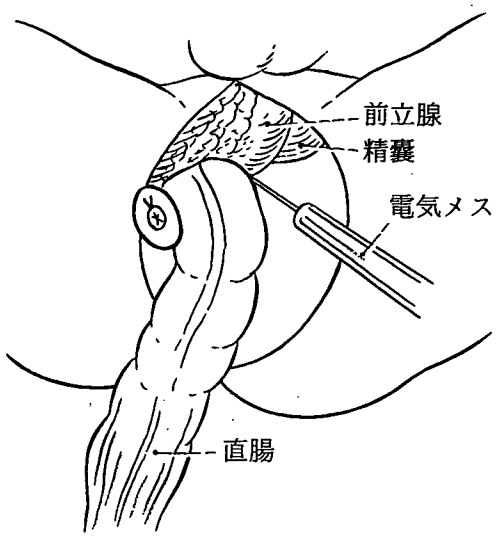

図 7　直腸前方の切離
直腸を創外へ脱転し，腹腔側からの切離に続けて電気メスで前立腺から切離し，肛門挙筋の残りを切離する。

て前方の前立腺（腟後壁）から直視下で直腸を電気メスで剝離し，ついで前方の肛門挙筋を切離して直腸の切離を完了する（**図 7**）。

留意点：前方の肛門挙筋の切離，すなわち前立腺部の剝離時がもっとも出血しやすいので，この部の切離を最後にする。万一出血しても直腸切断後にただちに止血操作に入れる。

前立腺部からの直腸の剝離は直腸を前方に持ち上げて，尾骨側から直腸と前立腺との剝離部を直視下に視認して鋭的に行い，指で剝離するなどけっして盲目的に行わないこと。

このように直視下で直腸前壁を切離すれば剝離層を誤ることはないが，直視下に操作できない場合には直腸を後方へ牽引し，前立腺（腟後壁）と直腸の間に示指を差し入れてガイドしながら前方の肛門挙筋，perineal body を切離する。

留意点：この場合剝離層が直腸側へ逸れると直腸壁を穿破し，外側へ逸れると男性なら外尿道，女性なら腟後壁を損傷するので注意する。

ていねいに止血し，抗生物質加生理食塩液または 4 倍イソジン液 2,000 m*l* で骨盤腔内を洗浄し，会陰創の皮膚を一層縫合で閉鎖する。

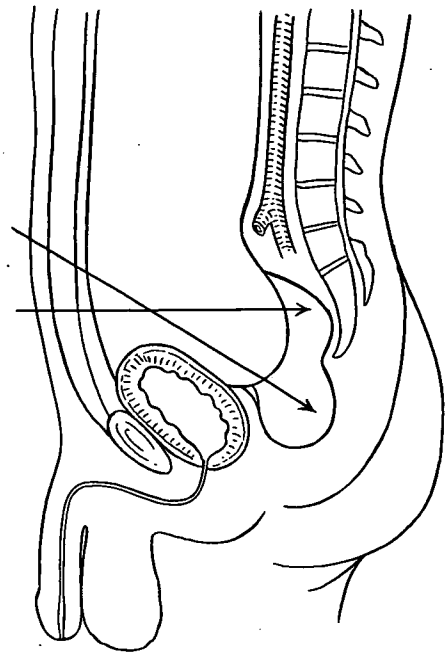

図 8　ドレーン先端の挿入位置
骨盤死腔は尾骨を境として頭側と尾側とに分かれるので，2 本のドレーンの先端はそれぞれの腔に置く。

側方リンパ節郭清を行う場合には，会陰操作が終わってから行う。

7. 人工肛門造設と骨盤底腹膜の閉鎖

術前に人工肛門作製部位に設定したサイトマーキング部に直径 3 cm の皮切を加え，円筒状に皮下脂肪をくりぬく。腹直筋膜を切開し，S 状結腸断端を経腹直筋的に腹膜外経路で腹壁に引き出す。骨盤底腹膜と後腹膜を閉鎖する。

留意点：骨盤底腹膜の閉鎖は膀胱や小腸の骨盤腔への落ち込みを防ぎ，術後万一骨盤内局所再発を起こした場合に，再手術や放射線治療を行いやすくするためである。残った腹膜が少なくて閉鎖できない場合には吸収性の polyglactin 910 メッシュを用いて閉鎖している[1]。

8. 閉　腹

左右の腹直筋外縁から骨盤腔内へ 8 mm ドレーンを後腹膜ルートから 2 本挿入する。骨盤死腔は**図 8** のように尾骨を境としてくびれができるので，ドレーンの先端は 1 本は仙骨前面

に，1本はくびれよりも会陰側におく。

留意点：ドレーナージは closed system の −50 mmHg の低圧持続吸引ポータブルバッグで行う。会陰側からドレーンを挿入した場合は自然落下でもドレナージでき，またドレーン抜去後の再挿入は容易であるが，座るとドレーンが触り痛みが強いことと，清潔度が保てない欠点がある[2]。

腹壁は吸収糸で3層に閉鎖するが，筋層縫合時に膀胱を腹壁側へ吊り上げるように膀胱漿膜を固定する。皮膚縫合は皮膚が膨隆してストーマ装具装着に障らないように3-0吸収糸で埋没縫合を行う。

おわりに

直腸癌では局所再発や肝転移再発などに対して再開腹・切除することを念頭において手術を行わなくてはならない。術後の癒着を防止するためには，術中の出血を少なくし，また確実に止血すること，挫滅組織を残さないこと，閉腹時に後腹膜および腹膜の断端を腹腔側へ出さないことが肝要である。

本稿では側方リンパ節郭清手技については省略した。拙著[3)4)]を見ていただきたい。

文　献

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2) 加藤知行ほか：大腸癌治療のプロトコール．臨床外科 55：99-106. 2000
3) 加藤知行ほか：直腸切断術．外科 58：1603-1608, 1996
4) 加藤知行ほか：下部直腸癌におけるリンパ節郭清のコツ；神経非温存．手術 47：2197-2204, 1993

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特集 Stage IV 大腸癌と診断したらどうするか

肝転移を伴う Stage IV 大腸癌の治療方針

Treatment policy for stage IV colorectal cancer with liver metastases

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肝転移を伴うStage IV大腸癌では，まず原発巣の治癒切除を行い，次いで肝転移巣に対する治療を行う．

肝転移に対する治療は転移巣の完全切除が第一選択である．完全切除が行えない症例では切除と凝固療法を併用したり，neoadjuvant chemotherapyを行い腫瘍縮小を待って切除を行う．残肝量が少なくなる症例では門脈塞栓を行って肝肥大を，あるいは二期手術を計画して転移巣の切除を目指す．

切除以外では，ラジオ波や凍結などの凝固療法が有望である．外国では放射線外照射も行われる．

はじめに

当院における1965～1999年の大腸癌手術例は3,235例で，そのうち414例(12.3%)が肝転移のためにStage IVとなった．これは全Stage IV症例中の56%に当たる．同時期の治癒切除例2,491例の術後の再発でも肝転移再発が7.5%で最も多い再発であり，肝転移への対応は大腸癌治療の上で重要な位置を占める．

肝転移を伴うStage IV大腸癌に対する治療戦略は，原発巣が切除できるものは原発巣による症状があるものはもちろん，症状がないものについても持続する出血や将来おこるであろう狭窄を予防するためにまず原発巣を切除する．原発巣を切除して，遺残する転移巣を肝転移のみにすれば，肝切除を初めとする局所療法や全身化学療法などいろいろな治療法を選択できる．原発巣を切除できない症例に対しては全身化学療法を行う．

本稿では原発巣を切除した上での肝転移に対する局所療法について解説する．

I. 肝 切 除

1．肝転移切除の現状

肝転移無治療例では5年生存は期待できず[1)2)]，非切除例の5年生存率が5%以下であるのに対し，肝切除例の5生率は20%～50%[3)]で原発巣と

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表1 肝転移切除後の予後不良因子(文献4より)

1．原発巣因子	3．肝転移切除後の予後因子：手術因子
根治度 C	断端陽性
リンパ節転移陽性(転移個数多)	tw＜10 mm
組織型 低分化／粘液	肝転移巣の遺残
ly 2～3	
budding あり	4．肝転移切除後の予後因子：背景因子
	術前遠隔転移
2．肝転移巣因子	肝転移時の他臓器転移
肝転移組織型 低分化／粘液	同時性
肝転移個数(多発)	無病期間＜1年
局在(両葉)	肝切除前CEA高値
肝転移程度(H_3)*	肝切除後CEA高値
腫瘍最大径	肝切除後CA19-9高値
衛星病変あり	
肝転移進展因子	
門脈腫瘍塞栓，肝静脈腫瘍塞栓	
胆管内腫瘍進展，門脈浸潤	
神経周囲浸潤	
腫瘍周囲偽皮膜形成	
liver cell entrapment	
肉眼型	
肝所属リンパ節転移陽性	

*大腸癌取扱い規約第6版による

同様に外科的完全切除以外に根治的な治療法はない．現在では肝以外の臓器に転移があっても，それが完全切除できれば肝転移，他臓器転移ともに切除することが多い．

肝切除後の再発は残肝再発が40%，次いで肺転移が20%に見られて[4]，肝切除後はこの2つの再発の予防法が現在の課題である．

肝切除後の予後に関係する因子を表1に示した．切除後の予後不良因子として異論がないのは，剝離断端の癌露出，肝所属リンパ節転移陽性，衛星病変など肝転移進展因子陽性，肝外転移であり，さらに予後に大きく影響を与える因子として肝転移個数，肝切除断端距離(tw)，肝転移切除後のCEA値とCA19-9値などがあげられる．大腸癌取扱い規約第7版で新たに採用された肝転移の進行度分類における予後規定因子は，肝転移巣の転移個数と最大径および原発巣のリンパ節転移度である[3)5)]．

肝転移巣切除後の残肝再発についても根治の可能性があれば，初回手術と同じ基準で切除の対象となる．再肝切除の成績は5生率は30～50%[6)-9)]と良好であり再肝切除は肝転移の治療成績を向上させる重要な因子である．

2．肝切除の適応

肝切除の適応基準として，①外科切除のリスクが良いこと，②原発巣がコントロールされていること，③適度な残肝量を残して肝転移巣が完全に切除できること，④肝転移以外の遠隔転移がないこと，⑤肝所属リンパ節転移がないことが一般にあげられ，さらに肝転移巣の条件として，⑥肝転移個数4個以下，⑦切除断端距離(tw)を10 mm以上切除できることが手術のstandard criteriaとされてきた．肝転移症例のうち，切除可能なものは25～50%である[10)]．

以下，手術に関係するいくつかの問題点について考察する．

3．切除時期

同時性肝転移に対しては，原発巣と同時に切除する者と，まず原発巣を切除して，その後3ヵ月ほど待って肝転移巣を切除する者とがある．

同時切除を行う理由は，①経過観察をしても予後に変わりはなく，②術中超音波検査で小病巣も

把握できるから遅らせる必要はない，③3ヵ月遅らせることで肝転移巣からの二次転移の危険性がある，④多発肝転移に対し3ヵ月遅らせることで肝切の時期を逃すなどである．

異時切除を行う理由は，①肝転移状況の精査，②肝外転移の精査，③肝切除を同時に行うことによる死亡率や合併症率が高い，④同時に行うと微小転移を診断できないことがあるので隠れた転移巣が明らかになるまで待って肝切除を行う，というものである．また，最大径2 cm 以下の小さな病変では他に検査で描出できない微小病変が隠れていることがあるので，3ヵ月待って新しい病変の出現を待って一括して切除し，一方最大径5 cm 以上のものや肝静脈，下大静脈，肝門に近いものは切除の機会を逃さず直に手術する意見もある[11]．また安野ら[11]は，1年以内の再発例も3ヵ月間経過観察をするとしている．

Capussotti ら[12]は予後不良因子(男，原発巣リンパ節転移3個以上，隣接臓器浸潤)を有する症例では neoadjuvant chemomtherapy を行い，肝転移の進展がない症例に肝切除を行うとしている．

原発巣切除は肝転移巣切除に先行して，あるいは同時に行われるのが一般的であるが，Mentha ら[13]は多発肝転移や大きな肝転移では原発巣手術後に肝転移巣が進展する危険性があるので，大腸癌の狭窄症状がない症例ではまず neoadjuvant chemotherapy を行い，6コース(肝転移奏効例では3コース)後にまず肝切除を行い，その3～8週後に原発巣の手術を行う方法を提唱している．

4．肝転移個数と大きさから見た適応

一般に単発例は多発例よりも予後が良く，転移個数の多いものはそれ以下のものと比べて予後は不良である．Sasson ら[14]は転移個数が多いものでは切除断端距離を十分に取れないことが予後不良の理由ではないかと推測している．一方，転移個数と予後とは関係ないとする報告も少なくない．

肝転移巣最大径は3 cm ごと，4 cm で分類するもの，5 cm で分けるものなど幾つかの分類があり，小さいものの予後が良いとされるが，切除断端の距離(tw)を十分にとって切除すれば予後には関係ないとする報告もある．

5．切除断端距離(tw)

切除断端に癌が露出しているものの予後は不良である．さらに，切除断端が陰性でも tw が10 mm 以下の症例の予後は不良とされ，tw を10 mm 以上取ることは肝切除時の主要な目標であった．

一方，肝転移巣周辺の衛星病変の頻度は少なく[15][16]，存在する範囲も転移巣からわずかの距離であり，肝切除前の転移存在診断が確実に行えるようになった現在では断端(－)であれば切除距離には関係しないとする報告も多い[17]-[19]．

6．肝切除術式

肝切除術式は局所切除，区域切除，葉切除，拡大葉切除(3区域切除)などが行われ，大きく分けて，解剖学的肝系統切除と非解剖学的肝局所切除とに分類される．基本術式が解剖学的系統切除か非解剖学的部分切除かについては解決していない．

系統切除派の意見は，3 cm 以上の転移巣では衛星病変や肝転移進展因子が高頻度に出現するが，系統切除はこれらを一括して切除できて予後が良いとするものである．

部分切除派の意見は，肝転移巣では非連続性進展の頻度は低いので，術中超音波検査を行って断端(－)あるいは surgical margin を十分にとって局所切除を行えば局所切除で良く，残肝量を多くして再肝切除に備えるというものである．さらに部分切除の予後は系統切除と差はない，合併症が系統切除と比べて少ないと主張している．ただし局所切除症例は小さな転移巣が選ばれるという selection bias を考慮しなくてはならない．

7．肝所属リンパ節郭清

肝門部リンパ節転移は他部位へ転移している

signalであるとされその予後は不良で，1988年にUS Registry of Hepatic metastasesが肉眼的リンパ節転移(859例中24例) を切除して5生例が1例のみだったこと(Eliasら[20]より引用)から肝門リンパ節転移例は手術適応外とすることが世界的なconsensusとなった．

Eliasら[20]は最近の報告から肝所属リンパ節の転移率1～7%，転移切除例の5生率0～27%，系統的en blocリンパ節郭清を行った場合の転移率13～25%，5生率0～42%とまとめている．またRodgersら[21]は15報告例から145例の肝門部リンパ節転移陽性例を集積し，その5年生存率は5%であると報告した．Katoら[4]の報告では転移陽性18例の5生率は12.5%である．転移陽性例の5生率は低いものの肝門部リンパ節郭清を行うことで生存期間が延長するという報告も多い．Sakaguchiら[22]は初回手術のリンパ節転移陽性例の予後は悪いが，再肝切除時に肝門部リンパ節に転移がある例では郭清効果があるとしている．

Jaeckら[23]は160例に郭清を行い17例のリンパ節転移をArea 1(肝十二指腸靱帯・膵後部)とArea 2(総肝動脈・腹腔動脈転移)に分け，Area 1の転移例(8例)の3生率38%に対してArea 2の転移例(9例)では1年以上生存例がなかった．

郭清範囲について，Eliasら[24]は100例の肉眼的に転移陰性と判断したもののうち14例が顕微鏡的に転移陽性であり，肝門部～腹腔動脈まであらゆる部位に転移していたと報告しており，Kaneら[25]はisosulfan blue dyeを腫瘍周囲に注入して注入前に判らなかったリンパ節の染色を7例中3例(全例転移なし)に認めた．転移リンパ節が必ずしも腫大しているわけではないので郭清する以上はJaeckの言うAreal・2の系統的郭清を行うべきと考えられる．

転移の有無に関係なく郭清例と非郭清例の生存率を比べると両者間に差はなく[4]，予防的肝門部リンパ節郭清の意義についての評価は定まっていない．山本ら[26]はリンパ節転移例の予後は不良であり，予防的郭清は残肝再発が多い大腸癌肝転移では(再肝切除が行いにくくなり)むしろ弊害が多いと述べている．Jaeckら[23]は全例にroutineに行うのではなく，リンパ節転移の危険性が高い転移個数3個以上，転移巣がsegment 4および5に存在するもの，低分化腺癌例に行うとしている．

8．補助療法

肝切除後の再発は残肝再発が最も多く次いで肺転移再発が多い．したがって，肝切除後の残肝再発と，肺転移を主とした他臓器転移の予防が重要となる．肝切除後の補助療法は主に残肝再発の予防を目的として5-FUを主体とした肝動注療法が行われてきたが，肝局所再発は抑えるものの全身転移が押さえることができず，補助療法を行わなかったものと生存率は変わらなくて有効性は確立していない．そこで肝動注と全身化学療法の併用が試みられているが，まだ長期経過例で有用性を示す報告はない．厚生労働省の第三次対がん総合戦略事業・がん臨床研究事業(H16-032)では，肝切除後の補助療法として現在進行大腸癌に対して最も有効とされる5-FU／leucovorin／oxaliplatin併用療法(mFOLFOX6)の有効性を検証する比較試験を開始した．

9．切除不能肝転移例の対応

1) Neoadjuvant chemotherapy

Bismuthら[27]は5FU／folinic acid／oxaliplatin併用療法を切除不能53例に行い，腫瘍の縮小を待って局所的に根治切除が可能となった時点で肝切除を行った．46例は肉眼的根治切除が行え，7例は非治癒切除となったので門脈塞栓術により残肝の増大を図り化学療法を続けて第2期切除を行った結果5生率は40%，残肝再発66%，肝外再発47%と報告した．

これ以後，腫瘍が大きい，多発肝転移，転移場所が悪いなどの理由で切除不能となった症例に対してneoadjuvant chemotherapyを行い，腫瘍の縮小が得られたものに切除を行った多くの報告がある．Neoadjuvant chemotherapyによる肝転移の切除率は腫瘍縮小率に相関し，完全切除率

は3.4～47%である[28)29)]．肝動注療法は全身化学療法と比べて延命効果では変わりはないが，腫瘍縮小率が高いのでneoadjuvantとして有効である[30)]．全身化学療法と肝動注療法の併用の報告も多い．最近の報告では，全身化学療法に用いられる薬剤は5-FU, leucovorin, oxaliplatin, CRT-11, gefitinibなどであり[28)29)]，肝動注ではFUDR／dexamethason[31)]，mitomycin C[32)]，CDDP[33)]などである．Neoadjuvant chemotherapy後に肝切除可能となった症例でははじめから切除可能だった症例よりも再発率は高い[34)]．

2）残肝量が少ない症例の対応

肝切除の適応に残肝の予備能が十分であることがある．残肝容積が25%以下の場合90%が肝機能障害を起こし[35)]，肝炎や肝硬変など慢性の肝疾患がある患者，あるいは大量の化学療法を受けた患者では40%以上の残肝量が要るとされる[36)]．正常肝の場合，非腫瘍部の肝を40%以上温存できない場合[37)]，あるいは非癌部肝切除率が50～70%[38)]に及ぶ場合には術前に片側の門脈塞栓術あるいは門脈結紮を行い予定残肝容積増大を促す適応となる．術前に門脈塞栓を行った場合の肝増量は8%である[36)]．Selznerら[39)]は門脈結紮と肝動注を行い，6ヵ月後も腫瘍の増大がなくて11例中4例に完全切除が可能となったと報告している．

3）Two stage operation

Bismuthら[27)]およびAdamら[40)]は多発肝転移で切除不能と思われる症例に対して術前化学療法，門脈塞栓術を行ってもone stageで完全切除ができない場合はtwo stage手術を提唱している．第I期手術ではできるだけ多くの転移巣を切除して，残肝の肥大を待つ間全身化療で遺残腫瘍の増大と転移を防ぎ，肝肥大が起きて完全切除ができるようになれば第II期手術を行うものである．3生率は35%であるが第I期手術ではなかった術死が15%にあり合併症もII期手術では多い．

4）肝切除と凝固療法の併用

多発転移に対しては，RFAと肝切除の併用が行われており，Curleyら[41)]の報告では肝切除の5生率65%，肝切除とRFAの併用36%，RFA単独22%であった．注意しなくてはならないのは併用療法が肝切除単独やRFA単独治療と比べて術後の合併症が20%前後と高く，手術死亡もあることである[41)42)]．

Eliasら[43)]は肝切除量が大きくなって残肝機能を維持できない21症例に対して，切除線上の転移巣をRFAで焼灼して壊死させ，その壊死部上で肝を切除し，小転移巣はRFAで焼灼した結果，術死の1例を除いて切離線上の局所再発はなかった(median follow up 19.4ヵ月)と報告している．

II. 凝固療法

凝固療法にはエタノール注入，マイクロ波熱凝固療法，ラジオ波熱凝固療法(RFA)，凍結療法などがある．

熱凝固療法は，本邦では1990年頃からマイクロ波熱凝固療法が行われていたが，1995年以降は主にRFAが行われるようになった[44)]．RFAの1回の凝固で治療できる範囲は3 cm以下であり[45)]，それよりも大きいものは凝固を繰り返すこと[41)]で対応する．3 cm以下の症例の局所再発率は低い[46)]．転移巣への到達ルートは経皮的，腹腔鏡下，開腹の3ルートがある．経皮的は侵襲が少ないが再発率が高く，合併症率も高い．完全凝固できた場合の5年生存率は20%前後[41)47)]で，Machiら[48)]は30%と高い生存率を報告しており，現在では肝切除に次ぐ治癒率が期待されている．

凍結療法は本邦ではあまり行われないが，Seifertら[49)]の報告では凍結療法単独または凍結療法と手術の併用で26%の5年生存率を上げている．RFAよりも合併症率が高いが，RFAが3 cm以下のものが対象となるのに対して大きいものにも有効である[50)51)]．8 cm以上になると局所制御率は低くなる[50)]．

また，肝切除の補助療法として断端陽性例あるいはTWが1 cm未満の症例に同部の凍結療法，マイクロ波凝固壊死療法やラジオ波熱凝固療法などの凝固療法を行う報告もある．

III. 放射線外照射

肝転移に対する放射線外照射は全肝照射となるために本邦ではほとんど行われないが，外国では試みられており[52]，最近は転移巣に限局した高線量照射が可能となって[53]，肝動注あるいは全身化学療法を併用して良い成績をあげている[54)55)]．Malikら[52]のreviewによれば，照射による肝障害は35 Gy以上で出現し，重度な肝障害の出現を5%以下に留める照射線量は肝の照射範囲が1／3で55 Gy，全肝照射では40 Gyとされ，全肝照射の安全域は30～35 Gyである．外照射30 Gyでは奏効率90%，生存期間4ヵ月であり，外照射25 Gy＋5FUでは奏効率90%，生存期間10ヵ月であることから，Malikら[52]は全肝に30 Gy＋腫瘍部に10～20 Gyの照射と5FUの併用を推奨している．その他，腫瘍部への陽子線治療[56]あるいは放射線sphereを肝動脈内へ投与・塞栓する報告もある[57]．

おわりに

肝転移を伴うStage IV大腸癌に対してはまず原発巣を切除し，肝転移巣に対しては切除を第一選択とする．肝転移切除不能例に対しては抗がん剤の肝動注療法を行うのが今までの治療戦略だった．現在の検討課題はいかにして切除例の治癒度を高め，いかにして切除不能例を治癒切除が可能な状況にするかという点である．

抗がん剤の肝動注療法はneoadjuvant therapyも含めて肝転移に対する重要な局所治療であるが，他稿に譲った．

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Total splenic vein thrombosis after laparoscopic splenectomy: a possible candidate for treatment

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Abstract

Background: Portal or splenic vein thrombosis (PSVT) is a common disorder after laparoscopic splenectomy (LS). Splenomegaly is a well-known risk factor for PSVT. However, no treatment strategy for PSVT has been established.

Methods: Thirty-three consecutive patients who had undergone LS and postoperative imaging surveillance were examined. PSVT was classified according to the site of thrombosis. We evaluated patient background, operative factors, and clinical symptoms.

Results: Spleen weight of patients with PSVT (n = 17, median 218 g) was greater than that of patients without PSVT (n = 16, median 101 g). Seven patients developed thrombosis involving the entire splenic vein (total splenic vein thrombosis), and 4 of them had clinical symptoms (fever >38°C and/or abdominal pain). The incidence of clinical symptoms was significantly more frequent in patients with than without total SVT. Operation time, blood loss, and spleen weight were also significantly greater in patients with total SVT. Multiple logistic regression analysis demonstrated spleen weight was the strongest predictor of PSVT and total SVT.

Conclusion: Patients with total SVT have greater risk factors for PSVT and frequently have clinical symptoms. They are candidates for anticoagulation therapy.

Keywords: Laparoscopic splenectomy; Portal or splenic vein thrombosis; Total splenic vein thrombosis; Anticoagulant therapy; Contrast-enhanced computed tomography scan

Portal or splenic vein thrombosis (PSVT) is a rare but serious complication after splenectomy [1,2]. However, with the improvement in diagnostic modalities and increased interest in this disease entity, it is becoming apparent that the incidence of PSVT is greater than clinically appreciated, especially in patients with splenomegaly [3,4]. We recently conducted a study to compare the incidence of PSVT after open splenectomy to laparoscopic splenectomy, and demonstrated that PSVT is a common complication after elective laparoscopic splenectomy (LS) [5].

Though common, it is still not clear who should have postoperative image surveillance, whether prophylactic anticoagulation is effective, and the exact type of PSVT that

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Table 1
Comparison of clinical characteristics of patients with or without PSVT

	No PSVT (n = 16)	PSVT (n = 17)	*P* value
Age (y): median, range	56, 17–73	50, 18–75	.51
Sex (M:F)	4:12	2:15	.32
Type of disease			.06
Idiopathic thrombocytopenic purpura	14	6	
Hypersplenism due to cirrhosis	0	4	
Malignant lymphoma	1	2	
Splenic lymphangioma/hemangioma	0	2	
Autoimmune hemolytic anemia	0	1	
Hereditary spherocytosis	0	1	
Evans syndrome	0	1	
Splenic cyst	1	0	
Surgical approach, LS/HALS	16/0	12/5	.044
Operating time (min): median, range	112, 65–165	120, 55–349	.54
Blood loss (mL): median, range	40, 10–180	30, 10–5600	.78
Spleen weight (g): median, range	101, 11–350	218, 61–2315	.01
Platelet count at POD 7 ($\times 10^4/\mu L$): median, range	23.8, 1.7–66.9	27.8, 2.9–75.7	.68

PSVT = portal or splenic vein thrombosis; LS = laparoscopic splenectomy; HALS = hand-assisted laparoscopic splenectomy; POD = postoperative day.

requires treatment [6]. Because patients with PSVT-related clinical symptoms often have poor prognosis [1,2], the aim of the current study was to examine the relationship between clinical symptoms and extent of thrombus propagation, and assess the predisposing factors to establish a treatment strategy for PSVT.

Patients and Methods

Patient population

This study was conducted in a consecutive series of 33 patients who were scheduled for elective LS at our institution between April 2001 and March 2004. Data of 22 of these patients have been reported previously [5]. Table 1 lists the clinical characteristics of the 33 patients. Intermittent pneumatic foot pump was used for perioperative prophylaxis of deep venous thrombosis until full ambulation, but no anticoagulant was used in any of the patients. The operative technique was purely laparoscopic in 28 cases, while hand-assisted laparoscopic splenectomy (HALS) was performed in the other 5 patients as described previously [5].

Detection and diagnosis of PSVT

All patients except those with renal dysfunction and hypersensitivity to the intravenous contrast media underwent preoperative and postoperative helical computed tomography (CT). Two patients were excluded and were evaluated by Doppler ultrasonography or magnetic resonance imaging (MRI). Imaging analysis was performed after splenectomy between postoperative days (POD) 3 to 11 (median 6.0 POD) except for 1 patient, who underwent CT on POD 23 because of clinical condition and CT availability. Detection of PSVT was based on the criteria defined previously [5,7]. In brief, PSVT was diagnosed when an unenhanced region was detected in a dilated splenoportal system, which was otherwise free of any abnormality in the preoperative CT. PSVT was classified into 5 types according to the location of the thrombus [5]. Distal splenic vein thrombosis (dSVT) was defined as thrombosis located in the splenic vein distal to the junction of inferior mesenteric vein (IMV). Thrombi between the portal vein and IMV were diagnosed as proximal SVT (pSVT). Total splenic vein thrombosis (SVT) (pSVT + dSVT) was defined as thrombus involvement along with the entire splenic vein. In case of IMV directed towards the superior mesenteric vein (SMV), splenic vein thrombosis was identified as dSVT. Thrombi in SMV, intra- and extrahepatic portal vein were diagnosed as SMVT, iPVT, and ePVT, respectively. Two patients with dSVT only were included among patients without PSVT. Figure 1A and B demonstrates CT images and schematic drawing of iPVT and dSVT, respectively. The CT image of a patient with total SVT is shown in Figure 1C, and a schematic drawing of total SVT is shown in Figure 1D.

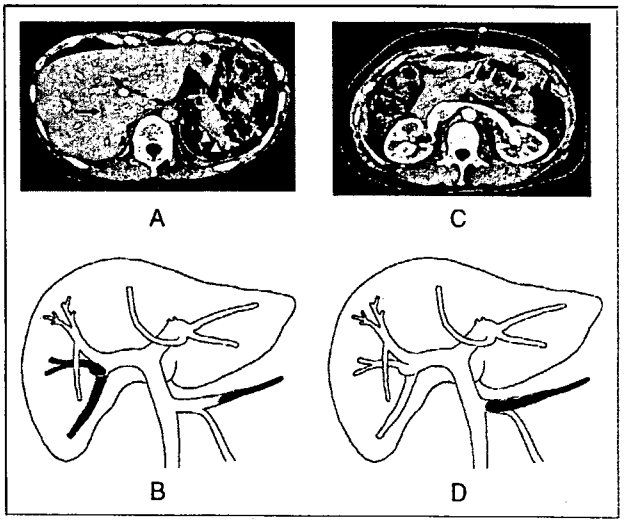

Fig. 1. (**A**) A 23-year-old woman with idiopathic thrombocytopenic purpura. Contrast-enhanced CT demonstrates thrombus in the posterior branch of the intrahepatic portal vein (arrow) and distal splenic vein (arrowheads). (**B**) Schematic drawing of portal and splenic vein thrombosis of the patient shown in (**A**). (**C**) A 57-year-old woman with hypersplenism due to liver cirrhosis. Contrast CT scan shows total splenic vein thrombosis (arrows). (**D**) Schematic drawing of total splenic vein thrombosis of the patient shown in (**C**).

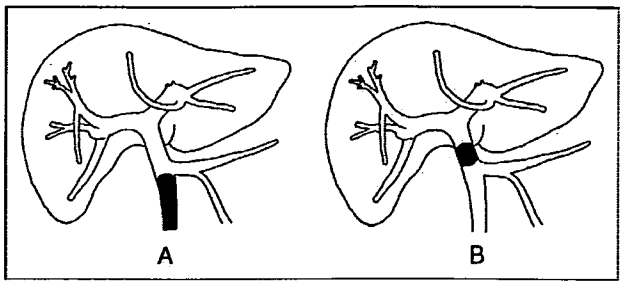

Fig. 2. Schematic drawing of superior mesenteric vein thrombosis (**A**) and extrahepatic portal vein thrombosis (**B**).

Furthermore, Figure 2A and B show schematic drawings of SMVT and e-PVT, respectively.

Clinical features of PSVT are nonspecific and it is impossible to screen patients based on clinical signs or symptoms. Among the clinical signs, fever is the most important and frequently observed in PSVT [3,4,7–9]. We reviewed the data of 53 patients who elected to undergo LS or HALS before this study specifically in terms of fever after POD 2. We found unexplained fever greater than 38°C in 3 patients. Since the beginning of this study, we found no patients having unknown cause of fever greater than 38°C after POD 2 without evidence of PSVT. Therefore, we considered fever of unknown origin greater than 38°C after POD 2 as fever due to PSVT. Abdominal pain, another important clinical symptom, was considered pain due to PSVT when no other reason for abdominal pain such as wound pain could be deduced.

Statistical analysis

Continuous data are expressed as median and range, unless otherwise specified. Statistical analysis was performed using the chi-square test or Fisher exact test for categorical data and Mann-Whitney *U* test for nonparametric data. The relationships between the patient's clinical features and the occurrence of PSVT or total SVT were examined by multiple logistic regression analysis. Variables (age, sex, operating time, blood loss, spleen weight, platelet count at POD 7) were divided into 2 categories by the respective median value, and subjected to multiple logistic regression analysis. All statistical analyses were completed using StatView 5.0J (SAS Institute Inc, Cary, NC). A P value less than .05 was considered significant.

Results

Seventeen of 33 (52%) patients developed PSVT after elective LS. No significant differences were noted in age, gender, operating time, blood loss during operation, and platelet count at POD 7 between patients who developed PSVT and those who did not (Table 1). In terms of type of primary disease, all 4 patients with hypersplenism due to cirrhosis developed PSVT and 2 of 3 patients with malignant lymphoma had PSVT, but the difference between the 2 groups was not significant. Spleen weight was significantly greater in patients who developed PSVT. All patients who underwent HALS developed PSVT. Five patients developed iPVT only and 5 patients had iPVT and dSVT. Three patients developed total SVT only, 2 patients had total SVT and iPVT, 1 patient had total SVT and ePVT, and 1 patient had total SVT, ePVT, and SMVT (Table 2).

Table 2
Location of thrombus and clinical features in 17 patients with PSVT

Site of thrombus	No. of patients	Clinical symptoms (fever*/abdominal pain)
iPVT	5	1/1†
iPVT + dSVT	5	—
Total SVT	3	2/0
Total SVT + iPVT	2	1/1†
Total SVT + ePVT	1	1/0
Total SVT + ePVT + SMVT	1	—

iPVT = intrahepatic portal vein thrombosis; dSVT = distal splenic vein thrombosis; total SVT = total splenic vein thrombosis (including distal and proximal splenic veins); ePVT = extrahepatic portal vein thrombosis; SMVT = superior mesenteric vein thrombosis.

* Fever body temperature >38.0°C.

† Patient also had fever.

Since poor prognosis has been reported in patients with complete obstruction of SMV, and isolated portal vein thrombosis is associated with good prognosis [1,2], we divided patients into 2 groups according to the total SVT, which could occlude SMV by thrombus propagation. Ten patients had iPVT alone or iPVT plus dSVT, and 7 patients had total SVT (Table 2). As shown in Table 3, all 7 patients with total SVT were females. Patients with idiopathic thrombocytopenic purpura (ITP) did not develop total SVT, and all 4 patients with hypersplenism due to liver cirrhosis developed total SVT, 2 of whom had ePVT and 1 had SMVT. One patient with Evans syndrome and total SVT had a prothrombotic disorder, abnormal lupus anticoagulant. The median operating time, blood loss, and spleen weight were significantly greater in patients with than those without total SVT. Four patients with total SVT had fever, 1 of whom had abdominal pain postoperatively, whereas only 1 patient without total SVT had fever and abdominal pain due to PSVT (Table 2). None of the patients developed diffuse abdominal pain suggestive of severe bowel ischemia. Multiple logistic regression analysis was performed to find the most important factors for predicting PSVT and total SVT, and spleen weight was identified as the only significant risk factor for PSVT (P = .016) and total SVT (P = .023).

Anticoagulant therapy was initiated immediately after the detection of PSVT. We did not use antiplatelet agents, such as aspirin. Sixteen patients received warfarin for at least 3 months, and 4 patients also received heparin. Only one patient did not receive warfarin or heparin based on a decision made by the attending physician. All PSVT, except for dSVT in 2 patients, resolved after 3 to 6 months of anticoagulant therapy. There were no significant adverse events during anticoagulant therapy.

Comments

PSVT is a well-recognized but rare complication after splenectomy; however, it is becoming apparent that it is a common disorder after LS [3,5]. Many investigators demonstrated that the risk of PSVT is higher in patients with

Table 3
Comparison of patients with or without total splenic vein thrombosis

	No total SVT (n = 10)	Total SVT (n = 7)	*P* value
Age (y): median, range	41, 18–67	50, 25–75	.026
Sex (M:F)	2:8	0:7	.49
Type of disease			.02
Idiopathic thrombocytopenic purpura	6	0	
Hypersplenism due to cirrhosis	0	4	
Malignant lymphoma	2	0	
Splenic lymphangioma/hemangioma	1	1	
Autoimmune hemolytic anemia	1	0	
Hereditary spherocytosis	0	1	
Evans syndrome	0	1	
Surgical approach, LS/HALS	10/0	2/5	.0034
Operating time (min): median, range	97.5, 66–135	265, 55–349	.015
Blood loss (mL): median, range	20, 10–120	656, 10–5600	.018
Spleen weight (g): median, range	150, 61–260	495, 300–2315	.0006
Platelet count at POD 7 ($\times 10^4/\mu L$): median, range	26.0, 2.9–75.7	28.6, 13.3–58.7	.66

SVT = splenic vein thrombosis; LS = laparoscopic splenectomy; HALS = hand-assisted laparoscopic splenectomy; POD = postoperative day.

splenomegaly, hemolytic anemia, and myeloproliferative disorders [3–5,9,10]. In the present consecutive case series of 33 patients, we demonstrated, in agreement with other studies, that the risk for PSVT is high in those patients. However, there are no standardized protocols for effective treatment for symptomatic PSVT, including duration of anticoagulation and potential efficacy of prophylactic use of perioperative antiplatelet agents [11]. Another issue that remains to be discussed is the indications for treatment of image-detected asymptomatic PSVT [12]. On the other hand, De Cleva et al [13] and others [6,14] demonstrated spontaneous recanalization of PSVT without any treatment, and meta-analysis showed LS is associated with a significant reduction in splenectomy-related morbidity compared with open splenectomy [15]. These findings suggest that not all patients need to receive treatment. Thus, selection of patients who need postoperative image surveillance and establishment of criteria for treatment are the key issues at present.

The most significant complications of PSVT are bowel infarction and portal hypertension due to venous congestion. These serious consequences occur when the thrombus involves extrahepatic portal vein or superior mesenteric vein. Patients with total thrombus in the splenic vein, propagating into the superior mesenteric vein and extrahepatic vein, have a poor prognosis because the thrombus interferes with venous flow of both the inferior and superior mesenteric veins [1]. Therefore, patients with ePVT or SMVT require prompt anticoagulation, and patients with potential risk of such thrombosis are likely candidates for active treatment. Since patients with total SVT are at greater risk compared to patients with other PSVT and more frequently manifest clinical symptoms and signs, we recommend prompt anticoagulation of patients diagnosed with total SVT. We are currently planning a randomized trial to investigate the importance of treatment of patients with image-detected postsplenectomy PSVT stratified by the presence or absence of total SVT.

Six (30%) patients with ITP developed iPVT, but none had total SVT and only 1 patient presented with clinical symptoms. These data indicate that in patients with ITP, postoperative imaging surveillance might not be necessary and postsplenectomy PSVT can be followed by close observation only. However, 1 report described a patient with ITP whose PSVT required intensive treatment [11]. He had complete thrombosis in the portal vein extending into the SMV. In this case, protein S deficiency was suspected. Since PSVT develops due to concurrent multiple local and systemic predisposing factors, special attention should be paid to thrombophilia when selecting the criteria for postoperative imaging surveillance and anticoagulation [16]. Since the site and extent of thrombosis correlate directly with clinical features such as intestinal congestion and portal hypertension, indication for treatment should be more dependent on the site and extent of thrombosis rather than the primary disease.

The etiology of PSVT can be divided into 2 main causes: prothrombotic disorders and local precipitating factors [16]. PSVT occurs due to a combination of these risk factors [17]. Local precipitating factors include hepatic disorders, abdominal inflammation, malignancies, and abdominal intervention, such as splenectomy. Since these etiologic factors are closely related to systemic or local hypercoagulable state, the mainstay of treatment choice is anticoagulation. Condat et al [18] demonstrated that anticoagulation therapy consisting of unfractionated or low-molecular-weight heparin followed by oral anticoagulation resulted in high rate of complete or partial recanalization. Our results were similar to their findings. Postsplenectomy thrombocytosis has also been discussed as a cause of PSVT [19], and Gordon et al [20] employed aspirin and low-dose heparin to prevent this complication with good results. On the other hand, no substantial increase in the thromboembolic event was found in patients with thrombocytosis after splenectomy [21]. Because no standard use of antiplatelet agents has been proposed to date, their role in the treatment of PSVT must be evaluated and established.

In the present study, all 5 patients who had undergone HALS developed total SVT. Four patients had both splenomegaly and portal hypertension due to liver cirrhosis. PSVT

tends to develop in patients with hypersplenism because of portal hypertension, and can be fatal [22]. Liver cirrhosis is an established cause of PSVT and accounts for approximately 11.2% to 21.5% of cases [17,23], and a high incidence of postsplenectomy PSVT has been reported [24]. All 4 such patients developed total SVT. Three of the 4 patients who received anticoagulant therapy and the other 1 who was not treated had uneventful outcomes despite the serious occlusion of portal venous system. We speculate that preexisting portal venous bypass flow plays an integral role, and therefore concomitant interventions of the bypass flow during operation require attention [22]. The other single patient had a huge spleen (2315 g). Since flow stasis in the remnant dilated splenic vein is considered an underlying pathogenic mechanism of PSVT [5,20], these 5 patients who underwent HALS were prone to develop PSVT.

We reported previously that LS was associated with a significant incidence of PSVT [5]. The incidence of PSVT in the present series of patients was also high (52%). The Virchow's triad (venous stasis, hypercoagulable state, and endothelial cell injury) is a well-known etiologic factor for venous thrombosis [25]. The increased incidence of PSVT after laparoscopic surgery could be related to the hypercoagulable state and venous stasis by CO_2 pneumoperitoneum, including reduced pressure-mediated portal vein flow, hypercapnia-induced mesenteric vasoconstriction, and impaired coagulation [26]. Venous stasis in the splenic vein stump following ligation of splenic hilar vessels is also another factor. In LS, dissection of the pancreatic tail from the retroperitoneum for the application of endoscopic stapler is minimal, thus leaving a large stump of the splenic vein. The venous flow is also reduced following the simultaneous ligation of the splenic artery. Therefore, patients with large spleen, large splenic vein, and reduced splenic vein blood flow are at higher risk for PSVT. In fact, 16 patients with small spleens in our series did not develop PSVT.

In summary, PSVT after elective LS is a common disorder. Our data indicate that total SVT tends to occur in patients with splenomegaly, and can potentially induce serious complications. Patients with total SVT require prompt anticoagulation. Further studies are required to determine the relationship between the type of PSVT and poor prognosis, and the preferred prophylaxis and treatment.

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Evaluation of laser microdissection as a tool in cancer glycomic studies

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Abstract

Laser microdissection (LMD) is a recent development that enables the isolation of specific cell populations from tissue sections. This study focuses on the potential of LMD as a tool in cancer glycomics using colon cancer as a model. LMD was performed on hematoxylin and eosin stained frozen tissue sections. Tumor cells and normal epithelial cells were selectively microdissected. *N*-Glycans from the LMD- and the bulk tissue-derived samples were liberated by hydrazinolysis and then labeled with 2-aminopyridine. After sialidase digestion, the resulting asialo-*N*-glycans were analyzed by normal and reversed phase HPLC combined with mass spectrometry. Comparison of the various *N*-glycan profiles with the aid of LMD identified seven characteristic *N*-glycans with significantly different expression profiles between normal and cancerous cells that could not be detected by conventional analysis. Thus, LMD is a potent and useful tool for analyzing variations in the expression of *N*-glycans by overcoming the problem of tissue sample heterogeneity.

Keywords: Colon cancer; Glycomics; Microdissection; *N*-Glycans; Pyridylamination

It is well known that glycans on the cell surface or in the extracellular space play important roles in cellular differentiation, adhesion, and proliferation [1,2]. The biosynthesis of glycans is tissue-specific and is regulated not only by physiological conditions, but also by pathological conditions such as tumorgenesis [3–5]. Aberrant glycosylation of membrane components occurs in essentially all types of human cancers, and many glycosyl epitopes constitute tumor-associated carbohydrate antigens (TACAs) [6–8]. Many lines of evidence suggest that the TACAs function mainly as adhesion molecules and contribute to cancer metastasis [7,9–11]. Alteration of the expression profile of TACAs in certain types of cancer has prompted researchers to evaluate their potential use as diagnostic and/or prognostic tools.

The application of glycomics to cancer research can highlight changes in the expression profile of the glycans occurring during tumor development and progression, leading to the identification of new molecular markers or potential therapeutic targets. However, because cancer tissue is composed of multiple subpopulations of cells, including normal epithelial cells, stromal cells, inflammatory cells, and angiogenic elements, accurate molecular analysis requires isolation of the tumor cells. Laser microdissection (LMD) is a recently developed technique that permits the reliable procurement of specific cell populations from tissue sections under direct microscopic observation. The laser-assisted microdissection technique has already been extensively used to isolate specific types of cells for the molecular analysis of DNA, RNA, and protein. However, in the field of glycan research, only one application of this technique for the analysis of glycosaminoglycans in postmortem human LASIK corneas has been reported [12]. Because LMD is a highly time consuming technique, feasibility

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and usefulness of this procedure must be thoroughly evaluated prior to its application in the analysis of glycans in cancer specimens.

In this study, we have investigated the potential of LMD as a tool in cancer glycomic studies using colon cancer as a model. Asialo-PA-*N*-glycans were prepared from bulk colon cancer tissue, bulk normal colon tissue, and from both normal colonic epithelial cells and cancerous colonic cells isolated from the bulk tissue using LMD. The *N*-glycans were then analyzed by normal and reversed phase HPLC in combination with mass spectrometry. LMD enabled us to identify seven characteristic *N*-glycans which displayed remarkable differences in the expression profile between normal and cancerous colon cells that could not be detected by conventional techniques. Our results demonstrate the usefulness of LMD for the accurate analysis of *N*-glycans in cancer glycomic studies.

Materials and methods

Standard PA-oligosaccharides. The structures and abbreviations of the authentic PA-oligosaccharides used in this study are listed in Table 1. Authentic PA-sugars were obtained from the following suppliers: 224F from Takara (Shiga, Japan); 22bis, ag22bisF, $G_1$22bisF, and 22bisF from Seikagaku Co. (Tokyo, Japan). Ag22bis was prepared by digestion of 22bis with Jack bean β-galactosidase (Seikagaku Co.). The structure of ag22bis was verified by normal and reversed phase HPLC analyses, combined with successive exoglycosidase digestions and by mass spectrometric analysis.

Tissue. Paired samples of normal and cancerous colon were obtained from the same patient by a standard colectomy procedure. Areas of tissues examined were selected by an experienced gastrointestinal pathologist. The tissue was cut into blocks, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and stored at −80 °C until use. This study was approved by Local Ethics Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Informed consent was obtained from the patient.

Laser microdissection. Frozen tissue sections (8 μm thick) of either cancerous colon or normal colonic mucosa were cut on a cryostat, CM 1900 microtome (Leica, Milton Keynes, UK). Tissue sections were thaw mounted on to a film-coated glass slide (90FOIL-SL25, Leica), briefly air dried, and then fixed at room temperature in 95% ethanol for 1 min. Staining was performed by the following procedure. Sections were immersed in Mayer's hematoxylin solution (Muto Pure Chemicals, Tokyo, Japan) for 30 s at room temperature, washed with phosphate-buffered saline (PBS) until a vivid blue color appeared, and then immersed in pure eosin solution (Muto Pure Chemicals) for 2 s. The sections were then dehydrated in 100% ethanol for 30 s and air dried. Laser microdissection (LMD) was performed using a Leica AS LMD system.

Preparation of protein extracts. After microdissection, the microdissected cells were carefully transferred from a PCR tube to a glass centrifuge tube using water and a micropipette. The collected cells were lyophilized and then solubilized in a 1:1 mixture of hexafluoroisopropanol (HFIP) [13,14] and 0.2% acetic acid. Control samples, which had not been subjected to LMD, were prepared from the frozen tissue sections cut directly into the solubilization mixture. The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. A protein sample of 300 μg was collected, concentrated, and used for the preparation of PA-*N*-glycans.

Preparation of PA-N-glycans. *N*-Glycans were liberated from the glycoproteins by hydrazinolysis at 100 °C for 10 h and then re-*N*-acetylated with acetic anhydride in a saturated sodium bicarbonate solution as previously described [15]. The reducing ends of the liberated *N*-glycans were labeled with a fluorophore, 2-aminopyridine, by reductive amination [16]. The excess reagents were removed by phenol–chloroform extraction and cation-exchange chromatography [17]. The resulting PA-*N*-glycans were further purified by normal phase HPLC according to the method of Nakakita et al. [18] with minor modifications. Briefly, the lyophilized PA-*N*-glycans were dissolved in water and then injected into a TSKgel Amide-80 column (4.6 × 75 mm, Tosoh, Tokyo, Japan). The solvents used were

Table 1
Structures and elution positions in HPLC of standard PA-oligosaccharides

Abbreviation	Structure	Elution position in HPLC	
		RP (GU)	NP (GU)
ag22bis	GlcNAcβ1-2Manα1(6) GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1(3)	11.45	5.37
22bis	Galβ1-4GlcNAcβ1-2Manα1(6) GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1(3)	12.72	6.78
ag22bisF	GlcNAcβ1-2Manα1(6) Fucα1(6) GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1(3)	14.90	5.66
$G_1$22bisF	Galβ1-4GlcNAcβ1-2Manα1(6) Fucα1(6) GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1(3)	15.86	6.29
22bisF	Galβ1-4GlcNAcβ1-2Manα1(6) Fucα1(6) GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1(3)	16.80	7.02
224F	Galβ1-4GlcNAcβ1-2Manα1(6) Fucα1(6) Galβ1-4GlcNAcβ1(4)Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1(2)Manα1(3)	13.91	7.96

(A) 90% acetonitrile–3% acetic acid titrated to pH 7.3 with triethylamine and (B) 20% acetnitrile–3% acetic acid titrated to pH 7.3 with triethylamine. Elution was performed at a flow rate of 1.0 ml/min at 40 °C. The column was equilibrated with solvent A, and after injection of a sample, the following gradient was employed: 0–28% B over 7 min; 28–100% B over 5 min; 100% B for 4 min. PA-*N*-glycans were detected with a fluorescence spectrophotometer using excitation and emission wavelengths of 310 and 380 nm, respectively. The fraction between 7 and 16 min after the injection was collected, concentrated, and used for the structural studies.

Preparation and size-fractionation of asialo-PA-N-glycans. PA-*N*-glycans were dissolved in 50 μl of 100 mM ammonium acetate buffer (pH 5.0) and then digested with 2 U/ml of *Arthrobacter ureafaciens* neuraminidase (Nacalai Tesque, Kyoto, Japan) at 37 °C for 24 h. The reaction was terminated by boiling for 3 min, followed by centrifugation at 13,000*g* for 10 min. The resulting supernatant was injected into a normal phase HPLC apparatus equipped with a TSKgel Amide-80 column (4.6 × 75 mm), and the asialo-PA-*N*-glycans were size-fractionated into nine fractions from glucose unit (GU) 3 to GU12 at intervals of one glucose unit, according to the method of Fujimoto et al. [19] with some modifications. The solvents used were (A) 90% acetonitrile–0.6% acetic acid titrated to pH 7.3 with triethylamine and (B) 20% acetnitrile–0.6% acetic acid titrated to pH 7.3 with triethylamine. The elution was performed at 40 °C using a flow rate of 1 ml/min. The column was equilibrated with 5% solvent B, and after injection of a sample, solvent B was increased linearly to 75% in 25 min. The PA-*N*-glycans were detected using excitation and emission wavelengths of 310 and 380 nm, respectively.

HPLC for structural analysis. Reversed phase HPLC was performed at 30 °C on a Cosmosil $3C_{18}$-P column (2 × 100 mm, Nacalai Tesque) at a flow rate of 0.2 ml/min. The solvents used were (A) 20 mM ammonium acetate buffer pH 4.0 and (B) the same buffer containing 0.5% 1-butanol. The column was equilibrated with 5% solvent B, and after injection of a sample, solvent B was linearly increased to 100% over 50 min and then held at 100% for 3 min. Fluorescence was monitored using excitation and emission wavelengths of 320 and 400 nm, respectively.

Normal phase HPLC was performed at 40 °C on a TSKgel Amide-80 (2 × 150 mm, Tosoh) at a flow rate of 0.2 ml/min. The solvents used were (A) 90% acetonitrile–0.6% acetic acid titrated to pH 7.3 with triethylamine and (B) 20% acetnitrile–0.6% acetic acid titrated to pH 7.3 with triethylamine. The column was equilibrated with 5% solvent B, and after injection of a sample, solvent B was linearly increased to 75% over 40 min. Fluorescence was monitored using excitation and emission wavelengths of 310 and 380 nm, respectively.

The structures of the PA-glycans were assessed by two-dimensional sugar chain mapping [20–22]. The retention time of each of PA-glycans was given in glucose unit based on the elution time of the PA-isomaltooligosaccharides. The behavior of authentic PA-oligosaccharides on HPLC is shown in Table 1.

Glycosidase digestion for structural analysis. PA-glycans were digested in a volume of 20 μl for 16 h at 37 °C using the following enzymes: *Streptococcus pneumoniae* β-galactosidase (Prozyme, San Leandro, CA), specificity for β(1,4)Gal, 0.1 U/ml in 50 mM sodium acetate buffer, pH 5.6; *Streptomyces* sp.142 α-fucosidase (Takara), specificity for α(1,3/4)Fuc, 0.2 mU/ml in 50 mM potassium phosphate buffer, pH 6.0.

NanoESI ion-trap mass spectrometry. Mass spectra of the PA-glycans were observed on a Finnigan LCQ Deca XP ion-trap mass spectrometer (Thermo Electron Co., Waltham, MA) equipped with a nanoESI device (AMR, Inc., Tokyo, Japan) connected to a Paradigm MS4 μHPLC system (Michrom BioResources, Inc., Auburn, CA). Reversed phase HPLC was performed at room temperature on a Magic C18 column (5 μm, 0.2 × 50 mm, Michrom BioResources) with a FortisTip capillary needle (AMR, Inc.) at a flow rate of 2 μl/min. The solvents used were (A) 5 mM acetic acid titrated to pH 6.0 with triethylamine and (B) 50% (v/v) methanol. The column was pre-equilibrated with solvent A, and 3 min after injection of a sample, solvent B was increased to 100% over 1 min and then held at 100% for 10 min. The nanoESI voltage was set at 1.8 kV and the capillary temperature was 200 °C.

Results

Tissue preparation for LMD

Hematoxylin and eosin (H&E) are the most commonly used histochemical stains. Good recovery (85%) of pyridylamino (PA) *N*-glycans was obtained after H&E

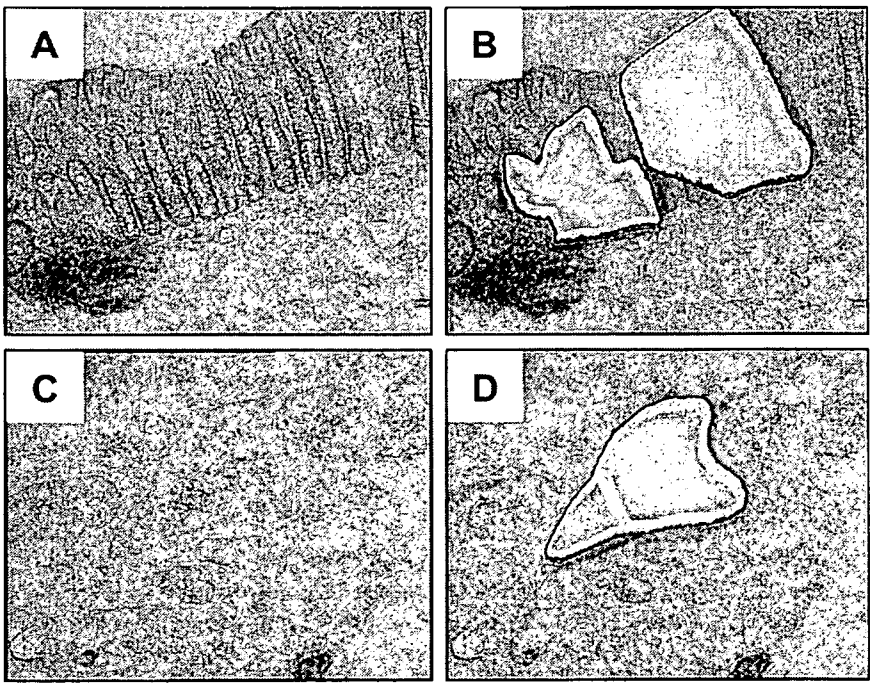

Fig. 1. Photomicrographs of laser microdissection of H&E stained tissue sections of normal colon (A,B) and cancerous colon (C,D). (A,C) Normal colon and cancerous colon, respectively, before microdissection. (B,D) Samples after successful microdissection. Representative data are shown.

staining and solubilization of the sections with 50% hexafluoroisopropanol (HFIP)/0.1% acetic acid mixture. Moreover, there was no gross effect on the resulting asialo-PA-*N*-glycans profile of colon cancer tissues (Supplementary Fig. 1). Thus, H&E staining and HFIP/acetic acid mixture were used for tissue preparation prior to LMD and for solubilization of samples, respectively. Colon cancer cells and normal colonic epithelial cells were successfully microdissected with the laser from the tumor and normal tissue sections, respectively, as shown in Fig. 1.

Comparison of N-glycan profiles from the LMD- and the bulk tissue-derived samples

PA-*N*-glycans were prepared from the LMD- or the bulk tissue-derived samples. After sialidase digestion, the resulting asialo-PA-*N*-glycans were separated into nine fractions (F1–F9) by normal phase HPLC (Fig. 2), in which oligosaccharides were separated in accordance with their molecular size. Each of the collected fractions was further separated by reversed phase HPLC. Comparison of the *N*-glycan profiles obtained from the LMD-derived samples with those from the bulk tissue-derived samples showed clear differences in fractions F2–F6 (Fig. 3). Although the expression profile of many *N*-glycans is different between normal and cancerous cells, we picked up 12 characteristic peaks (G1–G12) whose marked expression changes could only be detected using the LMD procedures. The detection of these peaks directly demonstrates the potential of using LMD to overcome problems associated with tissue heterogeneity. All 12 peaks displayed a substantial decrease in intensity in the cancerous colon cells relative to normal colon cells. Each of these peaks was fractionated and further purified by normal phase HPLC for structural analysis as described in Materials and methods. Elution positions of G1–G12 on normal and reversed phase HPLC are summarized in Fig. 4A as a two-dimensional map. Peaks G3, G5, G10, and G12 had the same positions on the map as G2, G4, G6, and G9, respectively, indicating that each paired peak possessed the same structure. These results presumably arose from incomplete separation during the initial size-fractionation step (Fig. 2). From the positions on the map corresponding to authentic PA-*N*-glycans (Table 1), G1, G2 (G3), G4 (G5), G6 (G10), and G9 (G12) were estimated to be ag22bis, ag22bisF, $G_1$22bisF, 22bisF, and 224F, respectively. These structures were also confirmed by mass analysis (Table 2). The structures of the peaks G7, G8, and G11 were determined by two-dimensional mapping combined with exoglycosidase digestion and mass spectrometry as follows.

Peaks G7 and G8, both of which had the same composition of $Hex_5HexNAc_5dHex_2$-PA (Table 2), were sequentially digested with exoglycosidases in following order: 1st *S. pneumoniae* β(1,4)-galactosidase, *S.* sp142 α(1,3/4)-fucosidase, and 2nd *S. pneumoniae* β(1,4)-galactosidase (Fig. 4B and Supplementary Table 1). A single residue was removed at each step, indicating the presence of one Lewis^x structure with fucose linked α(1,3) to GlcNAc (the presence of fucose rendering the galactose residue resistant to cleavage). Elution positions on the map of the digests were shifted to that corresponding to the authentic PA-*N*-glycans, $G_1$22bisF and ag22bisF, after digestion with the α-fucosidase and

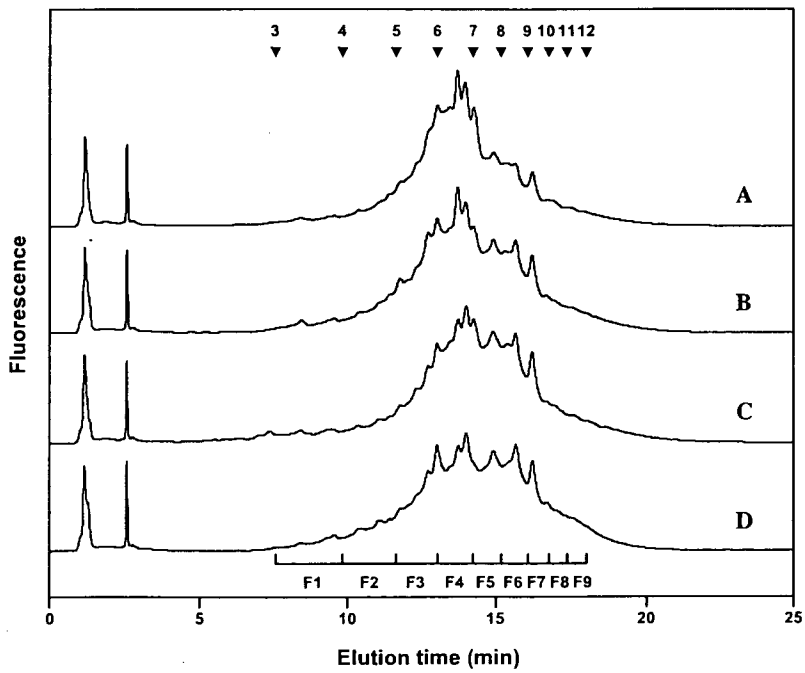

Fig. 2. Size-fractionation HPLC of asialo-PA-*N*-glycans from normal colon and colon cancer. (A,B) Normal and cancerous colon, respectively, which were not subjected to laser microdissection (LMD). (C,D) Normal and cancerous colon, respectively, which have been subjected to LMD to isolate normal epithelial cells and tumor cells. Numbered arrowheads indicate the elution position of PA-isomaltooligosaccharides with the corresponding degree of polymerization. Fractions F1–F9 were collected as indicated by the partitioned bars.

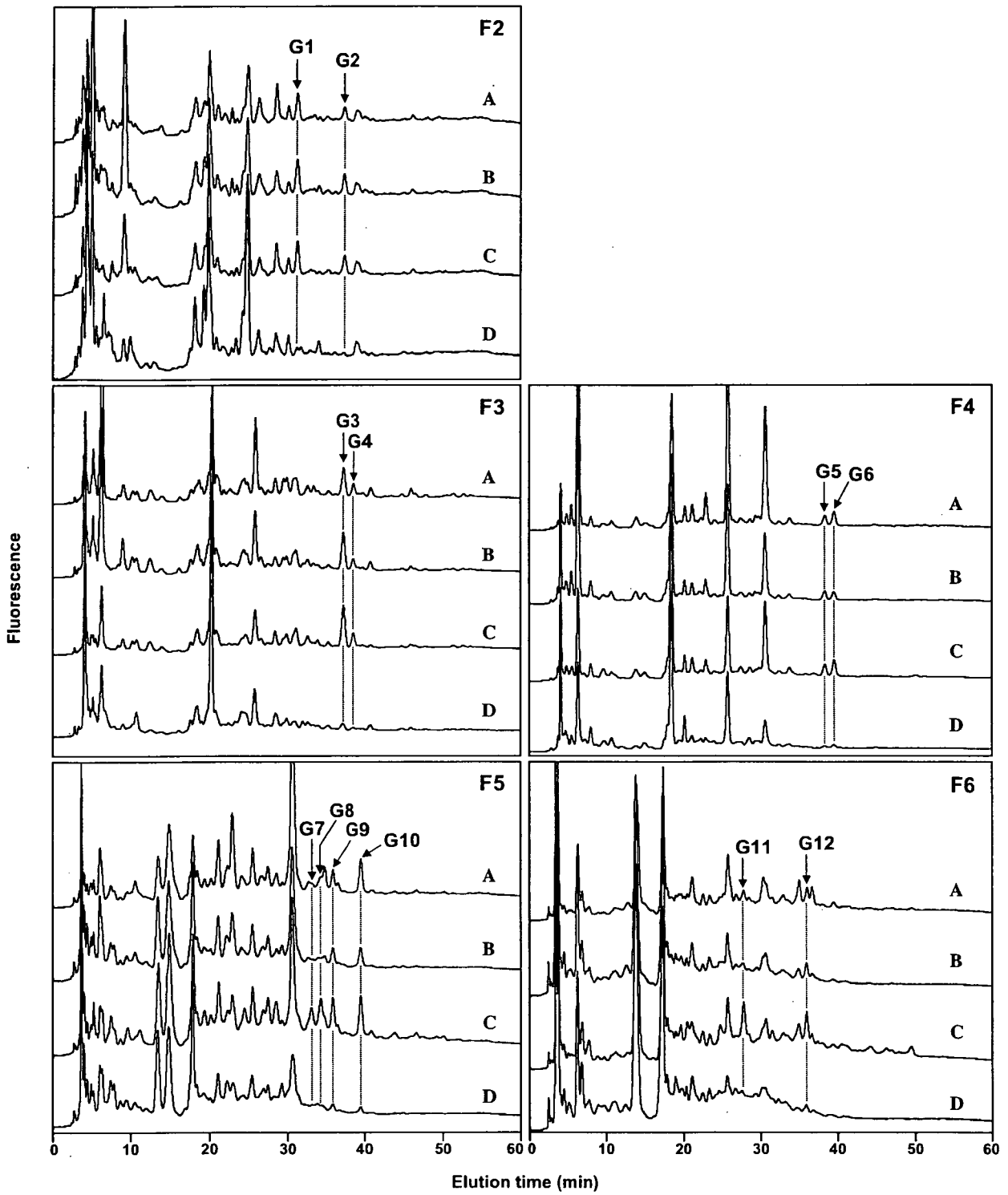

Fig. 3. Reversed phase HPLC profiles of the fractions F2–F6. (A,B) Normal and cancerous colon, respectively, which were not subjected to laser microdissection (LMD). (C,D) Normal and cancerous colon, respectively, which have been subjected to LMD. Twelve peaks (G1–G12) were collected.

the 2nd β-galactosidase, respectively. Thus the structures of these two peaks were estimated to be 22bisF with one Lewis^x structure.

Peak G11, which had the composition of $Hex_5HexNAc_5dHex_3$-PA (Table 2), was resistant to digestion with *S. pneumoniae* β(1,4)-galactosidase, but was sensitive to sequential digestion with *S.* sp142 α(1,3/4)-fucosidase followed by *S. pneumoniae* β(1,4)-galactosidase (Fig. 4B and Supplementary Table 1). Two residues were removed at each step indicating the presence of the two Lewis^x structures with fucose linked α(1,3) to GlcNAc. Elution positions on the map of the digests were shifted to that corresponding to the authentic PA-*N*-glycans, 22bisF and ag22bisF, after digestion with the α-fucosidase and the