨創の哆開が51%ともっとも多く，ついで骨盤死腔炎が39%であった。しかし，骨盤死腔炎の頻度は前期の72%から後期の23%へ有意（p=0.046）に減少を認めている。イレウスは5例で認めたが，すべて保存的に改善した。また，晩期合併症として小腸会陰瘻4例を認め，全例でバイパス手術が必要であった。回腸導管会陰瘻の1例は両側腎瘻が必要となったが，術後10年現在無再発生存中である。術後の殿部の疼痛は不可避で，MSコンチンの内服が効果的である。

多変量解析を行うと予後規定因子はfree surgical marginと血清CEA値で，R0手術を行った48症例の3年および5年無再発生存率はそれぞれ82，42%であった。

おわりに

TPESは骨盤壁に浸潤する局所再発直腸癌の患者にとって，完全切除の最後のチャンスであり，手術手技も確立され，比較的安全に行える治療となった。しかし，手術適応は厳格でなくてはならず，骨盤外科に精通した外科医のみが行うべき術式である。

文　献

1) 森谷冝皓ほか：局所再発に対する骨盤壁合併骨盤内臓全摘術，森谷冝皓編，メジカルビュー，149—155, 2001
2) 森谷冝皓ほか：直腸癌局所再発に対する仙骨合併骨盤内臓全摘術．手術 57：761—767, 2003
3) Wanebo HJ et al：Abdominal sacral resection of locally recurrent rectal cancer. Ann Surg 194：458—471, 1981
4) Moriya Y et al：Total pelvic exenteration with distal sacretomy for fixed recurrent tumor. Dis Col Rectum 47：2047—2053, 2004
5) Moriya Y et al：Total pelvic exenteration with distal sacretomy for fixed recurrent tumor. Surg Oncol Clin N Am 14：225—238, 2005

Extent of Mesorectal Tumor Invasion as a Prognostic Factor After Curative Surgery for T3 Rectal Cancer Patients

Masayoshi Miyoshi, MD, Hideki Ueno, MD,* Yojiro Hashiguchi, MD,* Hidetaka Mochizuki, MD,* and Ian C. Talbot, MD†*

Objective: To determine the significance of the extent of mesorectal tumor invasion as a prognostic factor for T3 rectal cancer patients.

Summary Background Data: There is controversy as to which primary lesion characteristics, other than regional lymph node involvement, in T3 rectal cancer are reliable prognostic factors.

Patients and Methods: The extent of mesorectal tumor invasion was evaluated using 2 data sets comprising 196 and 247 patients undergoing curative surgery at separate institutes. When the outer aspect of the muscular layer was not identifiable, an estimate was obtained by drawing a straight line between the 2 break points of the muscular layer.

Results: We selected 6 mm as the optimal value for subclassification of T3 rectal patients into 2 groups, based on the extent of mesorectal invasion, using the first data set. The overall 5-year survival rate was significantly higher in patients with <6 mm than in those with ≥6 mm of mesorectal invasion (72% versus 50%; $P < 0.01$). Similarly, in the second data set, the overall 5-year survival rates of patients with mesorectal invasion <6 mm and ≥6 mm were 59% and 37%, respectively ($P < 0.01$). In both data sets, multivariate analyses verified the extent of mesorectal invasion to be an independent prognostic factor, together with nodal involvement. Regarding positive nodal involvement and mesorectal invasion ≥6 mm as risk factors, the overall 5-year survival rates with none, one, and both of these factors were 84%, 61%, and 38%, respectively, in the first data set ($P < 0.01$). Prognostic results were similar for the second data set.

Conclusion: Extent of mesorectal invasion, based on a 6-mm cutoff value, is useful for subclassification of T3 rectal cancer patients.

(*Ann Surg* 2006;243: 492–498)

Recurrence of rectal cancer is very difficult to control. Thirty to fifty percent of patients who undergo potentially curative surgery alone ultimately experience recurrence in Western countries,[1–3] and one third of those receiving curative resections will die of the disease.[4] The most important factor impacting the survival of rectal cancer patients is tumor stage, determined by the depth of penetration through the bowel wall, and nodal involvement.[5]

Because of high failure rates, regardless of whether nodal involvement is present, postoperative adjuvant therapy has often been used to prevent local recurrence and improve survival rates for patients with transmurally invasive rectal cancers.[6–8] For example, in 1990, the National Institutes of Health Consensus Statement[9] recommended that patients with resected stage II and III rectal cancers receive adjuvant treatments such as chemotherapy and/or irradiation. However, some investigators hold the view that postoperative adjuvant therapy should be based not only on stage, but also other clinicopathologic factors and that adjuvant therapy may not be necessary for a group of T3 rectal cancer patients at very low risk of recurrence.[10]

The extent of mesorectal tumor invasion has been shown to be an independent risk factor for recurrence in some studies.[10–12] However, the TNM staging system[13] does not take into account of the extent of mesorectal tumor invasion, so long as there is no invasion of adjacent organs. Thus, the T3 tumor stage covers a spectrum encompassing minimal invasion beyond the muscularis propria (MP) to gross invasion into the mesorectum that stops short of invading an adjacent organ. Tumors at opposite ends of the T3 spectrum may have very different prognostic implications.

This study was designed to analyze factors impacting the survival of patients with T3 rectal cancer (stages II and III), and in particular to identify indicators for adjuvant therapy. The extent of mesorectal tumor invasion was a major focus of the present study.

MATERIALS AND METHODS

Patients

A total of 196 patients with primary rectal cancer located below the peritoneal reflection who underwent curative surgery at the Department of Surgery I, National Defense Medical College, Japan, between 1980 and 1997 constituted the first data set. No patients had evidence of distant metastasis, and all patients were confirmed to have undergone curative surgical resection, without circumferential resection margin involvement, based on histologic examination. We confined our analysis to 196 patients with T3 rectal cancer as determined by pathologic findings of surgically resected

From the *National Defense Medical College, Saitama, Japan; and †St. Mark's Hospital Academic Institute, Harrow, Middlesex, UK.

Supported by the National Defense Medical College.

Reprints: Masayoshi Miyoshi, MD, National Defense Medical College, Tokorozawa, Saitama, Japan. E-mail: miyoshi@e-mail.jp or miyoshi4155@yahoo.co.jp.

ISSN: 0003-4932/06/24304-0492
DOI: 10.1097/01.sla.0000205627.05769.08

specimens, who had not received preoperative neoadjuvant therapy. In this period, there were few patients underwent systematic intravenous postoperative adjuvant therapy at the National Defense Medical College. Overall, 87% of our cases were given only oral anticancer drugs such as 5-fluorouracil, 5′-DFUR, and HCFU. The patients consisted of 134 males and 62 females, with an average age 59.5 years at the time of surgery (range, 25–85 years). Mean follow-up for all patients was 77 months (range, 1–231 months).

A total of 247 patients with primary rectal cancer located in the lower two thirds of the rectum who underwent complete surgical resection of their tumors between 1960 and 1969 at St. Mark's Hospital comprised the second data set. These tumors were confirmed to be T3 and did not have circumferential resection margin involvement by pathologic findings of surgically resected specimens. None of these patients had synchronous cancers or cancers complicating familial adenomatous polyposis or inflammatory bowel disease. There were 164 males and 83 females with an average age at the time of surgery of 61.4 years (range, 33–89 years). All patients selected were followed up for at least 5 years (average, 140 months; range, 61–263 months) or until death. It was exceptionally rare for adjuvant therapy to be used at St. Mark's in the 1960s.

For these tumors, morphologic features of known prognostic importance such as tumor diameter, tumor differentiation, and nodal involvement had previously been recorded in pathology reports.

Extent of Mesorectal Tumor Invasion

All surgically resected specimens were opened in the operating theater by cutting along the antimesenteric border, carefully avoiding the tumor unless growth was circumferential. The specimens were pinned to a cork board and fixed in 20% formalin. One or more large longitudinal sections of the whole tumor were obtained at the point of maximum extension through the bowel wall, as judged macroscopically. The slice containing the deepest invasion was selected for division into blocks, which were then embedded and routinely processed for hematoxylin and eosin staining. In these sections, the extent of mesorectal tumor invasion, ie, the distance between the outer border of the MP and the outermost part of the tumor, was measured when the outer aspect of the muscular layer was identifiable (type A tumors).[10,12] When the outer aspect of the muscular layer was not identifiable because of destruction by the tumor or extensive inflammation (type B tumors), an estimate was obtained by drawing a straight line between the 2 break points of the muscular layer (Fig. 1). Hematoxylin and eosin-stained sections, containing the deepest tumor extension, in rectal tumor cases with and without a discernible outer muscular layer, are presented in Figure 1C and D.

Statistical Analysis

Statistical analyses were performed using StatView 4.11 software (Abacus Concepts, Berkeley, CA). Survival curves were drawn by the Kaplan-Meier method and were compared using the log-rank test. Overall survival was used for the survival analysis. Multivariate analyses to identify independent prognostic factors were performed using the Cox stepwise regression model.

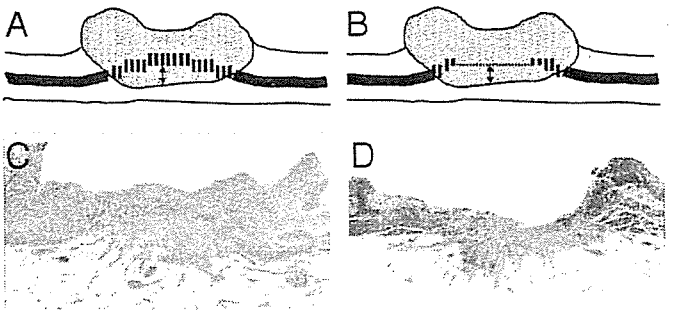

FIGURE 1. Method of measuring the depth of mesorectal tumor invasion. When the outer aspect of the muscular layer was identifiable, the distance between the outer border of the MP and the outermost portion of the tumor was measured (A, C). When the outer aspect of the muscular layer was not identifiable, an estimate was made by drawing a straight line between the 2 break points of the muscular layer (B, D).

RESULTS

Distribution of Extent of Mesorectal Invasion (First Data Set)

Tumor numbers were determined in 51 and 145 patients with type A and type B tumors, as shown in Fig. 1, respectively, for the first data set.

The distribution of the extent of mesorectal invasion (in millimeters) in 196 patients is shown in Fig. 2. The extent of mesorectal invasion in 55 patients (30% of all patients) ranged from 3 mm to 5 mm. The extent of mesorectal invasion was within 7 mm in 148 patients (75% of all patients), and the mean value for all patients was 5.2 mm (range, 0.1–22.1 mm).

Cutoff Value of Extent of Mesorectal Invasion (First Data Set)

For subclassification of T3 rectal cancer patients into 2 groups based on the extent of mesorectal tumor invasion, we attempted to identify the most clinically relevant cutoff value for extent of mesorectal tumor invasion by focusing on hazard ratios for the first data set. Among the 9 cutoff values (2, 3, 4, 5, 6, 7, 8, 9, 10 mm) examined, 6 mm was identified as the optimal value based on having the smallest P value for different outcomes (Table 1). The 5-year survival rate of patients with mesorectal invasion ≥6 mm was 50% and that of patients with mesorectal invasion <6 mm was 71% ($P < 0.01$)(Fig. 3).

For both type A and B tumors, patients with mesorectal invasion ≥6 mm had significantly poorer survival than those with mesorectal invasion <6 mm. In 51 type A tumor patients, the overall 5-year survival rate was 36% for those with mesorectal invasion ≥6 mm and 74% for those with mesorectal invasion <6 mm ($P = 0.0066$). In 145 type B tumor patients, the overall 5-year survival rate was 53% for those with mesorectal invasion ≥6 mm and 72% for those with mesorectal invasion <6 mm ($P = 0.0332$).

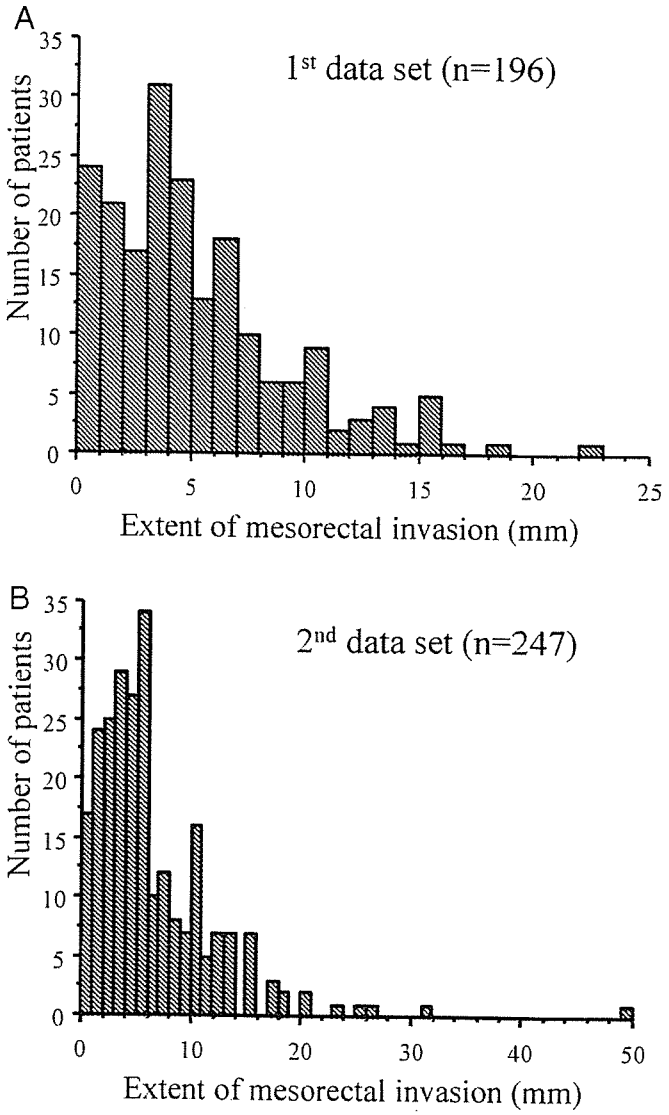

FIGURE 2. Distributions of the extent of mesorectal invasion.

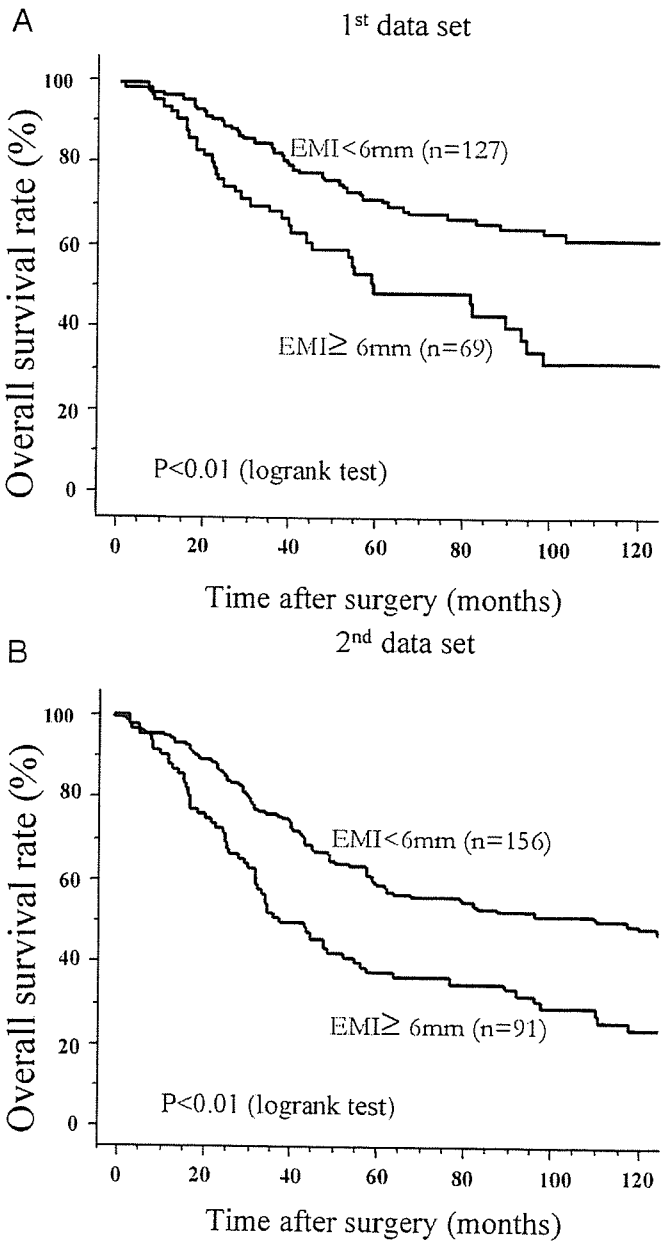

FIGURE 3. Overall survival curves by the extent of mesorectal invasion (EMI).

TABLE 1. Selection of the Optimal Cutoff Value for Extent of Mesorectal Invasion (EMI) Using the First Data Set

Cutoff Value for EMI (mm)	5-Year Survival Rate (no. of patients) Below Cutoff Value	At or Above Cutoff Value	Hazard Ratio	*P*
2	79% (43)	61% (153)	1.7	0.066
3	80% (62)	58% (134)	2.1	0.007
4	75% (90)	56% (106)	1.8	0.009
5	71% (144)	56% (82)	1.6	0.031
6	71% (127)	50% (69)	2.1	0.001
7	70% (145)	48% (51)	2.1	0.002
8	70% (156)	44% (40)	2.1	0.002
9	67% (162)	52% (34)	1.7	0.041
10	67% (169)	45% (27)	1.7	0.083

Distribution of Extent of Mesorectal Invasion (Second Data Set)

The distribution of the extent of mesorectal invasion (in millimeters) for the second data set is shown in Fig. 2. The distribution is similar to that of the first data set. The mean value for extent of mesorectal invasion for data set 2 was 6.1 mm (range, 0.1–50.0 mm).

Cutoff Value for Extent of Mesorectal Invasion (Second Data Set)

When the 6-mm cutoff value was tested on the second data set, patients with mesorectal invasion ≥6 mm had significantly poorer survival than those with mesorectal in-

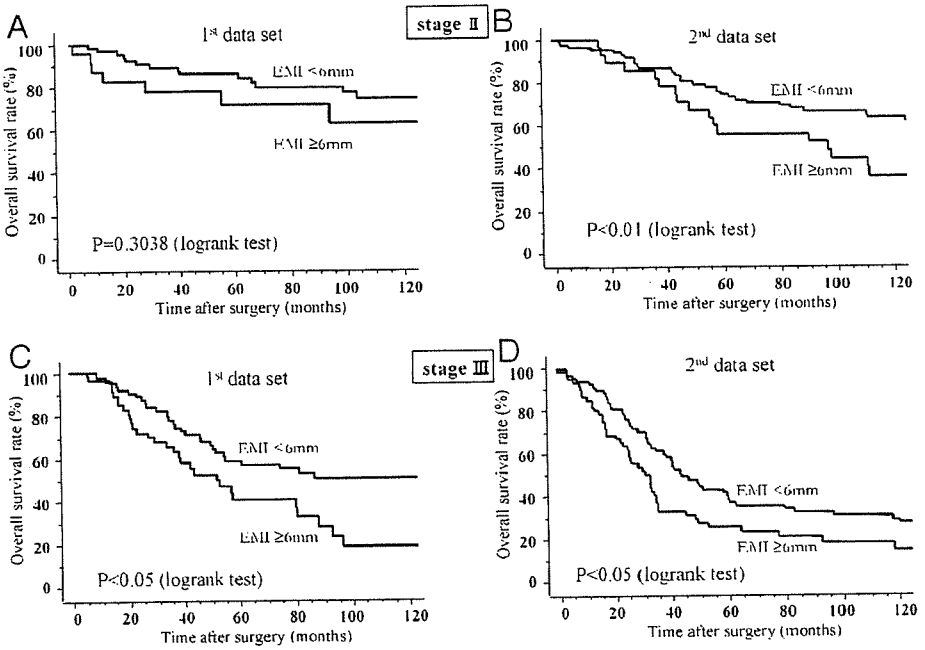

FIGURE 4. Prognostic significance of extent of mesorectal invasion (EMI) by TNM stage.

vasion <6 mm (37% versus 59%, 5-year survival; $P < 0.01$) (Fig. 3).

Prognostic Significance of Extent of Mesorectal Invasion According to TNM Stage (Both Data Sets)

The prognostic significance of the extent of mesorectal invasion according to the TNM stage is shown in Figure 4. Patients with stage II tumors, in the second data set, could be categorized into 2 groups with different prognoses by the extent of mesorectal invasion (52% versus 73%, 5-year survival; $P < 0.01$). Stage III tumor patients could be categorized into 2 groups in both data sets (first data set: 40% versus 58%, 5-year survival; $P < 0.05$; second data set: 27% versus 40%, 5-year survival; $P < 0.05$).

Multivariate Prognostic Analyses (Both Data Sets)

The results of univariate and multivariate prognostic analyses using parameters including extent of mesorectal invasion, tumor size, tumor differentiation, and nodal involvement are presented as Table 2. In both data sets, nodal involvement and extent of mesorectal invasion were identified as independent prognostic factors.

Nodal involvement and extent of mesorectal invasion had almost same impact for poor prognosis in second data set because each hazard ratio and P value were almost same (hazard ratio, 1.8, 1.9; P value: 0.0019, <.0001). Nodal involvement had stronger impact than extent of mesorectal invasion for poor prognosis in first data set (hazard ratio, 2.4, 1.8; P value: 0.0004, 0.0066).

Subclassification for Survival by Number of Risk Factors (Both Data Sets)

Taking nodal involvement and mesorectal invasion ≥6 mm as risk factors, the respective overall 5-year survival rates with none, one, and both of these factors were 84%, 61%, and 38% in the first data set ($P < 0.01$). The model was tested on the second data set and similar prognostic results were obtained (73%; 45%; 27% 5-year survival; $P < 0.01$).

DISCUSSION

Prognoses vary markedly in both stage II and stage III rectal cancer patients. To individualize the selection of optimal adjuvant therapy, subclassification of stages II and III rectal cancer patients based on other reliable prognostic factors may be needed.[14–23] The extent of mesorectal tumor invasion has been shown to be an independent risk factor in some studies.[10–12] However, in those studies, different cutoff values (2 mm or 4 mm) for the extent of mesorectal tumor invasion were used without detailed explanations as to how these cutoff values were determined.[10,12] Therefore, we attempted to identify the most clinically relevant cutoff value using our first data set. We examined 9 cutoff values (2, 3, 4, 5, 6, 7, 8, 9, and 10 mm), calculating a hazard ratio for each using the Cox proportional hazards model. Based on the results of this analysis, we concluded that 6 mm is the most useful cutoff value because the hazard ratio of 2.1 represented a sharp increase from the hazard ratio of 1.5 at 5 mm and the P value at 6 mm (0.0011) was the most significant of the 9 values examined.

Cawthorn et al used a 4-mm cutoff value in assessing the distribution of the extent of mesorectal spread in 167 patients.[12] However, they did not use a statistical analysis to determine this cutoff value, and their study included 50 T2 patients (absence of mesorectal invasion). Willett et al characterized tumors as showing minimum (through the MP with <2 mm invasion into perirectal fat), moderate (2–8 mm into perirectal fat), or extensive (≥8 mm into perirectal fat) invasion.[10] None of these earlier studies presented statistical analyses of their data. Steel et al divided tumors into mini-

TABLE 2. Univariate and Multivariate Prognostic Analyses

Variables	Univariate Analysis			Multivariate Analysis (stepwise method)	
		Prognosis			
	Patient No.	5-Year Survival (%)	*P*	HR	*P*
First data set					
Maximum tumor diameter					
<5 cm	74	67	0.3078	Not selected	
≥5 cm	122	63			
Tumor differentiation					
Well	60	79	0.2176	Not selected	
Moderate	124	58			
Others	12	63			
Extent of mesorectal invasion					
<6 mm	127	72	0.0008	1.8	0.0066
≥6 mm	69	50			
Lymph node involvement					
Negative	90	83	<.0001	2.4	0.0004
Positive	106	50			
Second data set					
Maximum tumor diameter					
<5 cm	100	59	0.0134	Not selected	
≥5 cm	147	39			
Tumor differentiation					
Well	40	67	<.0001	1.6	0.0034
Moderate	161	56			
Others	46	20			
Extent of mesorectal invasion					
<6 mm	156	59	<.0001	1.9	<0.0001
≥6 mm	91	37			
Lymph node involvement					
Negative	113	69	<.0001	1.8	0.0019
Positive	134	34			

mally invasive T3 (defined as invasion evident on microscopic examination only) and advanced T3 (defined as invasion discernible on macroscopic examination of the transected tumor).[11] They did not measure actual values and could not establish a cutoff value. Although we determined the best cutoff value to be 6 mm for the first data set of 196 patients, this value also fit the second data set of 247 patients well.

Cawthorn et al demonstrated poorer outcomes (overall 5-year survival rate) with extensive mesorectal invasion (25%) as compared with slight invasion (55%).[12] Willett et al, in a study of 117 patients, found a significant difference in local recurrence rates between patients with mesorectal invasion of ≥2 mm and those in whom invasion was less than 2 mm.[10] Steel et al demonstrated the depth of invasion into the

TABLE 3. Studies on Prognostic Significance of Mesorectal Invasion in Rectal Cancer Patients

Reference	Year	Cutoff Value	Local Recurrence	Overall Survival	No. of Patients	Patient Stage
Cawthorn et al	1990	4 mm	Not analyzed	Significant	167*	I, II, III (T2,T3)
Willett et al	1999	2 mm	Significant	Not analyzed	117	II (T3N0)
Steel et al	2002	Macroscopic vs. microscopic	Significant	Not analyzed	222	II, III (T3)
Present study	First data set	6 mm	Not significant	Significant	196	II, III (T3)
	Second data set	6 mm	Not analyzed	Significant	247	II, III (T3)

*Including 45 patients who received palliative operations.

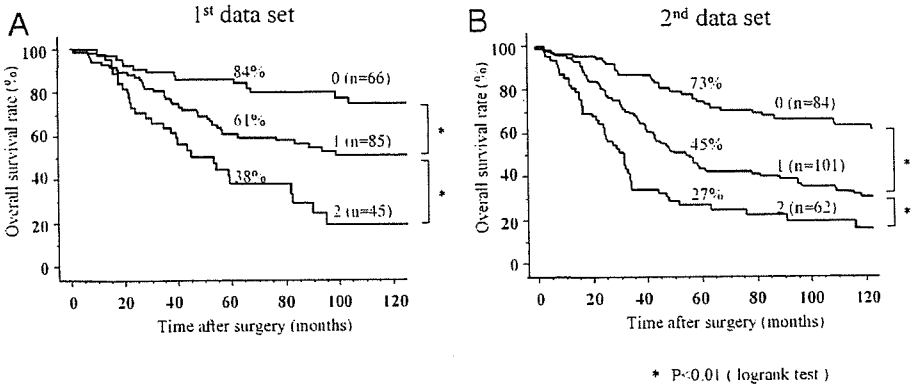

FIGURE 5. Overall survival curves according to the number of risk factors: lymph node involvement, extent of mesorectal invasion ≥6 mm.

mesorectum (macroscopic versus microscopic) to apparently be an independent prognostic factor for local recurrence in a cohort of 222 patients undergoing surgical resection.[11] These 3 studies are summarized in Table 3.

In 2 of the 3 studies, the extent of mesorectal invasion was shown to be a significant risk factor for local recurrence while having no impact on overall survival. In our first data set, the local recurrence rate is significantly higher for those with marked as compared with slight tumor invasion when the cutoff value is 7 mm or 8 mm. However, when the cutoff value is 6 mm, the value that is most significant statistically in terms of survival, the local recurrence rate does not differ markedly according the extent of mesorectal invasion. According to the recurrence-free survival curve in the first data set, patients with mesorectal invasion ≥6 mm have significantly lower recurrence-free survival rate than those with mesorectal invasion <6 mm (5-year recurrence-free survival rate: 55% versus 69%; $P < 0.05$). So, total recurrence rates significantly differ because of the extent of mesorectal invasion. Many previous studies found that the circumferential resection margin is very important for prediction of local recurrence.[24–29] The extent of mesorectal invasion appears to be more meaningful for survival than for local recurrence, however.

Only Cawthorn et al[12] found the extent of mesorectal invasion to be an independent factor predicting the overall survival rate by multivariate analysis. However, their study included 50 T2 rectal cancer patients and 45 patients who underwent palliative surgery. Thus, using 2 data sets comprising 196 and 247 patients, the present study more clearly shows the extent of mesorectal tumor invasion to be an independent factor predicting overall survival of T3 rectal cancer patients after curative surgery. Indeed, for both stage II and stage III tumors, patients in the second data set could be categorized into 2 groups with different prognoses based on the extent of mesorectal invasion. We examined the extent of mesorectal tumor invasion (cutoff value = 6 mm), tumor size, tumor differentiation, and nodal involvement in multivariate analysis using the Cox stepwise regression model. Extent of mesorectal invasion ≥6 mm and nodal involvement were found to be independent prognostic factors in T3 rectal cancer patients. Patients with T3 rectal cancer could clearly be categorized into 3 groups based on these risk factors (overall 5-year survival rates with none, one, and both risk factors: 84%, 61%, and 38%, respectively; $P < 0.01$ for the first data set, 73%, 45%, and 27%, respectively; $P < 0.01$ for the second data set) (Fig. 5).

CONCLUSION

The extent of mesorectal tumor invasion, in addition to nodal involvement, is useful for subclassifying T3 rectal cancer patients. These 2 factors were statistically independent of each other. Adjuvant therapies may not be necessary for T3 rectal cancer patients with none of these risk factors. Moreover, determination of criteria for neoadjuvant therapy may be possible by the use of ultrasound in current day staging to determine depth on invasion.

REFERENCES

1. Phillips RK, Hittinger R, Blesovsky L, et al. Local recurrence following 'curative' surgery for large bowel cancer: I. The overall picture. *Br J Surg.* 1984;71:12–16.
2. Heald RJ, Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. *Lancet.* 1986;1:1479–1482.
3. Pahlman L, Glimelius B. Local recurrences after surgical treatment for rectal carcinoma. *Acta Chir Scand.* 1984;150:331–335.
4. Olson RM, Perencevich NP, Malcolm AW, et al. Patterns of recurrence following curative resection of adenocarcinoma of the colon and rectum. *Cancer.* 1980;45:2969–2974.
5. Colon and rectum. In: *AJCC Cancer Staging Manual,* 5th ed. Philadelphia: Lippincott Raven, 1997:83–90.
6. Vigliotti A, Rich TA, Romsdahl M, et al. Postoperative adjuvant radiotherapy for adenocarcinoma of the rectum and rectosigmoid. *Int J Radiat Oncol Biol Phys.* 1987;13:999–1006.
7. Schild SE, Martenson JA Jr, Gunderson LL, et al. Postoperative adjuvant therapy of rectal cancer: an analysis of disease control, survival and prognostic factors. *Int J Radiat Oncol Biol Phys.* 1989;17:55–62.
8. Willett CG, Tepper JE, Kaufman DS, et al. Adjuvant postoperative radiation therapy for rectal adenocarcinoma. *Am J Clin Oncol.* 1992;15:371–375.
9. NIH Consensus Conference. Adjuvant therapy for patients with colorectal cancer. *JAMA.* 1990;264:1444–1450.
10. Willett CG, Badizadegan K, Ancukiewicz M, et al. Prognostic factors in stage T3N0 rectal cancer: do all patients require postoperative pelvic irradiation and chemotherapy? *Dis Colon Rectum.* 1999;42:167–173.
11. Steel MC, Woods R, Mackay JM, et al. Extent of mesorectal invasion is a prognostic indicator in T3 rectal carcinoma. *Aust NZ J Surg.* 2002;72:483–487.
12. Cawthorn SJ, Parums DV, Gibbs NM, et al. Extent of mesorectal spread and involvement of lateral resection margin as prognostic factors after surgery for rectal cancer. *Lancet.* 1990;335:1055–1059.
13. Greene FL, et al, eds. *AJCC Cancer Staging Manual,* 6th ed. New York: Springer-Verlag, 2002.
14. Chung DC. Molecular prognostic markers and colorectal cancer: the search goes on. *Gastroenterology.* 1998;114:1330–1332.

15. Hayashi N, Ito I, Yanagisawa A, et al. Genetic diagnosis of lymph-node metastasis in colorectal cancer. *Lancet*. 1995;345:1257–1259.
16. Gervaz P, Cerottini JP, Bouzourene H, et al. Comparison of microsatellite instability and chromosomal instability in predicting survival of patients with T3N0 colorectal cancer. *Surgery*. 2002;131:190–197.
17. Knutsen A, Adell G, Sun XF. Survivin expression is an independent prognostic factor in rectal cancer patients with and without preoperative radiotherapy. *Int J Radiat Oncol Biol Phys*. 2004;60:149–155.
18. Ueno H, Price AB, Wilkinson KH, et al. A new prognostic staging system for rectal cancer. *Ann Surg*. 2004;240:832–839.
19. Tanaka M, Hashiguchi Y, Ueno H, et al. Tumor budding at the invasive margin can predict patients at high risk of recurrence after curative surgery for stage II, T3 colon cancer. *Dis Colon Rectum*. 2003;46:1054–1059.
20. Compton CC. Pathologic prognostic factors in the recurrence of rectal cancer. *Clin Colorectal Cancer*. 2002;2:149–160.
21. Morodomi T, Isomoto H, Shirouzu K, et al. An index for estimating the probability of lymph node metastasis in rectal cancers: lymph node metastasis and the histopathology of actively invasive regions of cancer. *Cancer*. 1989;63:539–543.
22. Ueno H, Mochizuki H, Shinto E, et al. Histologic indices in biopsy specimens for estimating the probability of extended local spread in patients with rectal carcinoma. *Cancer*. 2002;94:2882–2891.
23. Ueno H, Murphy J, Jass JR, et al. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology*. 2002;40:127–132.
24. Quirke P, Durdey P, Dixon MF, et al. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection: histopathological study of lateral tumour spread and surgical excision. *Lancet*. 1986;2:996–999.
25. Wibe A, Rendedal PR, Svensson E, et al. Prognostic significance of the circumferential resection margin following total mesorectal excision for rectal cancer. *Br J Surg*. 2002;89:327–334.
26. Nagtegaal ID, Marijnen CA, Kranenbarg EK, et al. Circumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit. *Am J Surg Pathol*. 2002;26:350–357.
27. Dexter SP, Sue-Ling H, McMahon MJ, et al. Circumferential resection margin involvement: an independent predictor of survival following surgery for oesophageal cancer. *Gut*. 2001;48:667–670.
28. Hall NR, Finan PJ, al-Jaberi T, et al. Circumferential margin involvement after mesorectal excision of rectal cancer with curative intent: predictor of survival but not local recurrence? *Dis Colon Rectum*. 1998;41:979–983.
29. Adam IJ, Mohamdee MO, Martin IG, et al. Role of circumferential margin involvement in the local recurrence of rectal cancer. *Lancet*. 1994;344:707–711.

Diseases of the Colon & Rectum

Tumor Buds Show Reduced Expression of Laminin-5 Gamma 2 Chain in DNA Mismatch Repair Deficient Colorectal Cancer

Eiji Shinto, M.D.,[1,3] Kristi Baker, B.Sc.,[1] Hitoshi Tsuda, M.D.,[3] Hidetaka Mochizuki, M.D.,[4] Hideki Ueno, M.D.,[4] Osamu Matsubara, M.D.,[3] William D. Foulkes, M.D.,[2] Jeremy R. Jass, M.D.[1]

[1] *Department of Pathology, McGill University, Montreal, Canada*
[2] *Department of Human Genetics, McGill University, Montreal, Canada*
[3] *Department of Pathology II, National Defense Medical College, Tokorozawa, Japan*
[4] *Department of Surgery I, National Defense Medical College, Tokorozawa, Japan*

PURPOSE: Tumor budding at the invasive margin of colorectal cancer is an important adverse prognostic factor. The subset of colorectal cancer that is deficient in DNA mismatch repair has been associated with a good prognosis. It is hypothesized that tumor budding in this subset may lack biologic aggressiveness because it is not associated with aberrant expression of the independent prognostic factor, laminin-5 gamma 2. METHODS: Eighty colorectal cancers with high-grade tumor budding were studied, including nine sporadic colorectal cancers with immunohistochemical loss of expression of MLH1 (MLH1(−)), seven colorectal cancers from patients with hereditary nonpolyposis colorectal cancer, and 64 sporadic colorectal cancers expressing both MLH1 and MSH2 (MLH1(+)). Two regulatory mechanisms for laminin-5 gamma 2 expression were explored, including aberrant nuclear expression of β-catenin by immunohistochemistry and promoter methylation of laminin-5 gamma 2 by methylation-specific polymerase chain reaction. RESULTS: Only three of nine MLH1(−) colorectal cancers showed expression of laminin-5 gamma 2 compared with 46 of 64 MLH1(+) colorectal cancers (P = 0.05). Only two of seven hereditary nonpolyposis colorectal cancers expressed laminin-5 gamma 2 compared with MLH1(+) colorectal cancers (P = 0.03). Expression of nuclear β-catenin was more frequent (58 percent) in MLH1(+) colorectal cancers compared with MLH1(−) colorectal cancers (11 percent, P = 0.01). Methylation of laminin-5 gamma 2 was found in 5 of 38 (13 percent) cases but did not differ among colorectal cancer subsets. Four of five colorectal cancers with methylation of laminin-5 gamma 2 were scored as negative for laminin-5 gamma 2 by immunohistochemistry. CONCLUSIONS: The reduced expression of laminin-5 gamma 2 in colorectal cancers with deficient DNA mismatch repair may underlie a variant of tumor budding that is relatively nonaggressive. [Key words: Colorectal cancer; Tumor budding; MLH1; Laminin-5 gamma 2; Promoter methylation]

Morphologic features at the invasive front of colorectal cancer (CRC) are known to be associated with tumor aggressiveness. Two such features are tumor budding or dedifferentiation based on high-power microscopic examination[1,2] and growth pattern (infiltrating or expanding) based on low-power examination.[3] Importantly, tumor budding has been shown to be a reproducible prognostic factor that is independent of other features, including depth of invasion, lymph node spread, and distant metastases.[4,5] Tumor budding has been defined as the presence of isolated single cells

Supported by a grant from the Canadian Institutes of Health Research (grant no. MOP-67206).

Correspondence to: Eiji Shinto, M.D., Department of Pathology II, National Defense Medical College, 3-2, Namiki Tokorozawa, Saitama 359-8513, Japan, e-mail: eiji-c@zf6.so-net.ne.jp

Dis Colon Rectum 2006; 49: 1193–1202
DOI: 10.1007/s10350-006-0568-4

Published online: 15 June 2006

or small cell clusters (up to 4 cells) scattered in the stroma at the invasive tumor margin. Budding represents the summation of two features of malignancy: cellular discohesion, and active invasion by single or small groups of tumor cells.

Some dedifferentiated cancer cells (buds) are closely accompanied by showers of cytoplasmic fragments. These fragments were shown through three-dimensional reconstruction of serial sections to be cytoplasmic podia in direct continuity with budding cell bodies.[6] It was shown that the number of "cytoplasmic pseudofragments" increased with the expression of key components of the Wnt signaling pathway,[6] including nuclear expression of the transcriptional activator β-catenin and the downstream protein, laminin-5 gamma 2 chain (LN-5γ2).[7,8] Cancer cells in culture with Wnt signaling pathway disruption show cytoplasmic podia that allow the cell to contact surrounding structures for the purposes of migration.[9,10] We considered that cytoplasmic pseudofragments may serve to distinguish actively invading tumor buds with development of cytoplasmic podia and driven by Wnt signaling pathway activation from "passive" buds representing simple cell discohesion at the invasive tumor margin.[6]

LN-5γ2 chain has been shown to be expressed with high frequency in the cytoplasm of buds that are invading surrounding stromal tissues.[11–13] LN-5 is composed of α3, β3, and γ2 chains, and the presence of the γ2 chain is a characteristic feature of LN-5, with no other laminins having this chain.[14–17] A tight correlation between the grade of LN-5γ2 expression and tumor dedifferentiation has been shown,[18] and increasing numbers of reports have demonstrated a correlation between LN-5γ2 overexpression and the clinical behavior of cancers, including CRC.[18–20] A multivariate analysis of a previously published series of CRC with high-grade tumor budding[18] showed expression of LN-5γ2 to be an independent prognostic factor (hazard ratio, 3.3; 95 percent confidence interval (CI), 1.3–8.1; $P = 0.01$; raw data not shown).

Two mechanisms of LN-5γ2 regulation have been identified. With respect to its up-regulation, LN-5γ2 is positioned downstream of the Wnt signaling pathway, and its expression occurs as a consequence of nuclear translocation of the transcriptional activator β-catenin,[8] which binds with a member of the TCF/LEF family, which activates the transcription of multiple target genes by binding to their promoter sequences.[7,21] Down-regulation of LN-5γ2 by methylation of its promoter region was recently reported by Sathyanarayana *et al.*[22–25] in tumors of lung, breast, prostate, and bladder. They showed frequent epigenetic silencing of LN-5 encoding genes, including α3, β3, and γ2, in prostate cancer and correlated these observations with clinicopathologic features of poor prognosis.[24] However, methylation of *LN-5γ2* has not been reported in CRC. Because LN-5γ2 expression is a poor prognostic indicator in CRC, methylation of *LN-5γ2* might serve as a marker of reduced aggressiveness, in contrast to previous reports on other malignancies.

Microsatellite instability (MSI) is a tumor phenotype associated with loss of DNA mismatch repair proficiency. High-level microsatellite instability (MSI-H) CRC occurs in hereditary nonpolyposis colorectal cancer (HNPCC) in which there is germline mutation of a DNA mismatch repair gene, usually *MLH1* or *MSH2*. Sporadic MSI-H CRC is associated with methylation of the *MLH1* promoter region.[26] A recent large and comprehensive study comparing MSI testing and immunohistochemistry (IHC) for detection of MLH1 and MSH2 showed that IHC detection was 100 percent specific for MSI status in CRC.[27]

MSI-H CRCs are characterized by the microscopic findings of expanding or circumscribed growth, prominent lymphocytic infiltration, and larger proportion of poorly differentiated or mucinous cancers.[26,28,29] Additionally, IHC reveals a characteristic expression pattern of mucin core protein in which MUC2 and MUC5AC are more abundant in MSI-H cancers than in microsatellite stable (MSS) cancers.[30] With respect to pathologic features at the invasive front, sporadic MSI-H CRC has a low frequency of both tumor budding and aberrant nuclear expression of β-catenin.[29,31,32] In HNPCC, a comparatively low frequency of tumor budding is observed despite the finding of frequent nuclear accumulation of β-catenin.[29,31] However, these reports did not investigate LN-5γ2 expression according to CRC subsets. The findings in sporadic MSI-H CRC suggest that a normally regulated Wnt signaling pathway may modify the biologic significance of dedifferentiation in MSI-H CRC and thereby explain the improved prognosis of patients with this type of CRC.[33] Sporadic MSI-H CRC typically shows the CpG island methylator phenotype (CIMP). Reduced aggression could, therefore, be explained on the basis of *LN-5γ2* promoter methylation. An evaluation of LN-5γ2 expression, presence of cytoplasmic pseudofragments, and regulatory mechanisms underlying LN-

5γ2 expression in HNPCC may explain why budding is infrequent despite the nuclear accumulation of β-catenin.

Although tumor budding is a clinically useful adverse prognostic factor in CRC, the process may be more heterogeneous than previously recognized. It is important to clarify any differences in budding characteristics and to identify the underlying mechanisms. It is known that there are many differences in the biologic and pathologic features of MSI-H and MSS CRCs, and it is conceivable that the molecular and phenotypic diversity across these subtypes is relevant to the mechanism of tumor budding. In this study, we stratified 80 CRCs according to MLH1 and MSH2 expression status and assessed the potential aggressiveness of tumor buds by using LN-5γ2 expression and cytoplasmic pseudofragments as respective functional and structural biomarkers of tumor aggression. We also investigated the roles of β-catenin nuclear expression and *LN-5γ2* promoter methylation in the regulation of LN-5γ2 expression.

METHODS

The study material included 73 sporadic CRCs that were surgically resected from 1983 to 1994 at McGill University-related hospitals and 7 CRCs from patients with HNPCC and a proven germline mutation in *MLH1* or *MSH2*. In this article, HNPCC indicates HNPCC-Lynch syndrome diagnosed on the basis of family history and confirmed by the demonstration of a germline mutation in DNA mismatch repair gene. To be eligible for inclusion in the study, all tumors had to show the feature of high-grade tumor budding. The study was approved by the Institutional Review Board of the Faculty of Medicine of McGill University. Information relating to patient age, gender, tumor site, size, and stage was obtained from the pathologic and clinical records. There were 32 male and 48 female patients with a mean age of 71 (range, 34–96) years. Cancers were located in the proximal colon (35 cases), distal colon or rectum (41 cases), or site unknown (4 cases). All CRCs showed at least submucosal invasion and ranged in size from 9 to 100 (mean size, 44) mm. Tumors were staged according to the tumor-node-metastasis (TNM) staging system.[34] Tumor type was determined according to the criteria of the World Health Organization.[35] Growth pattern and lymphocytic infiltration at the advancing tumor margin were evaluated according to the criteria of Jass *et al.*[3] There was missing information with respect to tumor size (9 cases), tumor depth (2 cases), and lymph node metastasis (3 cases).

Immunohistochemistry

All cases were immunostained for MLH1 (BD Pharmingen™ purified anti-human MLH1 (IHC); clone G168-15; dilution 1:100; BD Biosciences Pharmingen, San Jose, CA), MSH2 (BD Pharmingen™ purified anti-human MSH-2; clone G219-1129; dilution 1:200; BD Biosciences Pharmingen), MUC2 (clone Ccp58; dilution 100 μg/100 ml; MONOSAN®, Uden, The Netherlands), MUC5AC (clone 45M1; dilution 100 μg/100 ml; MONOSAN®), β-catenin (monoclonal mouse anti-human beta-catenin; clone β-Catenin-1; dilution 1:200; DakoCytomation, Grostrup, Denmark), LN-5γ2 (monoclonal mouse anti-human laminin-5 gamma-2 chain; clone 4G1; dilution 1:50; DakoCytomation), and broad spectrum cytokeratin (monoclonal mouse anti-human cytokeratin; clone MNF116; dilution 1:50; DakoCytomation). Four-micrometer-thick sections were cut from representative blocks and mounted on silane-coated glass slides. After dewaxing and rehydration to dH_2O, sections for immunostaining except MUC5AC were subject to heat antigen retrieval in a microwave oven (1200 W, 15 minutes) in 0.001 mol/l of EDTA acid, pH 8.0 for MLH1, MSH2, and MUC2, in 0.01-mol/l citrate buffer, pH 7.0 for β-catenin or in purchased target retrieval solution, pH 9.0 (S2367, DakoCytomation) for cytokeratin and LN-5γ2. After cooling, nonspecific antibody binding was inhibited by incubating the sections in 4 percent nonfat milk. Endogenous peroxidase activity was blocked by using 0.5 percent H_2O_2. After transfer to a humidified chamber, the sections were incubated with 10 percent normal goat serum (X0501, DakoCytomation) for 20 minutes, and incubated with primary antibody at room temperature for 1 hour. Subsequently, the sections were incubated with peroxidase labeled polymer (K4005, EnVision™ + System-HRP(AEC); DakoCytomation) for 30 minutes at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole+ substrate-chromogen (K4005, EnVision™ + System-HRP(AEC)) for 30 minutes, and counterstained lightly with Gill's hematoxylin.

Immunostaining for MLH1, MSH2, and β-catenin was assessed in the nucleus while staining for MUC2,

MUC5AC, and LN-5γ2 was assessed in the cytoplasm of cancer cells. The extent of β-catenin and LN-5γ2 expression was based on the percentage of immunopositive cells among cancer cells at the invasive front of the tumor. Scoring for MUC2 and MUC5AC were based on the percentages of immunopositive cells among all cancer cells included in the section. Immunoreactivity was evaluated semiquantatively and divided into immunopositive or immunonegative based on the following cutoff values for the percentage of cells with immunopositivity: MUC2, >50 percent; MUC5AC, >5 percent; β-catenin, >10 percent; LN-5γ2, >20 percent. With respect to MLH1 and MSH2, loss of expression was recorded when nuclear staining was absent from all malignant cells but preserved in normal epithelial and stroma cells. Interobserver reproducibility (ES and KB) with respect to MLH1 and β-catenin was assessed by means of kappa (κ) test.[36] Based on the criteria of Landis and Koch,[37] κ values were assigned to a scale of strength of agreement. When κ values were < 0, 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8, and 0.81 to 1, the strength of agreement was judged as poor, slight, fair, moderate, substantial, and almost perfect, respectively.

Tumor Budding and Cytoplasmic Pseudofragments

Tumor budding was assessed as described previously.[2] Briefly, a focus of tumor budding was defined as a single isolated cancer cell or a cluster composed of up to four cancer cells (Fig. 1). To determine eligible cases with high-grade tumor budding, CRCs were initially divided into two groups according to the number of tumor budding foci in the densest field using a ×20 objective lens. Counts of 0 to 9 were termed low grade and these cases were excluded. The remaining 80 CRCs had counts of 10+ and were regarded as having high-grade budding. High-grade budding was further divided into counts of 10 to 19 (moderate) and 20+ (severe).

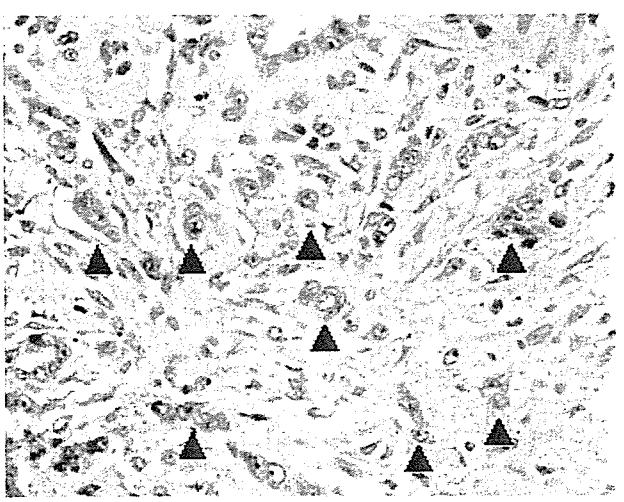

Figure 1. Characteristic high-power microscopic appearance of tumor budding (arrowheads) (an isolated single cell or a cluster of up to 4 cancer cells) at the invasive front (hematoxylin and eosin staining).

Figure 2. Multiple cytoplasmic pseudofragments (arrowheads) (round cytokeratin-immunostained spots without nucleus) are demonstrated around tumor budding foci (cytokeratin immunostaining).

Using cytokeratin-immunostained sections, small nonnucleated cytoplasmic pseudofragments were detected around tumor budding foci at the invasive tumor margin (Fig. 2).[6] To be counted as present, fragments had to be at least 2 μm in diameter, nonnucleated, lacking in evidence of nuclear fragmentation, uniformly positive for cytokeratin, smoothly contoured, and free of surrounding inflammatory cells. Scores were based on the highest number of fragments in a ×20 objective lens field. Low-grade cancers had 0 to 9 fragments, and high-grade cancers had 10+ fragments.

DNA Extract and Methylation-specific Polymerase Chain Reaction

Three paraffin sections, 8-μm-thick, were retrieved from 40 cases (24 MLH1-expressing, 9 MLH1-loss sporadic CRCs, and 7 HNPCC) for manual microdissection to select areas containing tumors. DNA was extracted by use of the QIAamp® DNA Mini kit (Qiagen, Valencia, CA) after digested with proteinase

K solution. Genomic DNA was modified with sodium bisulfite as described previously.[38] Methylation-specific polymerase chain reaction (MSP) exploits the effect of sodium bisulfite on DNA, which efficiently converts unmethylated cytosine to uracil but leaves methylated cytosine unchanged. This allows methylation status to be determined by use of specific primers. The bisulfite-modified DNA samples were amplified by primers specific for methylated or unmethylated sequences. Primer sequences for methylated (M) and unmethylated (U) were based on previous published reports by Sathyanarayana *et al.*[22] or Miyakura *et al.*[39,40] (*MLH1* region D, forward 5′-ataggaagagcggatagc-3′ and reverse 5′-caatacctcg tactcacg-3′ (M), forward 5′-attaataggaagagtggatagt-3′ and reverse 5′-atcacctcaatacctcatac-3′ (U); *LN-5γ2* forward 5′-aggtgtgcgttttttcgttgc -3′ and reverse 5′-taca aaaatcgctacccgacg-3′ (M), forward 5′-ttaggtgtgtgtt tttttgttgt -3′ and reverse 5′-actacaaaaatcactacccaaca-3′ (U)), and we followed their polymerase chain reaction (PCR) procedures.[22,39] Positive control (CpGenome™ Universal Methylated DNA, Chemicon, Temecula, CA) and negative control (distilled water without DNA template) were included in each amplification process. PCR products were analyzed in 10-percent acrylamide gel stained by SYBR® Gold nucleic acid gel stain (Molecular probes, Eugene, OR). Methylation status was established by the presence of a distinct band of methylated or unmethylated product. When both bands were detected, it was assumed that some normal tissue was included in the sample, and the cancer was assigned to the methylation-positive group. Samples that did not show either band were excluded from the study. All experiments were performed in duplicate. If a discrepancy was found between two experiments, a third and deciding test was performed (Fig. 3).

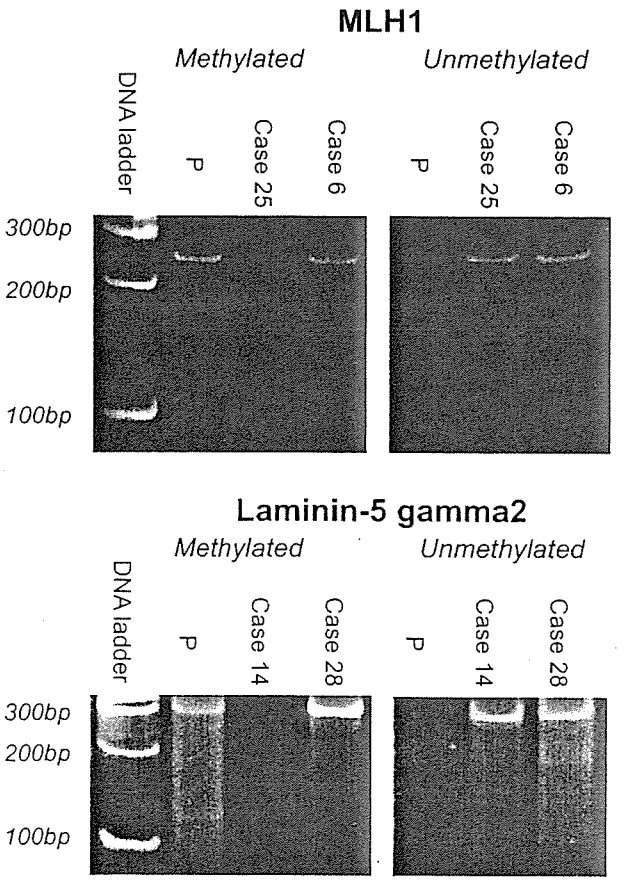

Figure 3. Methylation-specific PCR (MSP) analyses of the *MLH1* promoter region D and *LN-5γ2* promoter region. Primers were designed for unmethylated or methylated DNA sequences (*MLH1* promoter region D, from −286 to −53; *LN-5γ2*, from −303 to −32) as described previously. P = universally methylated positive control.

Statistical Analysis

Comparisons between groups were performed by using the chi-squared test or Fisher's exact method. Differences at $P < 0.05$ were considered significant.

RESULTS

Clinicopathologic Features According to MLH1/MSH2 Status

The levels of interobserver agreement were perfect for the scoring of MLH1 immunostaining ($\kappa = 1$; concordance rate, 100 percent) and substantial for β-catenin ($\kappa = 0.72$; concordance rate, 88 percent). All cancers expressed MSH2 except one in HNPCC, which showed loss of MSH2 and expression of MLH1. This CRC was known to be derived from a subject with a germline mutation of MSH2. Clinicopathologic features of three CRC groups are shown in Table 1. Comparisons between MLH1-expressing and MLH1-loss sporadic CRCs demonstrated that loss of MLH1 expression was associated with location in proximal colon ($P < 0.001$), mucinous/signet-ring cell carcinoma ($P < 0.001$), medullary carcinoma ($P < 0.001$), MUC2 expression ($P = 0.01$), MUC5AC expression ($P = 0.003$), expanding growth pattern ($P = 0.03$), peritumoral lymphocytic infiltration ($P = 0.002$), and negatively associated with high-grade cytoplasmic pseudofragments ($P = 0.003$) and LN-5γ2 expression ($P = 0.05$). There was a trend toward a positive relationship with female gender ($P = 0.08$).

Table 1.
Clinicopathologic Features in the Three Subsets of CRC

Variable	MLH1(+) (n = 64)	MLH1(−) (n = 9)	*P* Value[a]	HNPCC (n = 7)	*P* Value[a]
Age ≥70 years	41/64 (64%)	8/9 (89%)	0.26	1/7 (14%)	0.016
Female gender	36/64 (56%)	8/9 (89%)	0.078	4/7 (57%)	0.99
Right-sided	23/62 (37%)	9/9 (100%)	0.0004	3/5 (60%)	0.37
Maximum diameter > 50 mm	23/58 (40%)	4/8 (50%)	0.71	3/5 (60%)	0.64
Mucinous/signet-ring cell carcinoma > 20%	7/64 (11%)	4/9 (44%)	0.025	2/7 (29%)	0.21
Medullary carcinoma > 20%	1/64 (2%)	5/9 (56%)	0.0001	4/7 (57%)	0.0002
Positive MUC2 expression	9/64 (14%)	5/9 (56%)	0.011	3/7 (43%)	0.089
Positive MUC5AC expression	6/64 (9%)	5/9 (56%)	0.0028	2/7 (29%)	0.18
Tumor depth, T4	11/64 (17%)	2/9 (22%)	0.66	1/5 (20%)	0.99
Positive node metastasis	36/64 (56%)	2/8 (25%)	0.14	3/5 (60%)	0.99
Positive vessel invasion	49/64 (77%)	5/9 (56%)	0.23	5/7 (71%)	0.7
Infiltrating growth pattern	35/64 (55%)	1/9 (11%)	0.028	2/7 (29%)	0.25
Peritumoral lymphocytic infiltration present	9/64 (14%)	6/9 (67%)	0.0017	3/7 (43%)	0.089
Severe tumor budding	29/64 (45%)	3/9 (33%)	0.72	3/7 (43%)	0.99
High-grade cytoplasmic pseudofragments	48/64 (75%)	2/9 (22%)	0.0034	2/7 (29%)	0.021
Positive laminin-5 gamma 2 expression	46/64 (72%)	3/9 (33%)	0.051	2/7 (29%)	0.032

CRC = colorectal cancer; HNPCC = hereditary nonpolyposis colorectal cancer.
[a]*vs.* MLH1(+) cases.

Comparisons between sporadic CRCs expressing MLH1 and HNPCC revealed that CRCs in HNPCC were positively associated with lower age ($P = 0.02$), medullary carcinoma ($P < 0.001$), and negatively associated with LN-5γ2 expression ($P = 0.03$) and high-grade cytoplasmic pseudofragments ($P = 0.02$). Additionally, CRCs in HNPCC showed a trend toward MUC2 expression ($P = 0.09$) and peritumoral lymphocytic infiltration ($P = 0.09$).

MLH1 methylation status was established by using the MSP technique. Eight of 9 MLH1-loss sporadic CRCs and 1 case among 24 MLH1-expressing sporadic CRCs showed a methylated band. Twenty-three MLH1-expressing cases and six of seven HNPCC were assigned as unmethylated. There was insufficient DNA for assessing *MLH1* methylation status in two cases.

Markers Related to LN-5γ2 Expression

Possible relationships between 1) LN-5γ2 up-regulation and nuclear β-catenin expression, and 2) LN-5γ2 down-regulation and *LN-5γ2* promoter methylation were assessed within the three subsets of CRC, comparing sporadic MLH1-expressing CRCs first with sporadic MLH1-loss CRCs and then with HNPCC tumors (Table 2). In sporadic MLH1-loss CRCs, β-catenin nuclear expression (11 percent (1/9) and LN-5γ2 expression (33 percent (3/9)) were both infrequent, and one tumor showed expression of

Table 2.
Interactions Between β-catenin Expression, *LN-5γ2* Methylation and Expression of LN5γ2 in Three Subsets of CRC

Variable	Total (n = 80)	MLH1(+) (n = 64)	MLH1(−) (n = 9)	*P* Value[a]	HNPCC (n = 7)	*P*-Value[a]
Positive β-catenin expression	40/80 (50%)	37/64 (58%)	1/9 (11%)	0.012	2/7 (29%)	0.23
Positive LN-5γ2 expression with β-catenin expression	28/80 (35%)	27/64 (42%)	1/9 (11%)	0.14	0/7 (0%)	0.039
Positive *LN-5γ2* promoter methylation	5/38 (13%)	3/24 (13%)	0/8 (0%)	0.54	2/6 (33%)	0.25
Negative LN-5γ2 expression with promoter methylation	4/38 (11%)	2/24 (8%)	0/8 (0%)	0.99	2/6 (33%)	0.17

LN-5γ2 = laminin-5 gamma 2; CRC = colorectal cancer; HNPCC = hereditary nonpolyposis colorectal cancer.
[a]*vs.* MLH1(+) cases.

both markers. In HNPCC, β-catenin nuclear expression may be more frequent than in sporadic MLH1-loss (29 percent (2/7)) but was not associated with LN-5γ2 expression. LN-5γ2 overexpression in association with β-catenin nuclear expression occurred less frequently in HNPCC (0 percent (0/7)) than in MLH1-expressing sporadic CRCs (42 percent (27/64); P = 0.04). *LN-5γ2* promoter methylation was demonstrated in only 5 of 38 cases (13 percent). Two of 40 tumors that were assessed for methylation failed to show methylated or unmethylated bands. None of the five cases with methylation of *LN-5γ2* occurred in sporadic MLH1-loss cancers, which would be expected to show the CpG island methylator phenotype. However, four of five methylation-positive cases were scored as negative for LN-5γ2 expression. Two of these tumors were positive for nuclear β-catenin expression.

DISCUSSION

Tumor budding is a morphologic marker and LN-5γ2 is a biologic marker of dedifferentiation at the invasive margin of CRC. Although both features have been shown to be prognostic markers, they are strongly correlated and one may not contribute independent information in the presence of the other. We, therefore, undertook a multivariate analysis of a previously published series of CRCs[18] with high-grade tumor budding and showed that expression of LN-5γ2 remained as an important independent prognostic factor (hazard ratio, 3.3; 95 percent CI, 1.3–8.1; P = 0.01; raw data not shown). This finding may indicate that tumor budding is not a homogeneous process but may arise through mechanisms other than dysregulation of the Wnt signaling pathway. Based on the finding that LN-5γ2 was an independent prognostic marker, we elected to use the expression of LN-5γ2 as a biologic aggressive marker in CRCs with high-grade tumor budding.

Multiple pathobiologic features were assessed in sporadic MLH1-expressing and MLH1-loss CRCs and in HNPCC. All CRCs in these groups shared the feature of high-grade tumor budding. Despite the deliberate selection of CRCs with high-grade tumor budding, the three groups of CRC showed the expected clinical and pathologic features (Table 1) described in the literature.[26,28–30] However, with respect to pathologic features at the invasive front, LN-5γ2 cytoplasmic immunostaining and the frequency of cytoplasmic pseudofragments were higher in sporadic MLH1-expressing *vs.* sporadic MLH1-loss CRC or HNPCC. These findings indicate that the tumor buds in MLH1-expressing CRCs were not only qualitatively different from those in CRCs deficient in DNA mismatch repair but may be more aggressive. This suggestion is consistent with the worse prognosis of patients with MSS CRC *vs.* MSI-H CRC.[33]

Aberrant nuclear translocation of the transcriptional activator β-catenin is associated with up-regulation of multiple gene products, including LN-5γ2.[8] β-catenin nuclear expression was more frequent in sporadic MLH1-expressing *vs.* sporadic MLH1-loss CRC, an observation that could account for the similar difference with respect to LN-5γ2 expression. Based on this observation, it can be deduced that dysregulation of Wnt signaling pathway is more likely to be linked mechanistically with tumor buds in MLH1-expressing cancer than in MLH1-loss CRC. Important mechanisms for Wnt pathway dysregulation include inactivating mutation of *APC* and activating mutation of *β-catenin*. The preceding deduction is supported by the fact that mutation of both *APC* and *β-catenin* is uncommon in sporadic MSI-H CRC.[41] The number of HNPCC CRCs in our study was small, but the higher frequency of nuclear β-catenin expression compared with sporadic MSI-H CRC fits with previous studies and also with the elevated frequency of mutation of both *APC* and *β-catenin* in HNPCC.[29,41] Nevertheless, in HNPCC, expression of LN-5γ2 and presence of cytoplasmic pseudofragments occurred less frequently than in MLH1-expressing CRCs. Although the numbers are low, we observed a negative association between β-catenin expression and downstream alterations of Wnt pathway signaling in the HNPCC subset. This paradoxical finding conflicts with the concept of Wnt signaling dysregulation commencing with nuclear translocation of β-catenin and the generation of an aggressive phenotype through the downstream expression of multiple proteins implicated in tumor invasiveness.[7] It is possible that the marked peritumoral lymphocytic infiltration associated with CRC in HNPCC might impede the development of epithelial: stromal interactions necessary for aggressive tumor budding. In the sporadic MLH1-expressing subset of CRCs, our results are consistent with the concept that Wnt pathway dysregulation drives an aggressive budding phenotype. However, the mechanisms underlying budding in MLH1-loss tumors and in CRCs occurring in HNPCC seem to be largely independent