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大腸癌肝転移の治療戦略：切除の適応と肝切除時期 —肝切除はいつ行うのか*—

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要約：大腸癌肝転移は，肝に限局しているか全身転移の一部分なのかを考えて，肝切除か化学療法かの治療選択が重要である。①肝転移が肝に限局，②十分な残肝機能を残して転移巣の完全切除が可能の条件が満たされれば肝切除が適応される。同時性肝転移では，原発巣の深達度が深く，リンパ節転移も高度で，生物学的悪性度が高く，肝病変の進行度が早い傾向があり，術前の肝病変や肝外病変の評価を十分に行わないと，早期の残肝再発，肝外再発を来すような肝切除効果の低い症例にも肝切除を行ってしまう可能性がある。当科では肝転移が生じて間もないと考えられる最大径 2 cm 以下の同時性肝転移例は，原則的に，原発巣切除後 3ヵ月経過観察し，新しい肝病変や肝外病変の出現の有無を確認し，肝切除の適応を判断している。ただし，腫瘍が肝門部や右肝静脈近傍，尾状葉にある症例では待機によって切除不能ないしは肝切除量が極端に大きくなる可能性があるため，同時切除が適応される。

Key words：大腸癌，肝転移，肝切除，同時切除

はじめに —大腸癌肝転移に対する治療戦略—

大腸癌患者の約 10～20％は，診断時にすでに遠隔転移を有する（表 1）。大腸癌の切除率は 85～95％であり，治癒切除率は 70～80％である。また，治癒切除後にも約 30％の患者が転移再発を来すことから，転移巣に対する治療は生存率の改善に重要である。

肝は，同時性に 10％，異時性に 15％転移が合併する最も頻度の高い臓器である。遠隔転移が肝に限局し，切除可能な場合は，手術切除が最も効果的な治療である。大腸癌血行性転移はカスケード理論[1)]，すなわち「大腸癌の大部分の症例では，まず肝に血行性転移が成立し，ある程度の大きさになるとそこから肺に転移する。さらに，肺から全身に癌細胞が散布される」に従うと考えると，肝に転移が限局している時期に肝転移巣をすべて切除できれば，根治も可能と考えられる。逆に，肝にとどまらずに全身へ癌細胞が散布されている時期の肝転移に対しては，肝切除は効果的ではない。肝に限局した病期なのか全身的に腫瘍が転移している時期なのかを考慮して，肝切除をするのか，化学療法を行うのかという治療戦略をたてることが重要である。

画像診断能には限界があり，肝，肝外病変ともに，微小な転移病変をとらえることは困難である。そこで，明らかな肝転移を診断しても，即座には肝切除を行わず，隠れている微小転移巣が増殖し，画像的に捉えられるまで待って，画像診断を再び行い，治療方法を決定するという戦略が考えられる。これに対し，肝切除を待機している間に，肝臓から二次的に肺を始めとする肝以外の全身の臓器へ転移を生じる可能性があるので，早期切除をするべきという対立意見もある。

同時性肝転移についての，原発巣と同時に肝転移を切除するべきなのか，原発巣切除後に 2～3ヵ月待って，肝転移をするのかという点では，手術合併症の面からの議論もある。

いずれにしても肝転移診断後の肝切除時期につい

* Surgical Strategy for the Treatment of Colorectal Liver Metastasis : Indication for Liver Resection and Timing of Surgery —Shynchronous or Staged ?—

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表 1　大腸癌同時性遠隔転移頻度

	肝転移	肺転移	腹膜播種	その他の遠隔転移				
				骨転移	脳転移	Virchow 転移	その他	合計
結腸癌	11.6% 383/3291	2.0% 62/3077	6.4% 211/3297	0.4%	0.1%	0.2%	0.4%	1.0% 31/3234
直腸癌	10.0% 220/2200	2.0% 42/2065	3.0% 66/2191	0.6%	0.1%	0%	0.6%	1.2% 27/2180
大腸癌全体	11.0% 603/5491	2.0% 104/5142	5.3% 277/5488	0.5%	0.1%	0.1%	0.4%	1.1% 58/5414

1998 年の症例　大腸癌研究会・全国登録委員会（2004 年 4 月発行）

て，未だ controversial である。本稿では主に同時性肝転移例を対象として，文献および筆者らの経験をもとに，肝切除はいつ行うべきなのかを考察する。

I．肝切除の適応と治療戦略

肝転移に対する治療効果は，肝切除，肝動注療法，全身化学療法の順である。図 1 に当科における治療別の生存曲線を示した。肝切除が最も治療効果が高い。本邦での多施設集計 585 例の肝切除後 5 生率は，39.2%であった[2]。

肝切除の適応は転移個数で決定されることが多い。現時点で，肝切除の適応基準は確立していないが，①肝転移が肝に限局している。②十分な残肝機能を残して転移巣の完全切除が可能である，の条件が満たされれば肝切除が適応されている[3]。個数や分布，大きさ，大小不同，同時性か異時性か，片葉転移か両葉転移かなどを考慮し，切除を検討する。1980 年以前は単発例のみが切除の対象であったが，1980 年代以降になると，肝転移巣が多発であっても，転移個数が 4〜5 個程度までなら，肝切除が行われるようになった。肝切除後の予後不良因子があっても，他の治療法と比べると切除の治療効果は高いので，相当高度な肝転移に対しても肝切除が行われることがある。また，同時に肝外転移病変があっても，それが切除可能あるいは良好な制御が期待できる場合には(特に肺)，肝切除を施行する場合もある[4]。肝切除後の残肝再発は 50%以上と高頻度だが，再肝切除により，比較的良好な予後が期待できる[5]。肝切除後は残肝，肝外再発が各々30%，肝と肝外の両方の転移再発が 30%であり，残肝，肝外ともに再々発が高率に起こる[6]。肝切後の補助療法として肝動注化学療法が行われることがある。しかし，補助動注化学療法は，残肝再発は抑制するが，生存率に寄与していないなど有効性は確立されていない[7]。肝外再発対策として，生物学的悪性度の高い症例（低分化癌，リンパ節転移高度）には，全身化学療法を肝動注

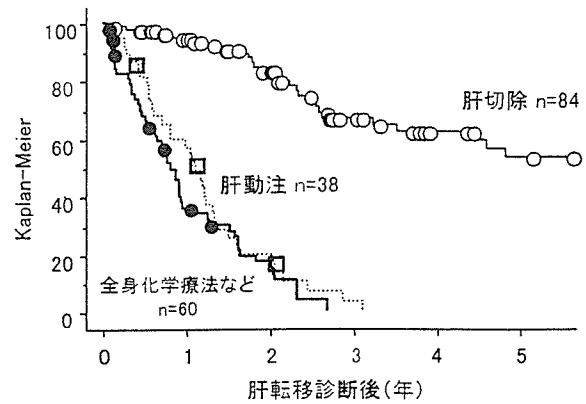


図 1　当科における大腸癌肝転移治療成績
(1992〜2004)

化学療法に加えて行うか，強力な全身化学療法を行ったほうが良いかは，controversial である。

II．同時性肝転移

同時性肝転移は，原発巣診断時にすでに肝転移を有していることから，異時性肝転移に比較して生物学的悪性度が高いといえる。肝転移切除後の予後に関して，同時性肝転移が異時性と比較し，不良との報告が多いが[8〜10]，差がないとする報告もあり[2]，同時性肝転移の臨床像には不明な点も多い。

大腸癌肝転移に積極的に肝切除や肝動注療法が始められた 1990〜2004 年に，当科で治療を行った大腸癌肝転移 210 例(同時：異時＝146：64 例，原発巣切除から 6ヵ月以上経過して診断されたものを異時性と定義)を分析した。同時性肝転移は，異時性より有意に肝転移個数が多く，肝転移区域も広汎であるなど，肝病変が高度であった。したがって，肝切除率は異時性の約 60%に対し，同時性では約 30%であった。同時性肝転移の予後は有意に不良であった（図 2)。同時性では，原発巣の深達度やリンパ節転移の進行した症例が有意に多く，同時性肝転移切除例では再々発時期が早い傾向はあるものの，肝切除後の予後では異時性肝切除例

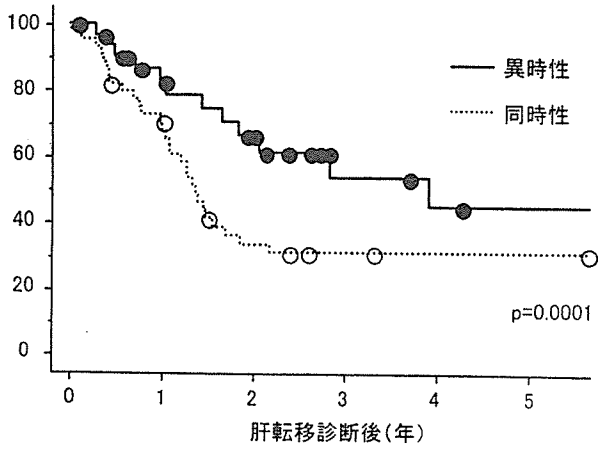


図 2　同時性肝転移/異時性肝転移一生存曲線一

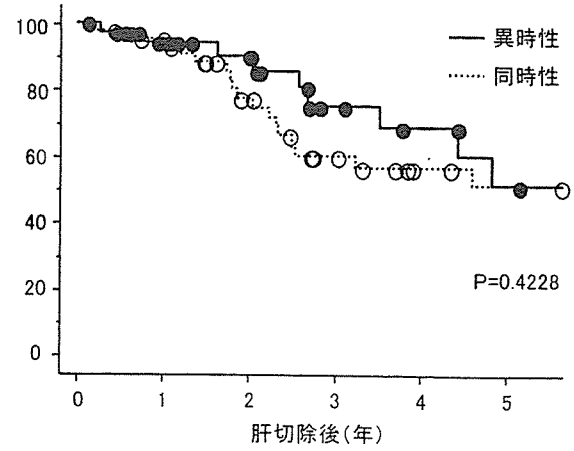
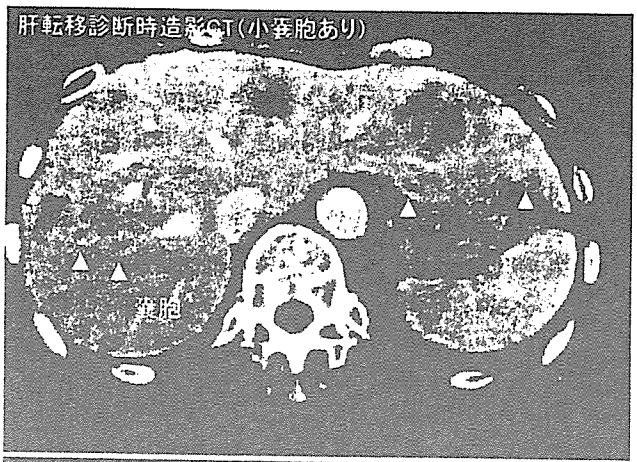
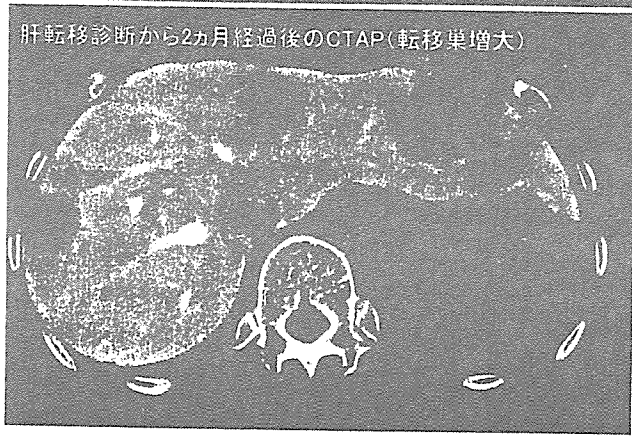


図 3　同時性肝転移/異時性肝転移切除例一生存曲線一





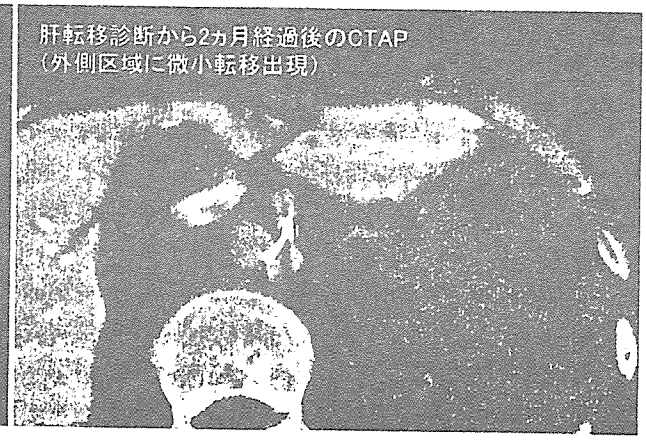


図 4　造影 CT（診断時）と CTAP（2ヵ月後）

と同等の生存期間が得られた（図 3)。

当科では，同時性肝転移例において，肝病変の進行度の評価，肝外病変の合併の有無を正確に診断するため，肝病変には CTAP，CTHA (SPIO MRI)，肝外病変には CT，MRI (PET) などの診断能の高い画像診断を組み合わせて行い，肝切除の適応を判断している。この判断を誤ると，肝切除の治療効果の低い症例を肝切除してしまう恐れがある。

図 4 は原発巣切除後 8ヵ月に肝転移を来し，当科に紹介された症例である。紹介時の CT では肝転移は 4 個で切除可能であった。早期に肝転移を来したこと，CEA doubling time が約 30 日と短いことで悪性度が高いことが示唆され，即座には肝切除を行わず 2ヵ月後に CTAP 検査を行った。先の 4 個の転移巣の著しい増大，新たな微小な転移巣を多数両葉に認めて，肝切除の適応とはならなかった。

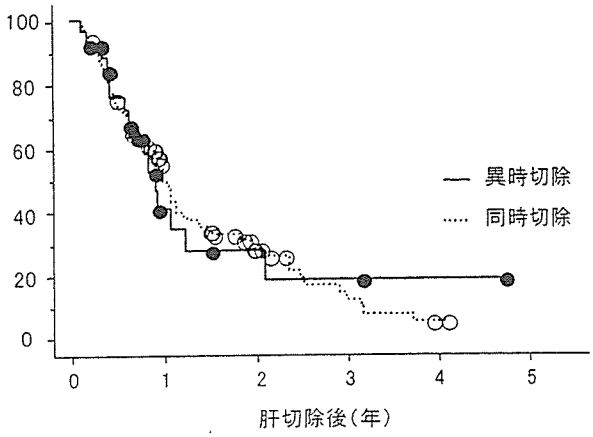


図 5　同時性肝転移：残肝無再発生存曲線
—同時切除 vs 異時切除—

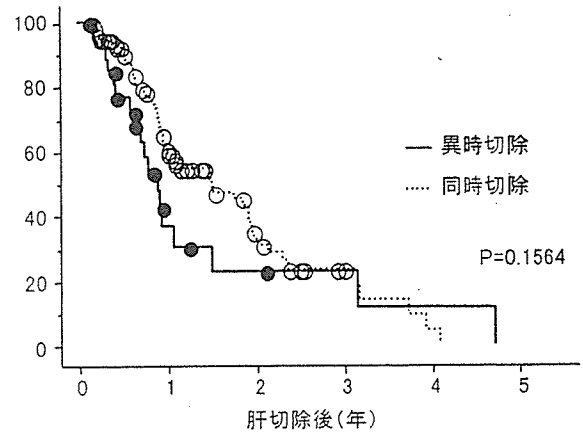


図 6　同時性肝転移：肝外無再発生存曲線
—同時切除 vs 異時切除—

III. 同時性肝転移切除の時期に関する議論のポイント

同時性肝転移に対しては，原発巣と同時に切除するという意見と，まず，原発巣を切除してその後3ヵ月程度待ってから，肝転移を切除するという意見がある。この同時切除か異時切除かの議論は，外科的側面と腫瘍学的側面からなされる。前者は，肝切除が開始された初期の頃，議論なされたポイントである。原発巣に加えて肝切除を同時に行うと，手術侵襲が大きくなる。そのため，1980年代の肝切除の合併症が多かった時期には，原発巣切除後に二期的に肝転移巣を切除することが多かった。肝切除が安全に行われるようになった近年では，同時切除が行われることが増えている。後者の腫瘍学的側面は，次に述べる，近年における同時切除か異時切除かの議論のポイントである。

同時性肝転移は，原発巣の深達度が深くリンパ節転移も高度で，生物学的悪性度が高く，肝病変の進行度が早い傾向があり，術前の肝病変や肝外病変の評価を十分に行わないと，早期の残肝再発，肝外再発を来すような肝切除効果の低い症例にも肝切除を行ってしまう可能がある。

精度の高い画像診断ツールであるヘリカルCTやMRIなどを駆使して，肝内病変の程度や肝外病変の有無を精査しても，その限界はあり，大きさ1cm以下の微小転移巣の診断は困難である。現在，鉄磁性体を用いたSPIO MRIは，5mmの肝病変でも描出できるが，それ以下の肝転移は捉えられない。微小肝転移巣は，画像診断の発達した今日でもすべて捉えることは不可能で，検査で描出されなかった病変は，切除されずに遺残し，残肝に再発する。結果，肝切除後には40%前後の高い頻度で肝再発が起こる。肝臓からは二次的に肺を始めとする肝以外の全身の臓器へ転移を生じ，肝切除後の肝外再発の頻度も高い。肝を経ずにリンパ行性，静脈性に全身性の転移を起こすこともある。これらの肝外転移病変の画像診断にも限界があり，大きさ1cm以下の微小転移巣の診断は困難である。

そこで，肝切除可能と判断しても即座には肝切除を行わず，微小転移巣が増大し画像的に捉えられるまでの期間を待って画像診断を再び行い，治療方法を決定するという戦略が考えられる。一方，肝臓から二次的に肺を始めとする肝以外の全身の臓器へ転移を生じる場合もあり，早期切除が原則であるとする考え方もある。ただし，大腸癌は，腫瘍学的に見れば原発巣，再発巣ともに局所に限局する傾向のある癌なので，短い一定期間を待って手術してもclinical outcomeには大きな影響は及ぼさないと思われる。一方，肝切除を待機中の短期間で，肝や肝外に新しい転移巣が出現したため，肝切除の適応とならなかった症例は，元来，生物学的悪性度が高く，原発巣とともに肝転移を同時切除したとしても，再々発を早期に来し，肝切除が無意味となった可能性が高く，効果の乏しい例が多い。

IV. 同時性肝転移は，原発巣と同時切除か，異時的に切除か

臨床の現場においては，同時性肝切除か異時性肝切除かは，個々の症例ごとに判断しなければならないことが多く，切除時期に関するコンセンサスは，簡単には得られない。しかし，この問題を考えるとき，参考となるいくつかの研究報告がある。

1．同時切除は術後合併症の危険を高めるか

同時切除では手術侵襲が大きいこと，肝切除のPrin-

gle 操作の門脈血遮断で，腸管にうっ血浮腫を来して縫合不全の率が高くなる危惧がある。Scheele らは 28 例の低位前方切除を含む 90 例の同時切除例において，13 例の縫合不全が見られて，そのうち 2 例が重篤な状態となって死亡したと報告した[11]。したがって，同時切除の場合は，部分切除などの小さな術式のみが許容されると述べている。一方，Elias らは，原発巣と肝転移の同時切除を行った 53 例—消化管の縫合箇所は 76 箇所，結腸・結腸 19 例，結腸・直腸 9 例，回腸・結腸 5 例，結腸・肛門 4 例（protective colostomy 4 例造設）を含む—の縫合不全率を検討した[12]。縫合不全は 2 箇所 3%（小腸・小腸 1 例，結腸・結腸 1 例）に見られたのみで，肝切除を同時に行っても，縫合不全率は高くならないと報告した。

2000 年代に入ると，Martin[13]と Lyass[14]の報告がある。Martin らは，同時性肝転移例に対して，原発巣と同時切除した 136 例と異時性切除を行った 106 例の術後合併症を比較した。同時性肝切除は，右側大腸癌，肝転移の数が少なく，最大径も小さい例が有意に多かったが，原発，肝切除合わせた合併症の率は，同時切除の 49%に対し，異時切除の 67%であった。合併症の重症度も重篤なものは少なく，肝，消化管の合併症の割合も同じであった。また，区域切除以上の肝切除が行われた subgroup 症例で比較しても，有意に同時切除のほうに合併症が少なかったので，原発・肝同時切除は安全に行えると報告している。Lyass ら[14]も，原発巣の部位や肝転移程度にかかわらず，同時と異時切除で，合併症(25%程度)，出血量，入院期間は同等であったと報告している。手術手技と周術期管理が確立した現在，閉塞性大腸炎などの特別な合併症がなければ，原発巣と肝転移の同時切除は安全に行えると言ってよい。

2．同時切除と異時切除で予後が異なるか

1985 年ごろより積極的に術中超音波検査(IOUS)が行われるようになった。IOUS は，CTAP のような診断能の高い術前精査を行った例でも，15～25%に新たに微小転移を見つけるといわれている[15]。Vogt らは，IOUS で微小な肝転移も pick up され，切除可能であるので，肝微小病変が増大発見されるのを待つ必要がなく，同時切除でも異時切除でも生存率は同じであると報告している[16]。Scheele[11]，Lyass[14]らも同時切除，異時切除で予後は変わらなかったと報告している。これに対し，Jenkins らは，肝切除後生存期間（中央値）が，同時切除で 24ヵ月，異時切除 39ヵ月と異時切除が良好と報告した[17]。さらに，Scheele[11]，Lyass[14]の報告も，症例数が少なく有意差が出ないだけで，それぞれの肝切除後の生存期間（中央値）は，Jenkins[17]のものと同様で異時切除のほうが予後良好であると述べている。

平成 10 年から 4 年間行われた厚生労働省がん助成金[10,11]「大腸がんの肝・肺転移例に対する治療法の確立に関する研究」（主任研究者：加藤知行）班で 12 施設から集積された症例のうち，同時性肝転移例切除は 402 例であった。同時性肝転移に対する異時性肝切除は 44 例（15.8%）に行われて，原発巣切除から肝転移切除までの期間は 15～476 日で中央値は 80 日であった。同時切除と異時切除で，生存期間に差はなかった。図 5，6 に残肝無再発生存曲線，肝外無再発生存曲線を示した。残肝再発は同様であるが，肝外再発（肺）が異時切除に早く生じる傾向があることから，肝切除を待っている間に肝から肝外へ転移した可能性も考えられる。

3．肝切除待機中に，新たな転移巣がどの程度見つかるのか

同時性肝転移例で原発巣切除後，肝転移巣の異時切除を待っている間に，どの程度の頻度で新たな病変が見つかるかについての記載は，Elias の論文にあるのみであった[18]。Elias は，3～6ヵ月の待機中に 5～10%に新病変が見つかると述べている。先の[10~11]「大腸がんの肝・肺転移例に対する治療法の確立に関する研究」（主任研究者：加藤知行）班で 12 施設から集積された症例において，最大径が 2 cm 程度で個数 1～2 個の症例の割合が，異時切除例の 20%に対して，同時切除では 30%程度と多い傾向にあった（$p=0.0973$）。待機中に，肝転移の数が増えることが，示唆される。

4．当科における肝転移切除時期

当科では，肝転移が生じて間もないと考えられる最大径 2 cm 以下の同時性肝転移例は，原則的に，原発巣切除後 3ヵ月経過観察し，新しい肝病変や肝外病変の出現の有無を確認し，肝切除の適応を判断している。高橋らの肝転移単発例の肝切除後の残肝再発率と肝転移最大径との関係の検討において，5 年残肝無再発率は，最大径 5 cm 以上の症例が 49.7%であったのに対し，最大径 2 cm 未満の症例では，14.7%であった。肝転移が小さな症例では，早期に肝転移が発見されたために，肝の他の部位に画像で捉えられない，微小転移が存在している可能性があると指摘している[19]。

肝転移の大きさと予後との関係では，5 cm 以上はそれより小さいものと比較して予後は不良であるが[8,10]，それより小さな転移巣では，1 cm でも 3 cm でも予後は変わらない[20]。Finlay は，肝転移の Doubling time (DT) は造影 CT により，臨床上明らかな転移の

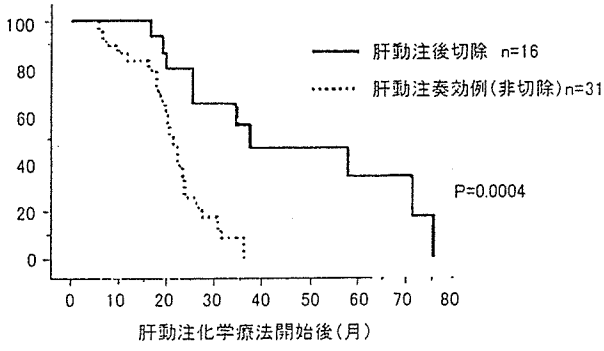


図 7 肝動注化学療法奏効例生存曲線

DT は 155 日，occult なものの DT は 86 日と報告した[21]。Tanaka らは，CTAP を用いて DT を測定し，46 日と報告している[22]。これらの DT からみると，2 cm 程度の肝転移巣が，予後の悪くなる 5 cm に達するには，3ヵ月程度はかかると考えられる。したがって，大きさ 2 cm 以内の肝転移例は原発巣切除後に 3ヵ月経過を観察し，新たな転移巣の出現を見極めてから，肝外病変の有無を判断し，肝切除を考慮しても遅くないと考えている。ただし，腫瘍が肝門部や右肝静脈近傍，ないし尾状葉にあるような症例では待機によって切除不能ないしは肝切除量が極端に大きくなる可能性があるため，同時切除が適応される。

異時性肝転移の場合，再発時期が 1 年以内のものは，予後が不良とされる[10,23]。1 年未満の再発は，同時性と同じ方針で肝，肝外病変の精査を行いながら 2〜3ヵ月待ってから，肝切除を行っている。

待機した結果，肝切除の対象が絞り込まれるためか，図 3 に示すように，肝切除後の生存期間は，他施設と比較して良好である。

5．術前化学療法後肝切除

切除不能肝転移で，転移が肝に限局する症例には肝動注化学療法が行われる。肝動注化学療法の奏効率は 60〜80%で，奏効例の 50%生存期間は約 2 年である[24]。肝動注化学療法が奏効し，肝転移巣の down stage（数や大きさの縮小）が得られ，肝の治癒切除が可能となった症例には，転移巣を切除することで予後が改善する可能性がある。筆者が経験した動注化学療法が奏効し，肝切除を施行した症例の生存曲線を図 7 に示した。

動注化学療法後の肝切除を行うという治療戦略は 1989 年より開始され，肝動注化学療法が奏効したものの切除を行わなかった症例は，1989 年以前の症例が多い。肝動注化学療法後に肝切除した症例は，肝転移巣が切除され，根治度 B になったと考えられる。この肝動注化学療法奏効後に，肝切除を行った症例の生存期間（中央値）は約 3 年であり，5 年生存率は 39%であった[25]。

Bismuth らは，高度肝転移症例は肝外病変を高頻度に合併することから，切除不能高度肝転移に 5FU＋LV＋oxaliplatin による systemic neoadjuvant chemotherapy を行い，奏効例に肝切除（一部は cryosurgery）を行って 5 年生存率 50%と良好な成績を示した[26]。

肝動注療法開始時に，肝外病変のない症例においても，約 20%に肝動注化学療法の奏効期間に肝外病変が出現する。切除不能な肝外病変を合併した例は，肝切除しても予後の改善が得られない。肝転移巣に対する治療効果と肝外病変の出現の有無の確認のために肝動注化学療法は 6〜8ヵ月継続し，肝機能の回復のために 1ヵ月の休薬の後，肝切除を行うのが良いと考える[27]。

おわりに

肝切除時期に関して，当科での方針とその根拠および文献的考察を行った。医療効率や患者の精神的負担も考えると，コンセンサスを決定するのは困難である。当科でも，患者側の肝切除を待つ間の心理的負担が大きい場合，患者の希望で同時切除を行うこともしばしばある。

個々の症例ごとに肝切除時期の判断をすることになろうが，本稿がその参考になれば幸いである。

参考文献

1) Weiss L, Grunddmann E, Hartveit F, et al.: Hematogenous metastatic patterns in colonic carcinoma: an analysis of 1541 necropsies. J Pathol 150: 195-203, 1986.
2) Kato T, Yasui K, Hirai T, et al.: Therapeutic results for hepatic metastasis of colorectal cancer with special reference to effectiveness of hepatectomy. Dis Colon Rectum 46(Suppl): S22-S31, 2003
3) 杉原健一：大腸癌肝転移の外科治療．日消外会誌 33：128-133, 2000.
4) Headrick IR, Miller DL, Nagorney DM, et al.: Surgical Treatment of hepatic and pulmonary metastases from colon cancer. Ann Thorac surg 71: 975-980, 2001.
5) Yamamoto J, Kosuge T, Shimada K, et al.: Repeat liver resection for recurrent colprectal liver resection matastases. Am J Surg 178: 275-281, 1999.
6) 高橋慶一，森　武生，安野正道：大腸癌肝転移に対する外科治療．日本外科系連合学会誌 22：229-236,

1997.
7) Kemeny N, Huang Y, Cohen A, et al. : Hepatic arterial infusion of chemotherapy after resection of hepatic metastases from colorectal cancer. N Engl J Med **341** : 2039-2048, 1999.
8) Sugihara K, Yamamoto : J surgical Treatment of colorectal liver metastases. Annales Chirurgiae et Gynaecologiae 89 : 221-224, 2000.
9) Sheele J, Stang R, Hofmann AA, et al. : Resection of colorectal liver matastases. World J Surg **19** : 59-71, 1995.
10) Fong Y, Cohen AM, Fortner JG : Liver resection for colorectal metastases. Journal of Clinical Oncology **15** : 938-946, 1997.
11) Scheele J, Stangl R, Altedorf HA, et al. : Indicators of prognosis after hepatic resection for colorectal secondaries. Surgery **103** : 278-288, 1991.
12) Elias D, Detroz B, Lasser P, et al. : Is simultaneous hepatectomy and intestional anastomosis safe? Am J Surg **169** : 254-260, 1995.
13) Martin R, Patey P, Fong Y, et al. : Simultaneous liver and colorectal resections are safe for synchronous colorectal liver metastases. J Am coll Surg **197** : 233-242, 2003.
14) Lyass S, Zamir G, Matot I, et al. : Combined colon and hepatic resection for synchronous colorectal liver metastases. Journal of Surgical Oncology **78** : 17-21, 2001.
15) Staren ED, Gambla M, Daziel DJ, et al. : Intraoperative ultrasound in the management of liver neoplasms. Am Surg **63** : 591-596, 1997.
16) Vogt P, Raab R, Ringe B, et al. : Resection of Synchronous liver metastases from colorectal cancer. World J Surg **15** : 62-67, 1991.
17) Jenkins LT, Millikan KW, Bines SD, et al. : Hepatic resection for metastatic colorectal cancer. Am Surg
18) Elias D : Impact of tumor doubling time on the therapeutic strategy : application to so-called synchronous metastases of colorectal cancers. Ann Chir **52** : 413-420, 1998 (Article in French).
19) 高橋慶一，森　武生，安野正道：大腸癌肝転移に対する切除の適応と限界．消化器科 **25** : 633-640, 1997.
20) Irie T, Itai Y, Hatsuse K, et al. : Does resection of small liver metastases from colorectal cancer improved survival of patients. The British Journal of Radiology **72** : 246-249, 1999.
21) Finlay IG, Meek D, Brunton F, et al. : Growth rate of hepatic metastases in colorectal carcinoma. Br J Surg **75**(**79** : 641-644, 1988.
22) Tanaka K, Shimada H, Miura M, et al. : Metastatic Tumor doubling time : Most important prehepatectomy predictor of survival and nonrecurrence of hepatic colorectal cancer metastasis. World J Surg **28** : 263-270, 2004.
23) Ueno H, Mochizuki H, Hatause K, et al. : Indicators for treatment strategies of colorectal liver metastases. Ann Surg **231** : 59-66, 2000.
24) Aral Y, Inaba Y, Takeuchi Y : Intermittent hepatic arterial infusion of high-dose 5-FU on a weekly schedule for liver metastases from colorectal cancer. Cancer Chemothera Pharmacol **40** : 526-530, 1997.
25) Miyanari N, Mori T, Yasuno M, et al. : Evaluation of aggressively treated patients with unresectable multiple liver metastases from colorectal cancer. Dis Colon Rectum **45** : 1503-1509, 2002.
26) Bismuth H : Neoadjuvant chemotherpy before resectIon of liver metastases from colon and rectal cancer. "Focus on Locoregional Cancer Therapy" : 113, 2000.
27) 安野正道，森　武生，高橋慶一：肝・肺転移の治療．大腸癌治療マニュアル，242-247，南江堂，2001.

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Expression of Dihydropyrimidine Dehydrogenase, Thymidylate Synthase, p53 and p21 in Metastatic Liver Tumor from Colorectal Cancer after 5-Fluorouracil-based Chemotherapy

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Abstract. *Background: The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated. However, little data is available on the expression patterns of these genes after chemotherapy. Patients and Methods: We investigated the expression of four genes, DPD, TS, p53 and p21, in the metastatic liver lesions obtained from colorectal cancer patients who had been treated with hepatic arterial infusions of 5-fluorouracil(5-FU)-based chemotherapy. Results: Expression of DPD, TS and p53 in the metastatic liver lesions was significantly higher in the chemotherapy-response group than in the no response group. In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of DPD, TS and p53. Conclusion: An analysis of genes involved in 5-FU sensitivity revealed that surviving tumor cells exhibited resistance characteristics, indicating that the chemotherapy regimen should be altered, even in partially responding cases, unless the response is pathologically complete.*

Pharmacogenetic markers of tumor cells have been intensively investigated using molecular biology technologies to predict chemosensitivity to 5-fluorouracil (5-FU) in colorectal cancer patients. Among them, *dihydropyrimidine dehydrogenase* (DPD) and *thymidylate synthase* (TS) are the most promising genes that have been used clinically in gastrointestinal cancer treatment. However, none of these genes are absolute predictors of 5-FU sensitivity.

Neoadjuvant chemotherapy has been proposed as an alternative approach to conventional surgery as an initial management strategy with the aim of improving the outcome of cancer patients. Strategies aimed at downstaging large or multifocal tumors and the control of micrometastases to enable curative resection by neoadjuvant chemotherapy have attracted much attention. Recently, in the field of breast cancer research, a sub-analysis of the National Surgical Adjuvant Breast and Bowel Project B-18 (NSABP B-18) trial revealed that a pathological complete response was the only reliable marker for selecting cases that were sensitive to a specific drug, resulting in an improved survival period (1).

In our institution, the resection of liver metastases for colorectal cancer has been actively performed after 5-FU-based chemotherapy *via* hepatic artery infusion. Although the survival advantage of hepatic arterial infusion over systemic therapy has been debated, the efficacy of this treatment with regard to tumor reduction was shown to be advantageous. We have used this procedure to treat patients with primarily unresectable metastases confined to the liver.

In this study, we examined the expression of *DPD, TS, p53* and *p21* in surgical specimens of liver metastases obtained from patients after chemotherapy.

Patients and Methods

Samples. Surgical specimens of synchronous or metachronous bilobular multiple liver metastatic tumors from 12 patients were obtained at Yokohama City University, Japan. The patients comprised 5 males and 7 females with a median age of 57.8 years (range, 41-74 years) (Table I). Since none of the 12 patients exhibited metastases at sites other than the liver, surgical resection was performed after 5-FU-based chemotherapy *via* hepatic arterial infusion. The treatment regimen was as follows: an infusion of 5-FU (500 mg/body) or 5-FU (500 mg/ body) + l-Leucovorin (150 mg/

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Key Words: Colorectal cancer, liver metastasis, chemotherapy, gene.

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Table I. Patient characteristics.

Primary tumor	Age	Gender	Response	Depth	n	DPD pri	DPD meta	TS pri	TS meta	p53 pri	p53 meta	p21 pri	p21 meta
rectum	59	F	PD+NC	se	1	2	0	0	0	0	0	1	0
rectum	41	F	PD+NC	ss	4	2	0	0	0	4	0	0	0
colon	55	M	PD+NC	se	1	0	2	0	0	0	0	0	0
colon	45	M	PD+NC	se	1	2	0	2	0	6	0	0	0
colon	51	F	PD+NC	mp	1	-	3	-	1	-	3	-	0
colon	74	M	PD+NC	si	0	1	3	1	1	0	0	0	0
colon	67	F	PR	ss	2	2	6	1	4	0	0	2	2
colon	65	F	PR	se	4	0	4	0	2	12	8	0	0
colon	54	F	PR	ss	2	1	4	1	1	9	9	2	1
rectum	69	M	PR	ss	0	0	4	2	6	9	12	6	1
colon	59	M	PR	ss	2	0	9	1	1	9	6	1	0
colon	55	F	PR	ss	1	4	4	0	3	0	0	1	0

pri: primary lesion
meta: metastatic lesion

body) + Cisplatinum (10 mg/ body) was administered every day for 5 days, and this cycle was repeated every 2 weeks for up to 4 cycles. None of the patients had received any other chemotherapy or radiotherapy treatments prior to the hepatic arterial infusions of 5-FU. Paraffin-embedded archival samples of their primary colorectal lesions were also examined. The study was approved by the institutional review board of the Yokohama City University School of Medicine, Japan.

Clinical evaluation. The chemotherapy response was determined by comparing the volume of the liver metastases before and after chemotherapy. A CT scan was performed after the 4 treatment cycles had been completed, and the results were evaluated using the World Health Organization (WHO) criteria (2). A complete response (CR) was defined as the complete disappearance of all intrahepatic tumor formation, and a partial response (PR) was defined as a reduction in the tumor volume by 50% or more, measured as the sum of the products of the two largest perpendicular diameters of all visible lesions. No change (NC) was defined as a reduction in tumor volume of less than 50% or an increase of less than 25%. An increase in tumor volume of 25% or more or the appearance of new liver lesions was defined as progressive disease (PD).

Antibodies. Rabbit anti-recombinant human *DPD* polyclonal antibody (dilution=1:500; The Second Cancer Laboratory, Taiho Pharmaceutical Co, Saitama, Japan.), *TS* polyclonal antibody (RTSSA, dilution=1:800; The Second Cancer Laboratory, Taiho Pharmaceutical Co.), mouse monoclonal antibody against *p53* protein (DO-7, dilution = 1:100; DAKO, Glostrup, Denmark) and *p21* protein (OP64, dilution=1:100; Oncogene Research Products, Cambridge MA, USA) were used as the primary antibodies for the immunohistochemical staining.

Immunohistochemistry

(i) DPD. Tissue sections (4 μm thick) were cut from each block, deparaffinized in xylene, rehydrated with graded ethanol and immersed in TBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 15 minutes. *DPD* protein expression was evaluated using the avidin-biotin complex immunohistochemical technique and a rabbit polyclonal antibody to recombinant human *DPD*. To block the nonspecific binding of the primary antibody, a normal rabbit serum (DAKO X901) dilution in TBS was used for 20 minutes. After removing the blocking solution, the *DPD* antibody (2 mg/ml) was applied for 60 minutes in a humidified chamber at room temperature. The sections were then incubated with biotin-conjugated swine anti-rabbit immunoglobulins for 20 minutes (DAKO-E353), followed by avidin-biotinylated peroxidase complex for 30 minutes. After developing the color reaction product with a freshly prepared 3,3'-diaminobenzidine chromogen solution for 5 minutes, the sections were counterstained with light hematoxylin for 10 seconds, dehydrated in a series of ethanols, cleared in xylene, mounted and covered with glass coverslips. Positive and negative controls were included in each experiment.

(ii) TS, p53 and p21. Tissue specimens (4 μm thick) were fixed in formalin and embedded in paraffin wax. After dewaxing, the sections were treated with 3% hydrogen peroxidase solution in methanol for 20 minutes to block endogenous peroxidase activity. The sections were then heated in a 0.01 M citrate buffer (pH 6.0) for 3-minute periods in a microwave oven for antigen retrieval. Non-specific antibody bindings were blocked using 10% normal bovine serum in PBS at 37°C for 15 minutes for the *p53* and *p21* staining procedures and normal goat serum for the *TS* staining procedure. The sections were then incubated with the primary antibodies described in the previous section. The sections were incubated at room temperature for 10 minutes with a byotinylated anti-mouse IgG + IgA + IgM (for monoclonal primary antibody) for *p53* and *p21* staining, and with a biotinylated anti-rabbit IgG for *TS* staining. The sections were then incubated at room temperature with peroxidase conjugated streptavidin and Elite ABC solution, respectively. The peroxidase reaction was developed using a 3,3'-deaminobenzidine tetrahydrochloride solution (Sigma Chemical Co, St. Louis, MO, USA) and 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated and mounted in a routine fashion. Positive controls and negative controls were always included in all experiments.

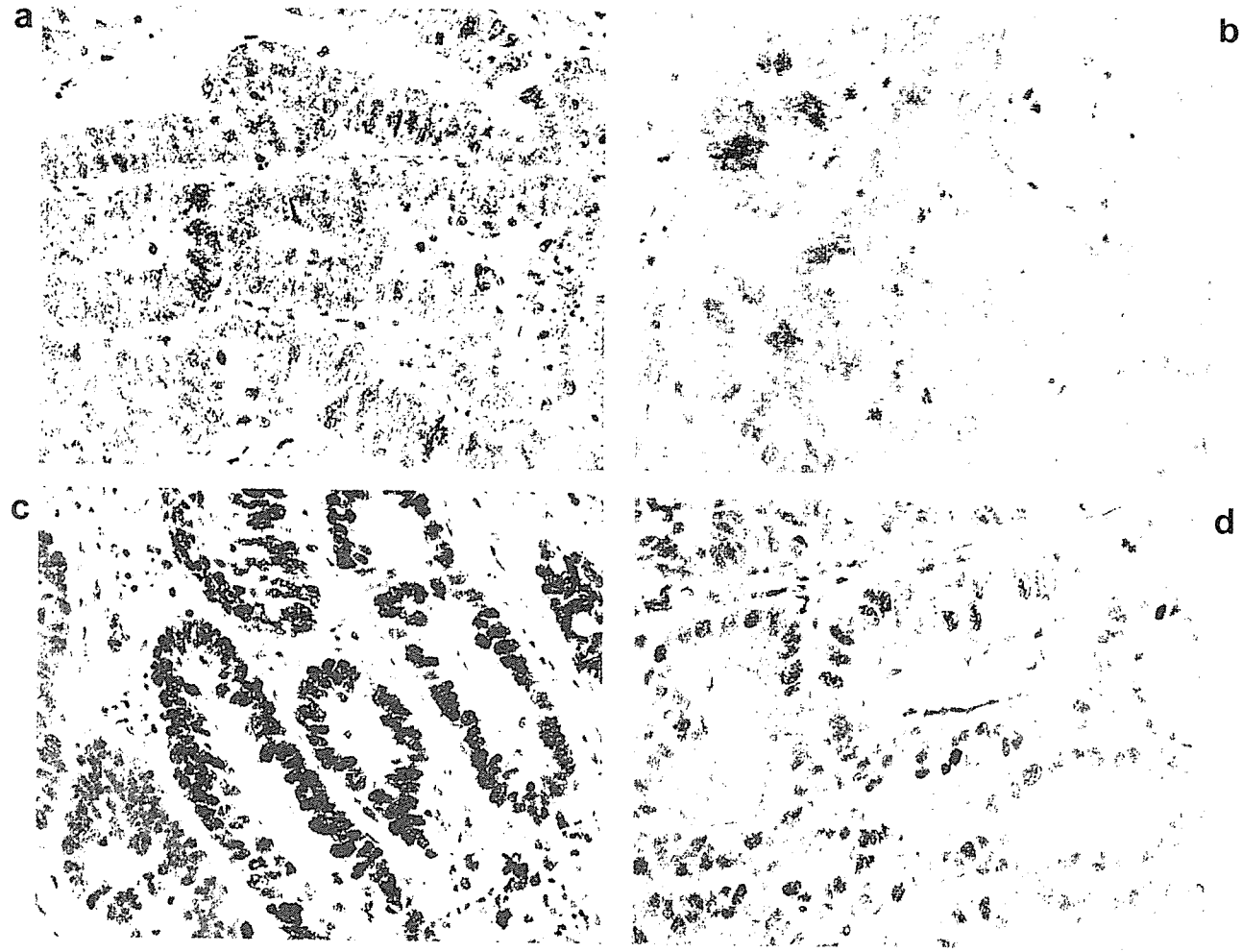

Figure 1. *Typical expression of DPD, TS, p53 and p21 genes after chemotherapy in surgical specimens of liver metastases. A: Expression of DPD, B: Expression of TS, C: Expression of p53, D: Expression of p21 (magnification, x400).*

(iii) Quantitation. Three representative fields were examined, more than 1000 tumor cells were randomly selected and the number of positive cells was counted at magnification x200. The expression of these proteins were evaluated according to the method described by Sinicrope *et al.* (3). In brief, positive-staining tumor cells were expressed as a percentage of the total number of tumor cells and assigned to one of the following five categories: class 0, ≤5%; class 1, 5% to 25%; class 2, 25% to 50%; class 3, 50% to 75%; and class 4, ≥75%. The intensity of the immunostaining was scored as follows: 1, weak; 2, moderate; 3, intense. These two scores were then multiplied. When heterogeneous levels of protein expression were found within a tumor (in multiple sections from different paraffin-embedded blocks of the same tumor), the highest protein expression score obtained for that lesion was used.

Statistical analyses. Dr. SPSS software for Windows was used for the statistical analyses; statistical significance was defined as $p<0.05$.

Results

Chemotherapy *via* hepatic arterial infusion was successfully performed in all 12 cases with no severe complications. The total 5-FU dosage was 3200±1500 mg (mean±SD). Clinical evaluations revealed that the liver tumors in 6 of the 12 patients partially responded to the treatment, although no complete responses occurred. In the remaining 6 cases, the tumors did not change in size or progressed after treatment (Table I).

Immunohistochemistry for the 4 genes was successfully performed in all 12 cases. *DPD* and *TS* were clearly observed in the cytoplasm of the cancer cells, while *p53* and *p21* were observed in the nuclei of the cancer cells. Representative cases are shown in Figure 1. Since half of the patients exhibited partial responses to the treatment,

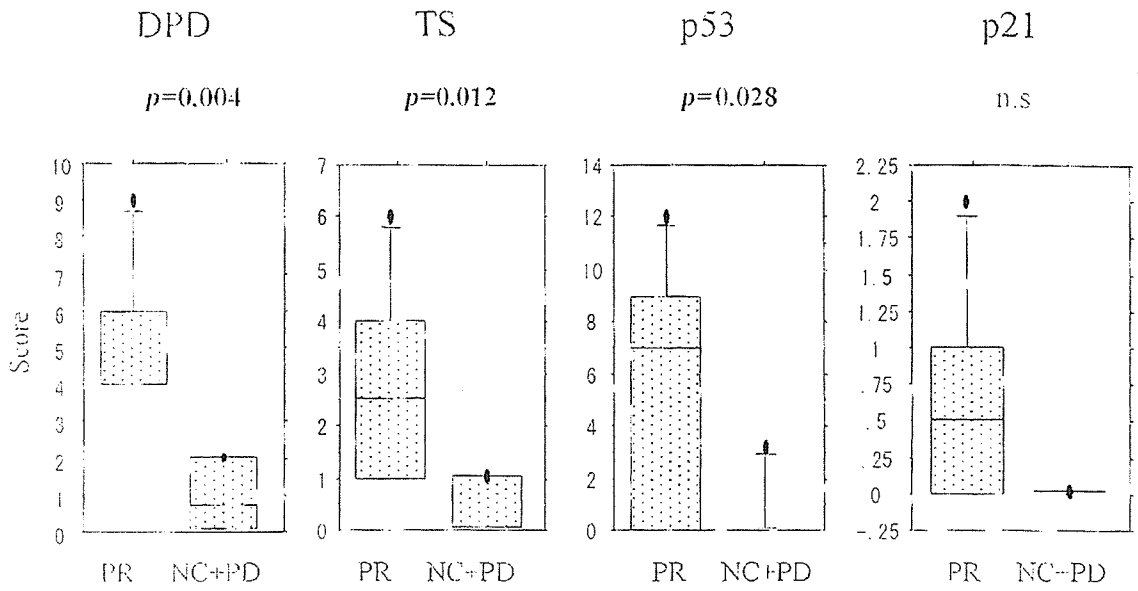

Figure 2. *Comparison of metastatic liver lesions in the PR group and the NC+PD group. The expression levels of DPD, TS and p53 were significantly higher in the PR group than in the NC+PD group ($p<0.05$).*

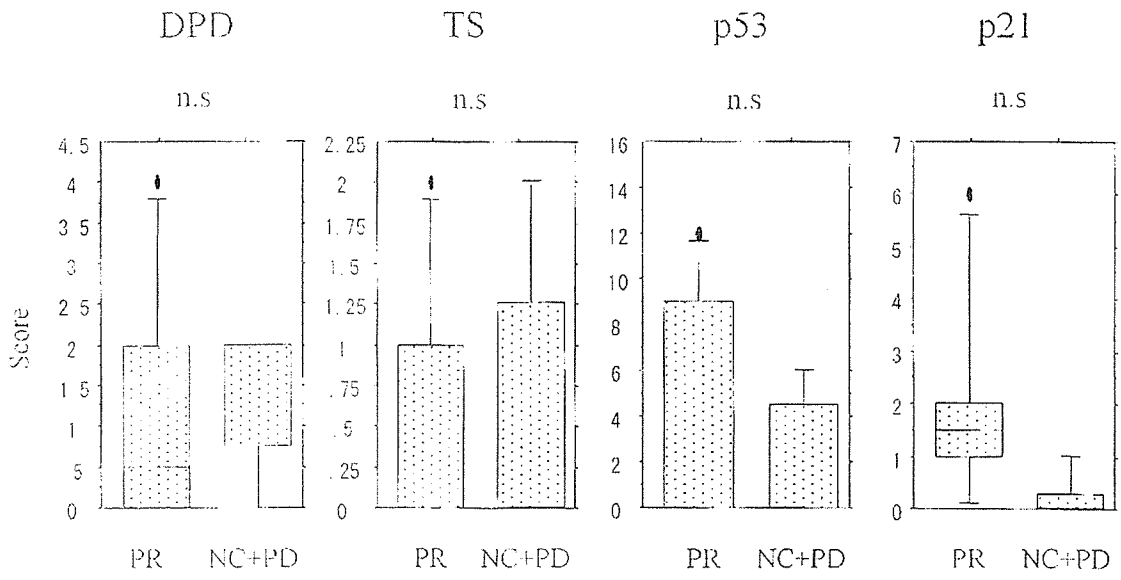

Figure 3. *Comparison of primary lesions in the PR and NC+PD groups. No significant differences in the expression levels of the 4 genes were seen between the PR and NC+PD groups.*

we compared the expression levels of these 4 genes in the chemotherapy response and no response groups. The mean scores for *DPD*, *TS* and *p53* immunoreactivity were significantly higher in the response group than in the no response group. All of the differences were statistically significant, as shown in Figure 2. However, the scores for *p21* were not significantly different between the two groups.

In the response group, viable cancer cell nests were seen in confined spaces surrounded by fibrous tissue. It was of interest that these cancer cells in the response group showed conspicuous immunoreactivity of *DPD*, *TS* and *p53*.

When the primary colorectal lesions from the archival samples were examined, no significant differences in the scores for any of the 4 genes were found between the response and no response groups (Figure 3).

Discussion

The expression of genes thought to be related to 5-FU chemosensitivity has been extensively investigated in the hope that methods for predicting 5-FU sensitivity might be established. However, little data is available on the expression patterns of these genes after chemotherapy. In our institute, the hepatic arterial infusion of 5-FU-based chemotherapy is routinely performed in patients with multiple liver metastases to improve the curative resection rate. Using surgical specimens, we examined the expression of 4 genes in liver tumors obtained from patients after chemotherapy. Although the number of cases in this study was limited, the expression rates of *DPD, TS* and *p53* were significantly higher in the response group than in the no response group. These findings suggest that the surviving tumor cells have both malignant and 5-FU-resistant characteristics.

DPD is the initial, rate-limiting enzyme in the catabolism of fluoropyrimidines, through which more than 80% of administered 5-FU is eliminated. Thus, the activity of this enzyme limits the efficacy of 5-FU treatment and is associated with tumor resistance to 5-FU (4,5). The intratumoral expression level of *TS* is considered a prognostic factor for survival in patients with colorectal cancer (6-8), although the ability of this marker to predict 5-FU chemosensitivity is controversial (9). The main pathway by which anticancer drugs induce apoptosis is a *p53*-dependent pathway (10,11). Normal *p53* protein has tumor-suppressing properties, and mutations in the *p53* gene result in the disruption of critical growth-regulating mechanisms (12-14). *p53* is also related to the malignancy of tumors and/or tumor resistance to chemotherapy. We previously reported that *p21* expression was correlated with the inhibiting activity of 5-FU (15), suggesting that *p21* may be a marker of 5-FU sensitivity.

These findings suggest two hypotheses: i) cells that are sensitive to 5-FU undergo apoptosis, but those that are resistant survive after chemotherapy, and ii) 5-FU exposure induces a mechanism that leads to drug resistance. The first hypothesis is feasible, but no direct evidence has been obtained to support this idea. However, Michael *et al.* examined *TS* expression in colorectal liver metastases after chemotherapy and found that previous fluorouracil exposure seemed to increase the resistance of the tumor cells to regional floxuridine *via TS* up-regulation (16). Nishiyama *et al.* performed an *in vitro* study on 5-FU exposure to examine changes in the expression of various genes, including *TS, DPD* and *MRP*. Although the results were very complicated, making their interpretation difficult, *DPD* and *TS* expression tended to increase in 5-FU-resistant cell lines after exposure to 5-FU (17). The mechanism of 5-FU chemoresistance is impossible to explain using the results of the present study alone.

However, the present findings may support the data obtained by the NSABP B-16 study on preoperative chemotherapy in patients with breast cancer (1). The outcome of chemotherapy was better in women whose tumors showed a pathological complete response than in women whose tumors exhibited a clinical partial response or a clinical no-response (relapse-free survival rates, 85.7%, 76.9% and 63.9%, respectively). Unless the cancer cells are totally killed by the drugs, remnant tumor cells survive and the prognosis of the patient does not improve. These findings strongly suggest that the initial chemotherapy treatment should be changed to one with a different mechanism, such as switching anthracycline to taxane, in patients with breast cancer who exhibit anything but a pathological complete response.

We also examined the expression of the 4 genes in the primary colorectal cancers obtained from this patient series, because liver metastases specimens obtained before chemotherapy were not available. No significant differences in the immunoreactivity of the 4 genes were seen between the response and the no response groups. Some differences in gene expression between the primary colorectal tumor cells and the metastatic liver tumor cells may exist. Therefore, it is not clear whether the 5-FU administration altered the expression of the 4 genes in the metastatic liver tumors after chemotherapy in the 2 groups.

Cancer chemotherapy has gradually improved with the production of new drugs exhibiting unique mechanisms and modifications. Hepatic resection after chemotherapy provides useful information enabling second-line chemotherapy treatments to be optimized. The results of this study suggest that a partial response may not be sufficient to improve the prognosis of colorectal cancer. Single-drug use limits the efficacy of treatment, while combination or sequential usage, like modified 5-FU, camptothecin and taxane regimens, may improve the prognosis of colorectal cancer patients.

References

1 Wolmark N, Wang J, Mamounas E, Bryant J and Fisher B: Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. J Natl Cancer Inst Monogr *30*: 96-102, 2001.

2 World Health Organization: WHO Handbook for Reporting Results of Cancer Treatment. WHO Offset Publication No. 48. Geneva, World Health Organization, 1979.

3 Sinicrope FA, Ruan SB, Cleary KR, Stephens LC, Lee JJ and Levin B: Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. Cancer Res *55*: 237-241, 1995.

4 Heggie GD, Sommadossi JP, Cross DS, Huster WJ and Diasio RB: Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res *47*: 2203-2206, 1987.

5 Diasio RB: The role of *dihydropyrimidine dehydrogenase* (DPD) modulation in 5-FU pharmacology. Oncology *12*: 23-27, 1998.

6 Johnston PG, Fisher ER, Rockette HE, Fisher B, Wolmark N, Drake JC, Chabner BA and Allegra CJ: The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. J Clin Oncol *12*: 2640-2647, 1994.

7 Lenz HJ, Hayashi K, Salonga D, Danenberg KD, Danenberg PV, Metzger R, Banerjee D, Bertino JR, Groshen S, Leichman LP and Leichman CG: p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. Clin Cancer Res *4*: 1243-1250, 1998.

8 Aschele C, Debernardis D, Casazza S, Antonelli G, Tunesi G, Baldo C, Lionetto R, Maley F and Sobrero A: Immunohistochemical quantitation of thymidylate synthase expression in colorectal cancer metastases predicts for clinical outcome to fluorouracil-based chemotherapy. J Clin Oncol *17*: 1760-1770, 1999.

9 Findlay MP, Cunningham D, Morgan G, Clinton S, Hardcastle A and Aherne GW: Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. Br J Cancer *75*: 903-909, 1997.

10 Takeda A, Nakajima K, Shimada H, Imaseki H, Takayama W, Hayashi H, Suzuki T, Ochiai T and Isono K: Clinical significance of serum p53 antibody detection on chemosensitivity assay in human colorectal cancer. J Surg Oncol *71*: 112-116, 1999.

11 Levine AJ: The p53 tumor-suppressor gene. N Engl J Med *326*: 1350-1352, 1992.

12 Chang F, Syrjanen S, Tervahauta A and Syrjanen K: Tumourigenesis associated with the p53 tumour suppressor gene. Br J Cancer *68*: 653-661, 1993.

13 Vogelstein B and Kinzler KW: p53 function and dysfunction. Cell *70*: 523-526, 1992.

14 el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell *75*: 817-825, 1993.

15 Hosaka N, Ichikawa Y, Ishikawa T, Nagashima Y, Kunisaki C, Takahashi M, Moriwaki Y, Akiyama H, Yamaguchi S, Ota M, Ooki S, Ike H and Shimada H: Correlation of immunohistochemical p53 labeling index with inhibition rate in chemosensitivity test in gastric and colon cancer. Anticancer Res *21*: 229-235, 2001.

16 Davies MM, Johnston PG, Kaur S and Allen-Mersh TG: Colorectal liver metastasis thymidylate synthase staining correlates with response to hepatic arterial floxuridine. Clin Cancer Res *5*: 325-328, 1999.

17 Nishiyama M, Yamamoto W, Park JS, Okamoto R, Hanaoka H, Takano H, Saito N, Matsukawa M, Shirasaka T and Kurihana M: Low-dose cisplatin and 5-fluorouracil in combination can repress increased gene expression of cellular resistance determinants to themselves. Clin Cancer Res *5*: 2620-2628, 1999.

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Clinical Trials

Feasibility of autonomic nerve-preserving surgery for advanced rectal cancer based on analysis of micrometastases

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Background: Autonomic nerve preservation has been advocated as a means of preserving urinary and sexual function after surgery for rectal cancer, but may compromise tumour clearance. The aim of this study was to determine the incidence of micrometastasis in the connective tissues surrounding the pelvic plexus.

Methods: The study included 20 consecutive patients who underwent rectal surgery with bilateral lymph node dissection for advanced cancer. A total of 78 connective tissues medial and lateral to the pelvic plexus and 387 lymph nodes were sampled during surgery. All connective tissue samples and 260 lymph nodes were examined for micrometastases by reverse transcriptase–polymerase chain reaction (RT–PCR) after operation. All patients were followed prospectively for a median of 36·0 months.

Results: Of 245 histologically negative lymph nodes, 38 (15·5 per cent) were shown by RT–PCR to harbour micrometastases. However, micrometastases to tissues surrounding the pelvic plexus were detected in only two (3 per cent) of 78 tissues, that is in two of 20 patients. Clinical follow-up showed that the two patients had a poor prognosis owing to distant metastases.

Conclusion: Autonomic nerve-preserving surgery may be feasible for advanced rectal cancer, but study of more patients positive for micrometastases is required.

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Introduction

The complex flow patterns of lymphatic fluid around the rectum were investigated in 1951 by Sauer and Bacon[1]. They regarded the lateral ligament as the tissue in which the inflow of lymphatic fluid from the rectum first occurs *en route* to the internal iliac artery. However, the high incidence of urinary and sexual dysfunction after rectal cancer surgery gradually led surgeons to realize that the lateral ligament plays an important role in postoperative urinary and sexual function[2]. The pelvic autonomic nerve system is comprised of the superior hypogastric plexus, hypogastric nerves, pelvic plexus (inferior hypogastric plexus) and pelvic splanchnic nerves[3]. The lateral ligament of the rectum consists of a neurovascular bundle derived from the combined redistributing sympathetic and parasympathetic nerves of the system, and dissection injury to these nerves may cause problems.

Inclusion of total mesorectal excision (TME) in rectal cancer surgery can improve the rate of cure[4]. Dissection of the rectum at the layer of TME allows preservation of the pelvic autonomic nerves, reducing postoperative urinary and sexual morbidity[5–7]. Extensive pelvic lymphadenectomy involving resection of both pelvic autonomic nerves and lateral lymph nodes was employed in Japan from the 1970s to the early 1980s with the aim of reducing local recurrence of rectal cancer[8]. However, postoperative urinary and sexual dysfunction invariably ensued, leading to marked reduction in quality of life. More recently, efforts have been made to preserve urinary and sexual function and to achieve local control of cancer, by a combination of autonomic nerve-preserving surgery and lateral lymph node dissection[9–12].

Recent pathological studies have warned against autonomic nerve preservation because histologically proven cancer foci might exist in the autonomic nerves

and surrounding connective tissues, and lead to local recurrence[13,14]. To aim of the present study was to examine the incidence of micrometastases in the region of the pelvic plexus by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, which is more sensitive than conventional histological diagnosis[15,16].

Patients and methods

The study included 20 consecutive patients who underwent rectal cancer surgery with bilateral lymph node dissection at the Graduate School of Medicine, Osaka University between October 1999 and May 2001. Demographic data are shown in *Table 1*. Tumours were located 0–10 (median 6·5) cm above the anal verge and diagnosed before surgery as advanced cancer (T2,T3 and T4)[17]. The operative procedures are summarized in *Table 1*. Curative resection was performed in all patients. Tumour stages determined by postoperative histological examination are shown in *Table 1*[17]. Two patients with lateral node metastasis without distant organ metastasis were included in stage III (*Table 2*). Nine patients with stage III or IV tumours were treated with 5-fluorouracil-based chemotherapy but none had radiation therapy. The patients were reviewed at least every 3 months after operation with blood tests such as measurement of carcinoembryonic antigen (CEA), and underwent computed tomography (CT) or magnetic resonance imaging (MRI) every 6 months. The study protocol was approved by the Human Ethics Review Committee of Osaka University Graduate School of Medicine and a signed consent form for the study was obtained from each patient.

Table 1 Clinical characteristics of patients

Median (range) age (years)	63·5 (35–92)
Sex ratio (M : F)	15 : 5
Surgical procedure	
Low anterior resection	9
Abdominoperineal resection	7
Hartmann resection	3
Total pelvic exenteration	1
Autonomic nerve preservation	
Bilateral	18
Unilateral	2
TNM stage	
I	4
II	7
III	6
IV	3
Histological type	
Well differentiated	1
Moderately differentiated	16
Poorly differentiated	2
Mucinous	1
Tumour location (cm)*	
< 6	9
≥ 6	11

*Distance from anal verge. TNM, tumour node metastasis.

All patients underwent radical resection with autonomic nerve preservation and lymph node dissection[11]. The following is a brief description of the surgical procedures, with schematic presentation in *Fig. 1*, focusing on the collection of specimens. After isolating the sigmoid colon, the mesorectum was dissected from the parietal fascia of the sacrum at the layer of TME to preserve the hypogastric nerves, and the medial side of the pelvic plexus was exposed. At this stage, the connective tissues of the medial and lateral sides of the pelvic plexus were meticulously sampled taking care not to injure the plexus, with four specimens being obtained from each patient. However, if the tumour had macroscopically infiltrated the pelvic plexus, only tissue from the lateral side was collected because the infiltrated pelvic plexus was dissected *en bloc* with the tumour. The specimens were promptly frozen in liquid nitrogen and stored at −70°C pending RNA extraction. A total of 78 connective tissues were obtained from 20 patients (*Table 2*).

After removing the tumour and performing lateral node dissection, the lymph nodes were sampled from the mesorectum and the lateral area along the internal iliac artery and the obturator nerve outside the boundaries of TME (*Fig. 1*). In 12 patients, each of the 260 lymph

Table 2 Metastasis to lymph nodes and tissues surrounding pelvic plexus

		Site of metastasis			Survival at 1 year	
TNM stage	No. of patients	Upper lymph nodes (HE)	Lateral lymph nodes (HE)	Tissues surrounding pelvic plexus (micrometastases)	Relapse free	Overall
I	4	0	0	0 (16)	4	4
II	7	0	0	0 (28)	6	7
III	6	6	2	0 (24)	5	6
IV	3	3	3	2 (10)	0	0

Values in parentheses are number of tissues; HE metastasis detected by histological examination with haematoxylin and eosin staining. TNM, tumour node metastasis.

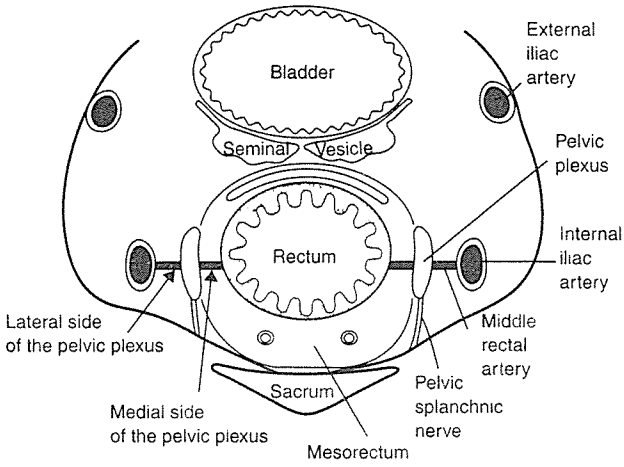

Fig. 1 Schematic presentation of the pelvic autonomic nerve system and connective tissues surrounding the pelvic plexus. The connective tissues comprise fat and lymphatic vessels including the middle rectal artery. The medial tissue is equivalent to the lateral ligament and the lateral tissue is located outside the boundaries of total mesorectal excision, between the plexus and internal iliac artery

node samples was halved, with one part being subjected to conventional histological diagnosis and the other being frozen in liquid nitrogen and stored at −70°C until extraction of RNA. In the other eight patients, 127 samples were collected and subjected to histological diagnosis only.

Total RNA was extracted by a single-step method as described previously[18]. Complementary DNA was generated with avian myeloblastosis virus RT using the procedure outlined by the supplier (Promega, Madison, Wisconsin, USA). *CEA* and *cytokeratin 20* (*CK-20*) transcripts were used as sensitive markers for micrometastases, and *phorphobilinogen deaminase* (*PBGD*) transcript was used to check for the presence of mRNA in samples[15]. PCR products were analysed by electrophoresis on 2 per cent agarose gels stained with ethidium bromide. The reproducibility of cDNA products was checked by repeated RT–PCR and gel electrophoresis. The sensitivity of PCR was determined by detecting *CEA* and *CK-20* transcripts in serial dilutions of a human colonic cancer cell line (HT29) mixed with human lymphocytes; the detection sensitivity was 100 HT29 cells among 10^6 lymphocytes.

Results

The presence of *PBGD* products was confirmed in all 78 connective tissue specimens and 260 lymph nodes examined for micrometastases by RT–PCR. The diagnosis was positive if bands specific for *CEA* or *CK-20* were present[15].

The 260 lymph nodes were collected from 12 patients (median 22 per patient). The appearance of both *CEA* and *CK-20* mRNAs was verified in all 15 histologically positive lymph nodes. Of the 245 histologically negative lymph nodes, 38 (15·5 per cent) harboured micrometastases (*Table 3*).

Metastases were identified histologically in the upper lymph nodes within the mesorectum in nine of 20 patients, and in the lateral lymph nodes along the internal iliac artery and the obturator nerve in five of 20, suggesting that many patients had very advanced cancer. Direct tumour invasion into the right or left pelvic plexus was observed macroscopically in two patients with stage IV disease. The pelvic plexus on the affected side was therefore resected *en bloc* with the tumour, whereas that on the other side was preserved.

Eighteen of 20 patients had neither *CEA* nor *CK20* mRNA in the four connective tissues surrounding the bilateral pelvic plexus. Two of three patients with stage IV disease were positive for both *CEA* and *CK20* mRNA in the lateral tissue from the resected pelvic plexus, but all other tissues surrounding the preserved pelvic plexus were negative. Micrometastases were identified in two (3 per cent) of 78 samples of connective tissue surrounding the pelvic plexus, that is in two of 20 patients (*Table 2*). All five patients with lateral node metastasis had metastasis to the upper lymph nodes, but only two had micrometastases to the connective tissues surrounding the pelvic plexus.

Median follow-up after surgery was 36·0 months. By 1 year after surgery, five of 20 patients had developed local recurrence or distant metastases, despite undergoing curative surgery and postoperative chemotherapy, and three had died from cancer (*Table 2*). Of three patients with pelvic recurrence, one patient showed relapse in the lateral area of the pelvis despite lateral node dissection, and two patients developed recurrent tumour in the rectum after a Hartmann's procedure. The autonomic

Table 3 Lymph node metastases detected by reverse transcriptase–polymerase chain reaction and histological examination with haematoxylin and eosin staining

	RT-PCR		
	Positive	Negative	Total
Haematoxylin and eosin			
Positive	15	0	15
Negative	38	207	245

RT–PCR, reverse transcriptase–polymerase chain reaction.

nerve system was preserved completely in 18 patients and unilaterally in two; no recurrence was found in this region by repeated follow-up CT and MRI. The overall survival rate, estimated by the Kaplan–Meier method, of the 18 patients without micrometastases surrounding the pelvic plexus was 94 per cent at 1 year and 88 per cent at 3 years. Neither of the patients with micrometastases was alive at 1 year after surgery.

Discussion

The lateral ligament is still regarded as a pathway of lymphatic vessels (middle lymphatic flow) from the lower rectum towards the lateral lymph nodes[1,19]. However, in the present study micrometastases to the connective tissues, including the lateral ligament, were identified by highly sensitive RT–PCR analysis in only two patients with distant metastases. Three of five patients with both upper and lateral lymph node metastases had no micrometastases in the connective tissues. A partial explanation for the discrepancy between the presence of lymph node metastases and the very low incidence of micrometastases to the connective tissues might be that lateral lymph node metastases developed via lower lymphatic flow rather than via middle lymphatic flow through the lateral ligament[19].

The autonomic nerve system was completely preserved in all but two patients in the present study, However, no local recurrence in the region of the preserved nerve system was observed by CT and MRI during follow-up. Contrary to expectation, micrometastases to the connective tissue surrounding the pelvic plexus were rare, verifying the feasibility of nerve preservation without oncological compromise in most patients.

Neither patient with micrometastases in the tissues surrounding the pelvic plexus survived for 1 year after surgery. Ueno *et al.*[14] performed complete dissection of the autonomic nerve system and pelvic lymph nodes with the aim of achieving local control in 61 patients with rectal cancer. They reported spread of cancer cells to the autonomic nerves in nine patients (15 per cent), six with Dukes' C and three with Dukes' 'D' lesions. The patients with Dukes' C tumours underwent curative radical resection, but all developed recurrence within 1 year and none survived for 4 years. The circumferential resection margin for TME is located inside the pelvic plexus whereas the pelvic nerve plexus and the lateral tissue are situated outside the margin. It has also been documented that TME in patients with tumour involvement of circumferential resection margin is associated with a poor prognosis[20].

The present results indicate that any patients with micrometastases in the preserved pelvic plexus already have advanced cancer, so their prognosis is unlikely to be affected by local recurrence that might develop if the autonomic nerves are preserved. Management of such patients should focus on maximizing the quality of remaining life.

The follow-up period in the present study was relatively short (median 36·0 months). However, some 50–80 per cent of local recurrences occur within 2 years after rectal cancer surgery, with a peak at 6–12 months[21]. The follow-up period should therefore have been sufficient for the analysis.

Based on examination of micrometastases, these results suggest that the autonomic nerve system should be preserved wherever possible, even in surgery for advanced rectal cancer. However, study of more patients positive for micrometastases is needed.

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References

1 Sauer I, Bacon HE. Influence of lateral spread of cancer of the rectum on radicability of operation and prognosis. *Am J Surg* 1951; **81**: 111–120.
2 Bernstein WC, Bernstein EF. Sexual dysfunction following radical surgery for cancer of the rectum. *Dis Colon Rectum* 1966; **9**: 328–332.
3 Lee JF, Maurer VM, Block GE. Anatomic relations of pelvic autonomic nerves to pelvic operations. *Arch Surg* 1973; **107**: 324–328.
4 Heald RJ, Ryall R. Recurrent cancer after restorative resection of the rectum. *Br. Med. J. (Clin Res Ed)* 1982; **284**: 826–827.
5 Murty M, Enker WE, Martz J. Current status of total mesorectal excision and autonomic nerve preservation in rectal cancer. *Semin Surg Oncol* 2000; **19**: 321–328.
6 Pocard M, Zinzindohoue F, Haab F, Caplin S, Parc R, Tiret E. A prospective study of sexual and urinary function before and after total mesorectal excision with autonomic nerve preservation for rectal cancer. *Surgery* 2002; **131**: 368–372.
7 Enker WE. Potency, cure, and local control in the operative treatment of rectal cancer. *Arch Surg* 1992; **127**: 1396–1401; discussion 1402.
8 Koyama Y, Moriya Y, Hojo K. Effects of extended systematic lymphadenectomy for adenocarcinoma of the rectum – significant improvement of survival rate and decrease of local recurrence. *Jpn J Clin Oncol* 1984; **14**: 623–632.
9 Matsuoka N, Moriya Y, Akasu T, Fujita S. Long-term outcome of urinary function after extended

lymphadenectomy in patients with distal rectal cancer. *Eur J Surg Oncol* 2001; **27**: 165–169.
10 Saito N, Koda K, Nobuhiro K, Takiguchi K, Oda K, Soda H *et al.* Nerve-sparing surgery for advanced rectal cancer patients: special reference to Dukes C patients. *World J Surg* 1999; **23**: 1062–1068.
11 Mori T, Takahashi K, Yasuno M. Radical resection with autonomic nerve preservation and lymph node dissection techniques in lower rectal cancer surgery and its results: the impact of lateral lymph node dissection. *Langenbecks Arch Surg* 1998; **383**: 409–415.
12 Maas CP, Moriya Y, Steup WH, Kiebert GM, Kranenbarg WM, van de Velde CJ. Radical and nerve-preserving surgery for rectal cancer in The Netherlands: a prospective study on morbidity and functional outcome. *Br J Surg* 1998; **85**: 92–97.
13 Yamakoshi H, Ike H, Oki S, Hara M, Shimada H. Metastasis of rectal cancer to lymph nodes and tissues around the autonomic nerves spared for urinary and sexual function. *Dis Colon Rectum* 1997; **40**: 1079–1084.
14 Ueno H, Mochizuki H, Fujimoto H, Hase K, Ichikura T. Autonomic nerve plexus involvement and prognosis in patients with rectal cancer. *Br J Surg* 2000; **87**: 92–96.
15 Miyake Y, Yamamoto H, Fujiwara Y, Ohue M, Sugita Y, Tomita N *et al.* Extensive micrometastases to lymph nodes as a marker for rapid recurrence of colorectal cancer: a study of lymphatic mapping. *Clin Cancer Res* 2001; **7**: 1350–1357.
16 Noura S, Yamamoto H, Ohnishi T, Masuda N, Matsumoto T, Takayama O *et al.* Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. *J Clin Oncol* 2002; **20**: 4232–4241.
17 Sobin LH, Wittekind C (eds). *UICC: TNM Classification of Malignant Tumours* (6th edn). Wiley-Liss: New York, 2002.
18 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
19 Takahashi T, Ueno M, Azekura K, Ohta H. Lateral ligament: its anatomy and clinical importance. *Semin Surg Oncol* 2000; **19**: 386–395.
20 Birbeck KF, Macklin CP, Tiffin NJ, Parsons W, Dixon MF, Mapstone NP *et al.* Rates of circumferential resection margin involvement vary between surgeons and predict outcomes in rectal cancer surgery. *Ann Surg* 2002; **235**: 449–457.
21 Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T *et al.* Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638–646.

Methylation and expression of p16^{INK4} tumor suppressor gene in primary colorectal cancer tissues

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Abstract. It is known that p16^{INK4} tumor suppressor gene expression in colon cancer cells is repressed by methylation at the CpG island of promoter, but *in vivo* silencing of p16 gene is not fully understood. Some studies showed that primary colorectal cancer (CRC) tissues often overexpress the p16 protein, while others showed the high incidence of p16 methylation. The aim of this study was to clarify p16 gene regulation *in vivo*. We used real-time methylation-specific PCR (MSP) to examine density of p16 methylation, and immunohistochemistry, Western blot analysis to determine p16 protein expression. Methylation was detected in 5 CRC cell lines tested and 9 of 21 (42.9%) CRCs. Four of 5 CRC cell lines did not express p16 mRNA, but 6 of 9 CRCs did express p16 mRNA even with methylation. Real-time MSP showed that CRC tissues had a wide variety in methylation density (methylation index: 0.28-0.91) and that highly methylated CRC tissues displayed significantly lower p16 mRNA expression than those with no-methylation or low-methylation. Immunohistochemistry showed that the majority of CRCs (53 of 55: 96.4%) overexpressed the p16 protein. Low p16 expression was associated with lymph node metastasis (p=0.003) and large tumor size (p=0.048). Western blot in a subset of non-tumor and tumor samples showed a consistent overexpression of the p16 protein. These results showed that CRC tissues displayed variable methylation density, which may be characteristics of p16 gene methylation *in vivo*. Our data suggest that a low p16 expression due to methylation may contribute to tumor enlargement and expansion of CRC.

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Key words: p16^{INK4}, colorectal cancer, methylation density, methylation-specific PCR

Introduction

The progression of the cell cycle is controlled by the cyclin-dependent kinase (CDK)/cyclin complex countered by CDK inhibitors (CKIs) (1,2). Cyclin D1/CDK4, CDK6 and cyclin E/CDK2 control the progression from the G1 to S phase of the cell cycle. Based on their structural and functional characteristics, CKIs are classified into two groups, the p21^{Waf1} family, which includes p27^{Kip1} and p57^{Kip2}, and another group, which consists of p16^{INK4a} (hereafter designated p16), p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. The p16 gene is localized on chromosome 9p21 and the p16 family can form complexes with CDK4, CDK6 and D-type cyclins (3-5). Overexpression of INK4 proteins can arrest cells in the G1 phase through inhibition of cyclin D/CDK activity (6). On the other hand, p16-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens, suggesting that p16 is a tumor suppressor gene (7). Indeed, deletions and mutations of the p16 gene are frequently present in primary cancers of the brain, biliary tract, lung, pancreas and esophagus (8-12), although a panel of cancer cell lines tends to retain p16 gene alterations more frequently (3). There is also evidence that p16 expression is down-regulated by *de novo* methylation of 5' CpG islands in the p16 promoter region (13,14).

In colorectal cancer (CRC) cell lines, expression of the p16 protein was reported to be undetectable and inactivation of p16 is thought to be a common alteration in CRC (15). It appears that deletion or loss of the p16 gene is rare in CRC (13,16,17) and the alternative pathway for inactivation of the p16 gene is thought to be methylation of its promoter (13,14). In CRC tissues, there is evidence that the p16 gene promoter is methylated in 29-55% of primary cancer tissues (13,18-22), whereas in non-neoplastic colonic mucosa the p16 promoter is hardly methylated (18,23). These findings invoke the notion that p16 gene inactivation via methylation may be involved in carcinogenesis of the colorectum.

However, recent immunohistochemical studies have shown that the majority of CRC tissues (64-82%) expressed the p16 protein, while normal mucosa displayed negative or very low expression (24,25). This overexpression of p16 in CRC tissues

is somewhat paradoxical in view of the possible role of p16 as a tumor suppressor gene, indicating that the above scenario on human CRC tumorigenesis via p16 inactivation may not be quite as simple *in vivo*. The question then arises as to how methylation and overexpression of the p16 gene can coexist in a significant fraction of CRC tissues? One clue to this puzzle might be the difference in methylation density of the CpG island in the p16 promoter between cell culture systems and primary CRC tissues, since it is reported that the level of transcriptional repression is dependent on methylation density (26,27). Although many studies have examined the incidence of p16 methylation alone or p16 expression alone *in vivo* (18-23), no study has yet simultaneously examined the degree of methylation and p16 gene expression in CRC tissues. To address this question, we examined p16 methylation and p16 gene expression in CRC tissue samples together with control experiments using CRC cell lines. Real-time methylation-specific PCR (MSP) was also performed in methylation-positive CRC tissue samples. An extended immunohistochemical analysis was conducted to elucidate the functional significance of the p16 protein in CRC tissues. Our data provide a rationale for methylation-associated regulation of p16 expression in primary CRC tissues.

Materials and methods

Cell lines and tissues. The human breast epithelial cell line HBL100, human CRC cell lines HCT116, SW480, LoVo, HT29, DLD1, human breast cancer cell line MDAMB468, and human glioblastoma cell line T98G were obtained from the Japanese Cancer Research Resources Bank and ATCC (American Type Culture Collection). These cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) or DMEM medium (Nikken Bio Medical Laboratory, Kyoto, Japan), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum at 37˚C in 5% CO_2.

A total of 55 samples of CRC tissues together with adjacent non-neoplastic mucosa were obtained from patients who underwent surgery at the Department of Surgery and Clinical Oncology, Osaka University, between 2000 and 2001. The age of the patients ranged from 28 to 81 years (mean age: 61 years). The resected surgical specimens were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin. Tissue sample of sufficient quantity was frozen immediately in liquid nitrogen and stored at -80˚C until use for reverse transcription-polymerase chain reaction (RT-PCR), methylation assay or immunoblotting. Samples of non-neoplastic mucosa were excised at least 5 cm lateral to the tumor. The study protocol was approved by the Human Ethics Review Committee of Osaka University, Graduate School of Medicine.

Immunostaining. The tissue specimens fixed in 10% buffered formalin were sliced into 4-µm thick sections, deparaffinized in xylene and rehydrated with graded concentrations of ethanol. Immunostaining was performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) after boiling for antigen retrieval, as described in our previous studies (28,29). Anti-$p16^{INK4}$ polyclonal antibody, which was raised against a full-length recombinant GST-p16 fusion protein, was purchased from PharMingen (San Diego, CA). The primary antibody was applied to the sections at a dilution of 1:400. The human breast epithelial cell line HBL100, which expresses a high level of p16, and the glioblastoma cell line T98G in which the p16 gene is inactivated by homozygous deletion, were used as positive and negative controls, respectively (30). For the absorption test, the immunogen was obtained from PharMingen.

Evaluation of immunohistochemistry. Inflammatory cells served as positive internal controls and nuclear staining for p16 was considered positive as reported previously (30-32). Ten fields in each specimen were randomly selected and examined under high power magnification and >500 cells were counted in order to determine the labeling index (LI), which represented the percentage of cells that were p16-positive. The samples were then classified into three groups according to the value of LI: group A consisted of tissue samples that contained >50% p16-positive cells, group B contained 10-50% p16-positive cells, and group C contained <10% p16-positive cells. Staining was repeated at least twice to eliminate possible technical errors, and the results were reproducible.

Western blot analysis. Approximately $5x10^6$ cells or 100 mg of tissue were homogenized and lysed in 1.0 ml of lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, and then placed on ice for 10 min. The lysates were clarified by centrifugation at 15,000 x g for 25 min at 4˚C. The protein samples were subjected to SDS-PAGE (15% gels) and immunoblotting was performed as described in our previous studies (28-30). The final dilution of the primary antibody was 1:1,000.

RNA extraction and RT-PCR analysis. Total RNA was extracted with a single-step method using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Semi-quantitative analysis for expression of p16 mRNA was performed by the multiplex RT-PCR technique, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal standard (33,34). To minimize the inter-PCR difference, PCR was performed with GAPDH and p16 primers in identical tubes under unsaturated conditions, as described in our previous studies (33,35,36). PCR reactions were performed in a total volume of 25 µl of reaction mixture containing 2 µl of cDNA template, 1X Universal PCR buffer, 2 mM deoxynucleotide triphosphates, 20 pmol of primer for p16, 4 pmol of each primer for GAPDH, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Alameda, CA). The primer set for p16 was designed to be localized in exon 1 and exon 2 of the p16 gene, flanking intron 1 and tested to ensure amplification of cDNA only, so that amplification of potentially contaminating genomic DNA could be avoided. The sequences of these PCR primers were as follows: p16 sense primer, 5'-AGC CTT CGG CTG ACT GGC TGG-3'; p16 antisense primer, 5'-CTG CCC ATC ATC ATG ACC TGG A-3'. The primers for GAPDH were synthesized as described previously (34). The sizes of the