厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

ヒト肝細胞がんにおけるGlypican-3(GPC3)免疫染色の有用性に関する研究

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研究要旨

Glypican-3(GPC3)の発現は肝細胞がん特異的であり、免疫組織化学的解析により、肝臓がんにおける肝細胞がん成分と胆管細胞がん成分の鑑別に、GPC3の免疫染色が極めて有用であることが示された。肝細胞がん107例において、GPC3染色陽性群(n=87)とGPC3染色陰性群(n=20)を比較すると、5年生存率は54.5%と87.7%であり、GPC3を発現しない肝細胞がんでは、発現するものに比べ、有意に予後が良好であった。また、今回GPC3染色を施行した肝細胞がんの約10%において、非癌部のごく狭い領域にGPC3染色陽性所見を認めた。肝細胞がんに対するGPC3免疫染色は、鑑別診断および予後予測に有用であることが示された。

A. 研究目的

近年、ヒト肝細胞がん(Hepatocellular carcinoma: HCC)において、Glypican-3(GPC3)が高率に発現することが報告されている。GPC3はヘパラン硫酸プロテオグリカンに属し、65kDのコアタンパクを有する膜タンパクである。細胞膜上ではGlycophosphatidylinositolを介してアンカーされている。このGPC3の機能は未だ解明されていない。今回、我々はHCCにおけるGPC3発現について、免疫染色の有用性とGPC3発現の意味について検討した。

B. 研究方法

国立がんセンター東病院にて肝切除を施行された症例を対象とし、切除標本のホルマリン固定パラフィンブロックを薄切して組織標本スライドを作製し、免疫染色により免疫組織化学的な検討を行った。まずGPC3免疫染色によるHCCと胆管細胞がん(ICC)の鑑別診断の有効性を検討するために、1992年から2006年の間に肝切除された症例のうち、年齢、性別、手術年代、腫瘍径、組織型をマッチングさせたHCC 46例とICC 28例およびCombined HCC and CCC 11例を対象とした。HCCについてはAFP免疫染色も行った。次にHCCのGPC3発現と予後との関連を検討した。対象は2001年1月から2002年12月に同じく当院にて肝切除が施行されたHCC 107例を対象とした。GPC3免疫染色には、マウス抗ヒトGPC3モノクローナル抗体(1G12, Biomosaic社)を用いた。

C. 研究結果

HCCのGPC3染色においては、膜型、びまん型、顆粒型の3つの染色パターンを呈した。組織型別では、高分化型9/15(60%)、中分化型16/18(89%)、低分化型11/13(85%)、計36/46(78%)であった。これに対し、AFP染色では、高分化型3/15(20%)、中分化型6/18(33%)、低分化型7/13(54%)、計16/46(35%)であった。ICCでは、高分化型0/8(0%)、中分化型0/10(0%)、低分化型0/10(0%)、計0/28(0%)であり、GPC3の発現を認めなかった。Combined HCC and CCC症例において、切除時に腫瘍割面が肉眼的にICCと診断された11例中3例のGPC3染色は陰性であったが、肉眼的にHCCあるいはCombined HCC and CCCと診断された8例では、組織学的HCCの部分にのみGPC3染色陽性所見を認めた。以上より、HCCとICCの鑑別にGPC3の免疫染色が極めて有用であることが示された。

GPC3染色性と予後の検討では、GPC3染色陽性は87/107(81%)であった。GPC3陽性群(n=87)とGPC3陰性群(n=20)を比較すると、組織型(高分化型/中分化型/低分化型)について、6/55/26と6/8/6であり、GPC3陰性群で高分化型が多い傾向があった(p = 0.010)。患者状態、肝機能、手術、腫瘍状況、病理組織などの各因子と予後についての解析を行った。単変量解析にて有意差をもって影響を及ぼす因子は、全生存期間についてHBsAg陽性(p = 0.0113)、HCV陽性(p = 0.0043)、ICG R15値(p = 0.0466)、術前PIVKA-II値(p = 0.016)、肝切除≧Segmentectomy(p = 0.005)、再発例(p = 0.0191)、腫瘍個数多発(p = 0.0091)、病理学的胆管侵襲あり(p = 0.0035)、肝内転移あり(p<0.0001)、切除断端陽性(p = 0.0045)と、GPC3染色陽性(p = 0.0253)であった。GPC3陽性群と陰性群の5年生存率は54.5%と87.7%であった。組織学分化度の違いにより予後に有意な差は認めなかった。HCC癌部でのGPC3発現と生存期間との関連が示唆された。これ

らの因子について多変量解析を行うと、GPC3 染色陽性(p = 0.0397)、腫瘍個数多発(p = 0.0149)、肝内転移あり(p = 0.0275)が有意な因子であり、GPC3 を発現しない HCC では、発現するものに比べ、有意に予後が良好であった。

また、今回 GPC3 染色を施行した全 HCC153 例中 15 例において、非癌部のごく狭い領域に染色陽性所見を認めた。

D. 考察

GPC3 を発現する HCC と発現しないものの網羅的遺伝子及び蛋白の発現解析を進めている。GPC3 の前癌病変での発現の報告もあるが、非癌部の GPC3 染色陽性を示した領域に注目し、同部位での遺伝子発現について解析を進めている。今後 HCC における GPC3 発現の意義についてさらなる検討が必要である。

E. 結論

HCC に対する GPC3 免疫染色は、鑑別診断および予後予測に有用であることが示された。

F. 健康危険情報　　なし

G. 研究発表

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H. 知的財産権の出願・登録状況

本年度研究によるものはない。

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分担研究報告書

がん免疫療法への応用を目指したヒトES細胞からの樹状細胞の作製に関する研究

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研究要旨

樹状細胞は、強力なT細胞刺激活性を有する抗原提示細胞であり、T細胞のプライミングに必須の細胞である。がんに対する抗原特異的な免疫療法を確立するためには、標的抗原となるがん関連タンパクの同定とともに、効果の強いワクチン法の開発が不可欠である。樹状細胞を用いる細胞ワクチンは、このための有力な手段であると考えられる。我々は、これまでの研究により、マウスとカニクイザル胚性幹（ES）細胞から、樹状細胞（ES-DC）を分化誘導する方法を開発している。また、マウスを用いた実験により、ES-DCが抗腫瘍免疫の誘導において非常に有用であることを証明している。今回、ヒトES細胞からES-DCを分化誘導する培養プロトコルを開発し、さらに、電気穿孔法を用いてヒトES細胞へ抗原の発現ベクターを導入した。そして、この遺伝子導入ES細胞から分化したES-DCにおいて、導入遺伝子が発現し機能することを確認した。

A. 研究目的

樹状細胞は、生体内における主要な抗原提示細胞であり、適切な免疫応答がなされるよう、免疫応答を制御する役割を担っている。腫瘍抗原に対する免疫応答を強力に誘導する手段として、生体外で培養し機能を修飾した樹状細胞に抗原を負荷して移入する細胞ワクチンが考えられる。我々は、以前より、細胞治療に用いる樹状細胞のソースとして胚性幹（ES）細胞を用いる方法を検討しており、マウスのES細胞から樹状細胞（ES-DC）を作製する方法を開発していた。さらに、各種の遺伝子改変を行ったマウスES-DCをマウス個体へ投与することにより、抗腫瘍免疫の誘導、あるいは、自己免疫疾患の予防と治療が可能であることを確認していた。

今回、ES-DCの将来の臨床応用を目指して、ヒトES細胞からES-DCの分化を誘導する培養プロトコルの開発を行った。さらに、ES細胞の段階で遺伝子導入を行い、遺伝子導入ES細胞からES-DCへの分化誘導を行うことにより、ウイルスベクターを用いることなくヒトの遺伝子改変樹状細胞を作製できるかどうか検討した。

B. 研究方法

本研究では、京都大学再生医学研究所において樹立されたヒトES細胞株（KhES-1およびKhES-3）を使用した。ヒトES細胞の血球細胞系への分化誘導には、これまでのマウスおよびカニクイザルのES細胞を用いた研究成果に準じて、フィーダー細胞共培養法を採用した。フィーダー細胞として、マウス由来ストローマ細胞である、OP9を使用した。樹状細胞への分化および樹状細胞の成熟を促す因子として、ヒトGM-CSF、M-CSF、IL-4、Flt-3Lなどのサイトカイン、およびLPSの効果を検討した。樹状細胞への分化の確認は、細胞の形態ならびにフローサイトメトリーによる細胞表面分子の解析により行った。

ヒトES-DCのT細胞への抗原提示機能については、まずHLA-DRを共有しない健康なドナーから分離したアロT細胞と、ヒトES-DCとの共培養による、アロ一次混合リンパ球反応(MLR)の誘導の有無について検討した。

さらに、ヒトES-DCにGAD65ペプチドあるいはGAD65蛋白質を負荷した後に、I型糖尿病患者由来のGAD65ペプチド特異的HLA-DR53拘束性$CD4^+$T細胞クローンと共培養し、その後のT細胞増殖反応の誘導を観察することにより、ヒトES-DCによる抗原のプロセシングと抗原提示機能を検討した。

GAD65抗原ペプチドをMHCクラスII分子上へ提示するベクター（CLIP置換型抗原提示ベクター）を電気穿孔法によりES細胞へ導入した。遺伝子導入細胞は、G418を用いて選択し、遺伝子導入後18-20日にES細胞クローンを単離した。高濃度のG418に耐性のクローンを分化誘導し、ES-DCへ分化した後、フローサイトメーターにより導入遺伝子の発現を確認した。

[倫理面への配慮]

本研究を開始するにあたって、熊本大学倫理委員会および文部科学省ヒトES細胞倫理委員会から、

ヒトES細胞使用研究の承認を得た。さらに、ヒトES細胞使用研究の遂行にあたっては、文部科学省のヒトES細胞指針および熊本大学の倫理規定を遵守しつつ、熊本大学倫理委員会ヒトES細胞分科会の監視のもとに、承認された使用計画書に沿って研究を行なった。

C. 研究結果

以下の手順によりヒトES細胞からES-DCを分化誘導できることを見出した。

まず、未分化状態のヒトES細胞をマイトマイシンC処理を行ったOP9フィーダー細胞上へ播種し、分化誘導培養を開始する。ES細胞の分化の進行を見ながら、3日に一度培養液を交換しつつ、通常14-18日間の培養を継続する。

この培養の結果として出現する非付着性の細胞を、新たに調整したOP9フィーダー細胞上へ播種する。この段階では、培養液にGM-CSFとM-CSFを添加する。その結果、浮遊性あるいは弱付着性の球形の細胞が出現し、徐々にその数が増加する。第2段階の培養は、ES細胞由来の浮遊細胞の数とOP9細胞の状態により、7-10日間継続する。

次に、出現したES細胞由来の浮遊細胞をピペッティング操作により回収し、GM-CSFとIL-4を含む培養液に浮遊させて細菌培養用のディッシュへ播種する。浮遊細胞の形態は、第2段階の後半から第3段階を通じて、球形から突起を有する不規則な形態へと徐々に変化する。第3段階開始後、2-4日目に成熟誘導因子として、TNF-α、LPS、OK432等を加えることにより、ES-DCの成熟が可能であった。

以上の培養法により、樹状細胞としての形態、細胞表面分子（CD80、CD86、CD40、HLA-DR）、IL-12などのサイトカイン産生能、アロT細胞への一次MLR刺激活性、および$CD4^+$T細胞クローンへの蛋白質ならびにペプチド抗原の提示機能を有するES-DCを分化誘導できた。

HLA-DRによりGAD65抗原ペプチドを提示するベクターを導入したES細胞に由来するES-DCは、GAD65抗原の非存在下にSA32.5の増殖応答を誘導できた。

D. 考察

ES細胞は適切な培養条件下では無限に増殖させることが可能であり、樹状細胞を体外で作製するためのソースとしてES細胞を用いることにより、アフェレーシス操作による単球分離を必要とせず、細胞ワクチン用の樹状細胞の大量生産が可能である。また、養子免疫療法のための腫瘍抗原特異的T細胞を刺激するための抗原提示細胞としても有用であると考えられる。

従来、樹状細胞への遺伝子導入には、ウイルスベクターの使用が不可欠であったが、本研究により、ウイルスベクターを使用することなく、電気穿孔法によりヒトの樹状細胞の遺伝子を改変する方法が確立された。以上より、本研究の成果は、樹状細胞療法の実用化をめざす上で大きな意義を有するものと考えられる。

今後の実用化への課題として、技術的にはアロ反応の問題の解決と、GMP対応の培養技術の開発が求められる。また、ES細胞由来の分化細胞のヒトへの投与について、倫理的な観点からの議論を行う必要があると考えられる。

E. 結論

抗原提示機能を有するヒトES-DCの分化誘導技術の開発に成功した。さらに、ウイルスベクターを使用することなく、電気穿孔法により遺伝子改変ES-DCを作製する方法を確立した。

F. 健康危険情報　　なし

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H. 知的財産権の出願・登録状況

本年度研究によるものはない。

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分担研究報告書

肝癌細胞における細胞死抵抗性の分子基盤の解明

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研究要旨

種々の癌治療により導かれる酸化ストレスは癌細胞に細胞死を誘導するが、癌細胞はもともと細胞死抵抗性を有しているため高い効果は期待できない。そこで肝癌細胞における細胞死抵抗性の分子機構を解析した。用いた複数の細胞株の中で、酸化ストレス刺激に最も抵抗性を示すHepG2細胞では、脱メチル化剤の処理により細胞死誘導性の遺伝子発現が亢進したことより、エピジェネテイックな変化（メチル化）が細胞死抵抗性の一因であることが示された。また酸化ストレス刺激後1時間、3時間で1.5倍以上の発現亢進を認めた遺伝子は各々約150個、約520個であり、それらの中にはアポトーシス関連遺伝子、細胞内情報伝達遺伝子、転写関連遺伝子が含まれていたことから、刺激により誘導されるこれらの遺伝子群も細胞死抵抗性に関与している可能性が示された。加えて、酸化ストレス刺激による蛋白質の燐酸化の変動が上記の遺伝子発現変化と連繋して、細胞死抵抗性の一翼を担っていることがpathway解析から明らかとなった。このように肝癌細胞ではエピジェネテイックな遺伝子発現の変化と、酸化ストレス刺激下での遺伝子発現や蛋白質燐酸化の変化が相まって、細胞死抵抗性をもたらしていることが示唆された。今後、候補責任分子群の発現や機能を制御することで治療標的となりうる責任分子を同定していく予定である。

A. 研究目的

化学療法や放射線治療などの種々の癌治療により細胞内に導かれる酸化ストレスは、癌細胞に細胞死を誘導する重要な働きを有している。一方、癌細胞はもともと細胞死抵抗性を生物学的特性のひとつとして備えており、その分子機構の詳細を解明することは癌治療成績を向上させるために必要である。そこで我々は、酸化ストレス刺激下の遺伝子発現や蛋白質発現・機能変化を網羅的に解析し、肝癌細胞における細胞死抵抗性の分子機構を検討した。

B. 研究方法

まず2種類のヒト肝癌細胞株(HepG2細胞、Huh7細胞)と1種類のヒト肝細胞株(Hc細胞)を対象に、酸化ストレスとしての過酸化水素刺激を加え、細胞数の変化をWST assayにて、アポトーシス（細胞死）の誘導をFACSにて解析した。次にエピジェネテイックな変化を確認するために、脱メチル化剤による遺伝子発現の変化を網羅的に解析した。さらに酸化ストレスと細胞死を結びつける細胞内応答を遺伝子解析と蛋白質機能解析により検討した。具体的には、脱メチル化剤あるいは過酸化水素刺激前後でRNAよりcDNAを作製し、Gene Chipにて遺伝子発現の網羅的解析を行った。また調整したcell lysateで2次元デイファレンシャル電気泳動を行い、蛋白質発現あるいは燐酸化の変化（機能変化）を評価した。さらに質量分析器にて蛋白質を同定した。最終的には情報統合プラットフォームを用いて遺伝子関連情報と蛋白質関連情報を統合して、細胞死抵抗性を担う責任分子群を絞り込んだ。

C. 研究結果

生細胞数の減少、あるいはFACSの結果から、HepG2細胞、Hc細胞、Huh7細胞の順に酸化ストレスに対する細胞死抵抗性が認められた。最も抵抗性を呈するHepG2細胞では、脱メチル化剤により多くの細胞死関連遺伝子の発現が亢進し、逆にGPC3の発現は低下していた。このようにエピジェネテイックな影響が細胞死関連遺伝子やGPC3の発現制御に関与している可能性が示された。また酸化ストレス刺激後1時間、3時間で1.5倍以上の発現亢進を認めた遺伝子は、各々約150個、約520個であり、それらの中でアポトーシス関連では刺激後1時間で8遺伝子、3時間後で14遺伝子、細胞内情報伝達関連では各々19遺伝子、35遺伝子、転写因子関連では各々14遺伝子、39遺伝子が含まれていた。他方、蛋白質機能の制御には翻訳後修飾が関与することから、プロテオーム解析として蛋白質発現に加え燐酸化の変化も解析した。刺激後1時間で燐酸化スポットは約30個であり、燐酸化が刺激により有意に変動する蛋白質は、細胞骨格や分子シャペロンに関与する蛋白であった。これらの結果を統合したpathway解析では、酸化ストレス刺激によりもたらされる蛋白質燐酸化の変化が、その下流に存在する分子群の変化を介して細胞死を調節することが示された。

その中で今回は Neucleophosmin (NPM)に焦点を当てた。NPM は核小体に存在する燐酸化蛋白質で、その働きとしては、細胞増殖の亢進、アポトーシスの抑制などが報告されている。上記の 3 種類の細胞株において、酸化ストレスによる NPM の発現量と燐酸化の変動を検討したところ、蛋白質発現量の変化はすべての細胞株で認められないものの、燐酸化は刺激後時間と共にすべての細胞で低下した。とりわけ Huh7 細胞や Hc 細胞では、HepG2 細胞に比べて燐酸化の低下が”より顕著”であった。また燐酸化 NPM の細胞内局在の変化による機能の変化の可能性が示唆された。

D. 考察と E. 結論

肝癌細胞では、エピジェネテイックな遺伝子発現の変化が細胞死抵抗性の一翼を担うことが明らかになった。さらに酸化ストレス刺激による遺伝子発現や蛋白質燐酸化の変化が相まって、細胞死抵抗性をもたらしていることも示唆された。とりわけ酸化ストレス刺激によりもたらされる早期の蛋白質燐酸化の変化が、その下流に存在する分子群の変化を介して細胞死を調節することが示された。今後、ヒト肝癌組織における候補責任分子の発現や燐酸化、メチル化を確認すると共に、siRNA や阻害剤等を用いて責任分子の重み付けを行う。最終的には、細胞死抵抗性の制御を介した新たな癌治療法の開発を目指す予定である。

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F. 健康危険情報　　なし

G. 研究発表

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H. 知的財産権の出願・登録状況

本年度研究によるものはない。

IV. 研究成果の刊行に関する一覧表

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V. 研究成果の刊行物・別刷

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Factors predicting survival in advanced T-staged hepatocellular carcinoma patients treated with reduction hepatectomy followed by transcatheter arterial chemoembolization

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Abstract

Aims: To evaluate the efficacy of reduction hepatectomy followed by transcatheter arterial chemoembolization (TACE) for advanced T-Staged hepatocellular carcinomas (HCCs).
Methods: A retrospective analysis of 39 consecutive patients who underwent reduction hepatectomy followed by TACE for advanced T-Staged HCCs was undertaken.
Results: Reduction hepatectomies, including 20 major ones, were performed. After a median interval of 30 days, the hepatectomies were followed by TACE using farmorubicin. Actual overall 3-year survival after surgery was 32%. Indocyanine green $R_{15} \geq 15\%$, preoperative AFP ≥ 2000 ng/ml, and tumour reduction rate <98% were predictive of decreased overall survival. When the three prognostic factors were used in a scoring system, with one point assigned for each factor, the 3-year survival rates of patients with scores of 0, 1, 2, and 3 were 71%, 40%, 0%, and 0% respectively.
Conclusions: Reduction hepatectomy followed by TACE is effective in patients with advanced T-Staged HCCs who have none of the 3 poor prognostic factors. Reduction surgery followed by TACE is one of the options for controlling advanced T-Staged HCCs in patients who are not candidates for curative resection or TACE alone.

Keywords: Hepatocellular carcinoma; Resection; Transcatheter arterial embolization; Transcatheter arterial chemoembolization; Prognosis

Introduction

Multiple hepatocellular carcinomas (HCCs) with huge tumours or tumour thrombi are associated with a poor prognosis.[1–3] No effective treatment for the disease has yet been identified. Although surgical resection offers the best chance for long-term survival,[4–6] many of the patients are not candidates for curative resection due to underlying liver dysfunction or the extent of the tumour. Moreover, transcatheter arterial chemoembolization (TACE), which is thought to be the first option for unresectable HCC,[7,8] is sometimes ineffective for controlling such advanced T-staged HCCs, since a large tumour burden or portal tumour thrombi can frequently coexist with the disease.[9,10]

Reduction surgery is a potential treatment for advanced T-staged HCCs that cannot be treated by either curative resection or TACE alone. Several studies have reported long-term survivors after reduction surgery.[11–16] Previously, we reported the results of reduction surgery followed by TACE for treatment of advanced T-staged HCC patients with tumours greater than 10 cm in size with preserved liver function and residual tumour accounting for less than 10% of the remnant liver.[17] However, the efficacy of this strategy is still uncertain, since the optimal patient selection criteria for the strategy have not been determined.

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The aims of this study were to confirm the efficacy of reduction surgery followed by TACE in the treatment of advanced T-staged HCC patients and to determine prognostic factors that could be used to identify those patients who would most benefit from reduction surgery. We focused particularly on the effect of the tumour volume ratio (volume of all tumours/volume of the whole liver × 100%) and the tumour reduction rate (volume of the resected tumours/volume of all tumours × 100%) on survival.

Patients and methods

Patient population

Three hundred and eighty-two patients with T3N0M0 HCC and 97 patients with T4N0M0 HCC were treated at the National Cancer Center Hospital East between November 1993 and November 2003. Among the T3N0M0 HCC patients, 173 had TACE, 122 had curative resection, 33 had ablation, 27 had hepatic arterial infusion (TAI), 15 had reduction hepatectomy followed by TACE, 8 had radiation, and 4 had systemic chemotherapy. Among the T4N0M0 HCC patients, 30 had TACE, 27 had TAI, 24 had reduction hepatectomy followed by TACE, 12 had curative resection, and 4 had radiation. The data of the 39 consecutive T3N0M0 HCC and T4N0M0 HCC patients who had reduction surgery followed by TACE were retrospectively examined. The patients consisted of 36 men and 3 women, ranging in age from 27 to 77 years (mean, 57 years). HCC staging was performed according to the staging criteria of the Japanese Liver Cancer Study Group.[18] The diagnosis of HCC was based on the pathological findings of the resected specimens.

The criteria for reduction hepatectomy were as follows: (1) the presence of multiple HCCs for which curative resection was not indicated and that appeared to be resistant to TACE due to tumour extent, tumour thrombus, or other factors; (2) no extrahepatic metastases; (3) sufficient liver function to tolerate the planned hepatectomy; and (4) written informed consent before treatment. All 39 patients had contrast-enhanced computed tomography (CT) scans, magnetic resonance imaging (MRI), hepatic angiography, abdominal ultrasonography, and chest X-Rays preoperatively to stage the HCCs and evaluate resectability. Liver function was assessed based on liver biochemistry tests, Child-Pugh grade,[19] and the indocyanine green retention rate at 15 min.[20] The patients' data were reviewed by hepatic surgeons, medical oncologists, and interventional radiologists during a conference to determine whether the patients met the aforementioned criteria.

Treatment procedure and follow up

First, large main tumours with satellite tumours that were obstacles for TACE were resected. Tumour thrombi in the portal or hepatic veins were also resected when they were recognized before or during the operation. Hepatic resection was performed by the forceps fracture method under inflow occlusion (Pringle's manoeuvre), and anatomic hepatectomy was performed whenever possible. All the resections were ultrasound-guided procedures.

Hepatectomy was followed by TACE as soon as liver function recovered during the postoperative period. TACE was repeated every 2–3 months until there was: complete remission of the remnant tumours; progressive disease despite treatment; or malfunction of the liver or other organs. The TACE procedure was performed by injecting a mixture of iodized oil (Lipiodol) 5 ml and farmorubicin 50 mg, followed by a gelatine sponge block (Gelfoam).

One month after treatment, the anti-tumour effects of TACE were assessed by CT. Subsequently, follow-up examinations, including CT, serum alpha-fetoprotein (AFP), and biochemistry assays, were conducted at least every 3 months. The median follow-up of the survivors was 23 months.

When disease progression was evident in the remnant liver, TACE was stopped and transcatheter arterial infusion chemotherapy with farmorubicin 50 mg was performed if possible. When disease progression was observed only outside the liver, TACE was continued if treatment to the hepatic tumour seemed to be beneficial.

Measurement of tumour volume ratio and tumour reduction rate

Tumour volumes were obtained from contrast-enhanced CT scans of the abdomen that were performed before hepatectomy using 5-mm collimation with administration of 120 cc of non-ionic intravenous contrast injected at 3 cc per second with 40-s, 60-s, and 3-min delays. Images were reconstructed at 5-mm intervals using a standard soft-tissue algorithm.

Tumours and the liver were outlined manually on each axial slice using a computer mouse. The tumour and whole liver volumes were calculated automatically by multiplying the sum of the areas from each slice by the reconstruction interval. Then, the tumour volume ratio (volume of all tumours/volume of the whole liver × 100%) and tumour reduction rate (volume of the resected tumours/volume of all tumours × 100%) were calculated. The resected part of the tumour was confirmed by both the surgical record and the findings of pre- and postoperative contrast-enhanced CT scans. All measurements were made by a radiologist.

Statistical analysis

Survival analyses were performed using the Kaplan–Meier method,[21] and differences between the curves were tested using the log-rank test. Factors related to survival were analyzed with the Cox proportional hazards regression model.[22] A P value of less than 0.05 was considered significant.

Results

Patient characteristics and clinicopathological features of HCCs

Hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody (HCVAb) were positive in 10 and 18 patients, respectively. Twelve patients tested negative for both HBsAg and HCVAb. The mean results of the preoperative liver function tests were: ALT, 71.3 IU/L; serum albumin, 3.7 g/L; prothrombin time, 81.8%; and ICG R_{15} 16.2%. Based on the Child-Pugh grading system, 31 patients were stage A, and 8 were stage B. Twenty patients had liver cirrhosis. The median maximum tumour size was 11.5 cm (range, 4.5–21.5 cm); 20 patients had 10 or more tumours. The average tumour volume rate was 41%. The average preoperative AFP level was 802 ng/ml. Macroscopic portal vein invasion (thrombus) that involved a major branch was found in 12 patients. Four patients had tumour thrombus in the trunk of the portal vein. Based on the Japanese Liver Cancer Study Group classification of tumour growth extent, 31 cases had expansive tumour growth, and 8 had infiltrative growth.

Reduction hepatectomy

Four patients had tri-segmentectomies (Fig. 1), two had central bi-segmentectomies, 14 had lobectomies, 11 had segmentectomies, and 8 had partial resections; 7 patients had simultaneous direct removal of the portal tumour thrombus.

The average resected tumour volume was 1488 ml; the average tumour volume left in the remnant liver was 53 ml. The average tumour reduction was 94.7%.

One patient died on the 15th day after reduction hepatectomy due to liver failure. The morbidity rate was 41%: 8 cases had a biliary leak; 5 had ascites; 4 had a wound infection; 3 had an intra-abdominal abscess; 2 had bleeding; and 2 had liver failure.

Transcatheter arterial chemoembolization

All but one patient who underwent reduction hepatectomy received TACE; postoperative liver failure prevented the one patient from receiving TACE. The median interval from reduction hepatectomy to the first TACE treatment was 30 days, and the average number of TACE treatments was 3.6 (range, 1–15).

Survival

Survival time was calculated from the date of reduction hepatectomy. Actual overall 3-year survival was 32%, with a median survival of 11 months (Fig. 2). Six patients survived more than 3 years.

Among the 39 patients, 27 developed disease progression. The location of the initial progression included: remnant liver, 19 patients; lungs, 7; bone, 6; lymph nodes, 1; and brain, 1.

Correlation between clinicopathological factors and overall survival

To determine the prognostic factors related to survival after reduction hepatectomy in patients with advanced T-staged HCCs, the clinicopathological factors and overall survival of the 39 patients were analyzed (Table 1). Serum albumin level <3.5 g/L ($P = 0.03$), indocyanine green (ICG) $R_{15} \geq 15\%$ ($P < 0.01$), preoperative alpha-fetoprotein (AFP) ≥ 2000 ng/ml ($P = 0.04$), tumour reduction rate $<98\%$ ($P = 0.02$), macroscopic portal vein invasion ($P < 0.01$), and infiltrative growth ($P < 0.01$) were

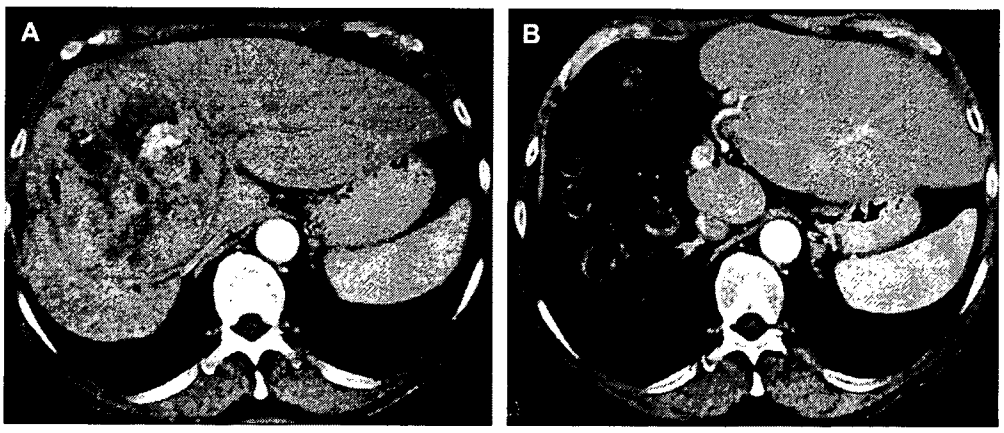

Figure 1. Contrast-enhanced computed tomography (CT) findings of a 66-year-old man with multiple hepatocellular carcinomas (HCCs). (A) CT before reduction hepatectomy demonstrates an HCC measuring 14 cm with many intrahepatic metastases throughout both lobes. The preoperative alpha-fetoprotein level was 6717 ng/ml, and no tumour thrombus was observed in the hepatic or portal veins. Liver function was preserved (ICG $R_{15} = 8.7\%$). The patient underwent reductive right tri-segmentectomy. (B) CT 4 years after reduction hepatectomy demonstrates small tumours well controlled by 10 successive transcatheter arterial chemoembolizations.

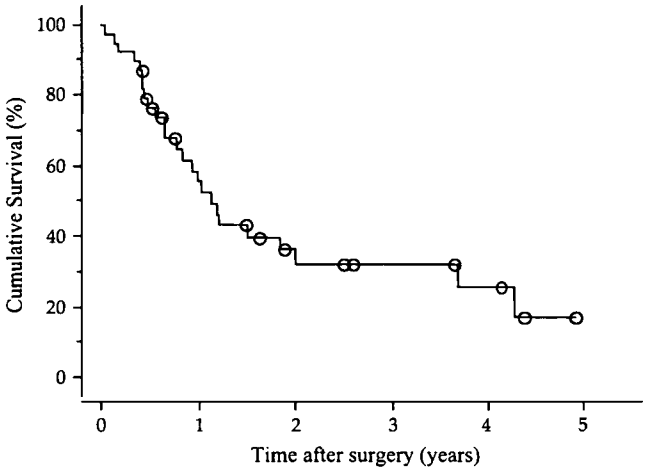

Figure 2. Cumulative survival curves after reduction hepatectomy followed by transcatheter arterial chemoembolization in patients with advanced T-staged HCCs.

significantly associated with poor overall survival. Neither the number nor size of tumours in the remnant liver was correlated with survival.

We examined the independent predictive value of the aforementioned factors for overall survival. The data were analyzed using a Cox regression model (Table 2). Serum albumin level <3.5 g/L was excluded from the analysis due to a possible correlation with ICG R_{15} ≥15%. ICG R_{15} ≥15% ($P < 0.01$; HR = 5.89; 95% CI, 1.98 to 17.5), preoperative AFP ≥2000 ng/ml ($P < 0.01$; HR = 5.85; 95% CI, 1.93 to 17.7), and tumour reduction rate <98% ($P < 0.01$; HR = 4.25; 95% CI, 1.55 to 11.7) were predictive for decreased overall survival.

When the three prognostic factors were used in a scoring system, with one point assigned for each factor, the total score was strongly correlated with survival after reduction hepatectomy ($P < 0.01$). The 3-year survival rates of patients with scores of 0 ($n = 7$), 1 ($n = 17$), 2 ($n = 13$), and 3 ($n = 2$) were 71%, 40%, 0%, and 0% respectively (Fig. 3). No patients with two or more criteria survived more than 15 months.

Table 1
Correlation between clinicopathologic factors and overall survival after reduction hepatectomy for multiple HCCs

	No. of patients	Median survival (mo)	P
HBsAg			
Negative	29	12.5	0.26
Positive	10	9.1	
HCVAb			
Negative	21	11.2	0.83
Positive	18	10.9	
ALT			
<70 IU/L	22	11.6	0.69
≥70 IU/L	17	9.3	
Albumin			
≥3.5 g/L	29	12.5	0.03
<3.5 g/L	10	5.0	
Prothrombin Time			
≥80%	22	11.0	0.65
<80%	17	11.2	
ICG R_{15}			
<15%	21	17.9	<0.01
≥15%	18	7.1	
Child-Pugh Stage			
A	31	11.9	0.61
B	8	7.3	
Tumor size			
<10 cm	19	7.9	0.17
≥10 cm	20	18.1	
Number of tumors			
<10	20	14.2	0.07
≥10	19	10.1	
AFP			
<2000 ng/ml	25	13.7	0.04
≥2000 ng/ml	14	6.1	
Tumor volume ratio[a]			
<50%	26	8.6	0.83
≥50%	13	12.5	
Tumor reduction rate[b]			
≥98%	21	17.9	0.02
<98%	18	7.1	
Macroscopical portal vein invasion			
Absent	27	13.7	<0.01
Present	12	5.8	
Growth type of tumor			
Expansive growth	31	13.7	<0.01
Infiltrative growth	8	6.9	

[a] Volume of all tumors/volume of whole liver × 100%.
[b] Volume of the resected tumors/volume of all tumors × 100%.

Discussion

The optimal treatment of patients with multiple HCCs who are not candidates for curative resection or TACE alone due to large tumours or tumour thrombi is unclear. Reduction surgery, which is also referred to as "debulking surgery", "tumour mass reduction surgery", and "tumour volume reduction surgery", is a potential treatment for patients with advanced T-staged HCCs.[11–17] Initially, large main tumours that are life-threatening and that can obstruct postoperative treatment are resected. Then, additional therapy, such as TACE or transcatheter arterial infusion chemotherapy, is given to treat the residual tumour in the remnant liver. Long-term survivors have been reported after reduction surgery for advanced HCCs. Several retrospective reports with small cohorts have shown that reduction surgery is superior to non-surgical treatment, such as TACE or transcatheter arterial infusion chemotherapy.[14,15] In a previous study, we reported the efficacy of reduction surgery followed by TACE in a small cohort of patients who had preserved liver function, tumour size greater than 10 cm, and residual tumour volume <10% of the remnant liver.[17] However, the indications for reduction hepatectomy followed by TACE are still unclear.

In the present study, all patients who underwent reduction hepatectomy were studied to confirm the efficacy of

Table 2
Multivariate analyses of factors affecting overall survival after reduction hepatectomy for multiple HCCs

	Hazard ratio	P
ICG R_{15} ≧15%	5.89 (1.98–17.5)	<0.01
AFP ≥2000 ng/ml	5.85 (1.93–17.7)	<0.01
Tumor reduction rate[a] <98%	4.25 (1.55–11.7)	<0.01
Macroscopical portal vein invasion	1.25 (0.38–4.07)	0.71
Infiltrative growth of tumor	2.19 (0.63–7.57)	0.22

Values in parentheses are 95 per cent confidence intervals.
[a] Volume of the resected tumors/volume of all tumors × 100%.

reduction surgery followed by TACE and to determine prognostic factors that might identify the patients who would most benefit from reduction hepatectomy. TACE was used as postoperative treatment since TACE is considered to be the first option for controlling unresectable multiple HCCs.

In this series, overall survival after reduction hepatectomy was 32% at 3 years and the results were not so much different from those of curative resection for stage IV HCC[23,24] while all our cases were classified as stage IVA according to the TNM classification system by UICC. Furthermore, patients who received the treatment were not a few because one quarter of patients with T4N0M0 HCC received the treatment.

Three factors associated with a poor prognosis were identified: ICG R_{15} ≥15%; preoperative AFP ≥2000 ng/ml; and tumour reduction rate <98%. These three factors capture the essence of the treatment strategy. First, the reduction hepatectomy tends to be a major hepatectomy due to the extent of tumour; however, postoperative liver failure or deterioration of liver function must be avoided, since this can delay the start of TACE. As well, sufficient liver function must be present preoperatively. Second, early

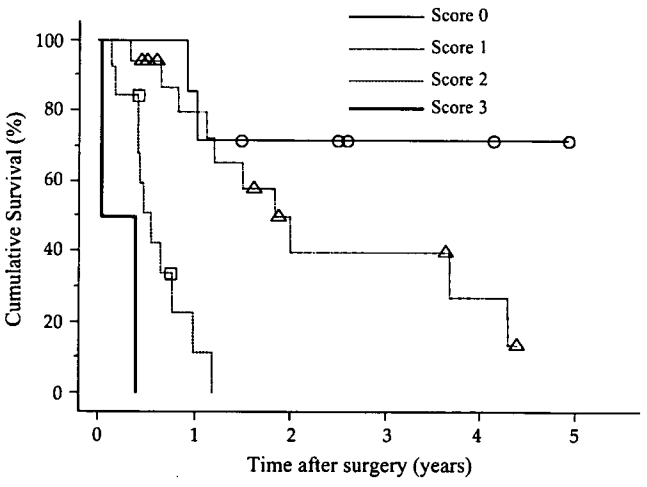

Figure 3. Cumulative survival curves after reduction hepatectomy followed by transcatheter arterial chemoembolization based on the scoring system. The scoring system was based on assigning one point for each of: ICG R_{15} ≥15%; preoperative alpha-fetoprotein ≥2000 ng/ml; and tumour reduction rate <98%.

progression is an issue with this treatment strategy; overall survival decreased to about 50% within a year in this series. A high preoperative AFP level has been reported to be a significant factor indicating a poor prognosis[2,25] and might be correlated with early death after reduction hepatectomy due to abrupt tumour progression inside or outside the liver. Third, control of the residual tumour in the remnant liver is necessary for long-term survival. A high tumour reduction rate indicates a low tumour burden in the remnant liver and may predict good control of the residual tumour with postoperative TACE.[9,10]

Extrahepatic disease,[12] residual tumour thrombus,[14] no effect of postoperative treatment,[14] and a remnant tumour index[13] have been reported as poor prognostic factors after reduction surgery for HCC. Yamamoto et al. proposed a remnant tumour index that is calculated based on the maximum diameter of the largest residual tumour (in cm) multiplied by the number of residual tumours; this index can be used to quantify the tumour burden in the remnant liver and select optimal candidates for reduction surgery.[13] The concept of a remnant tumour index is similar to that of the tumour reduction rate in the present study. However, the maximum diameter of the largest residual tumour, the number of residual tumours, and a remnant tumour index were not correlated with survival in the present study (data not shown). The tumour reduction rate may perhaps more accurately evaluate the efficacy of reduction surgery and the tumour burden in the remnant liver.

To select the best candidates for reduction hepatectomy, we would propose a scoring system that incorporates the aforementioned three factors for patients with multiple HCCs that are not treatable with curative resection. The score based on these factors was strongly correlated with survival after reduction hepatectomy; the 3-year survival rates of patients with scores of 0, 1, 2, and 3 were 71%, 40%, 0%, and 0% respectively. Patients with a score of 0 are good candidates for reduction hepatectomy followed by TACE. On the other hand, a score of 2 or 3 might be a contraindication for treatment, since there were no long-term survivors among patients who had scores of 2 or 3. Other non-surgical treatments, including experimental treatments, would be recommended for patients with a score of 2 or 3. Patients with a score of 1 sometimes survive a relatively long time; thus, patients with a score of 1 may be candidates for reduction hepatectomy, though other findings, such as cardiopulmonary function or performance status, should be considered in such patients.

Recently, intensive non-surgical treatments using transcatheter arterial infusion chemotherapy or systemic chemotherapy have improved the treatment of advanced T-staged HCCs.[26–31] A potential weakness of the present study is that our strategy using reduction hepatectomy followed by TACE did not take into account these new treatments. A clinical study comparing the efficacy of reduction hepatectomy followed by TACE with that of intensive non-surgical treatments in patients with advanced T-staged HCCs, especially

those with a score of 1, is needed. Moreover, the efficacy of reduction hepatectomy followed by one of these intensive non-surgical treatments should be investigated.

Since our study population was small, a prospective study is warranted to verify the validity of the scoring system.

In conclusion, reduction hepatectomy followed by TACE is effective in controlling advanced T-staged HCCs when the ICG R_{15} is <15%, the preoperative AFP is <2000 ng/ml, and the tumour reduction rate is ≥98%. Reduction hepatectomy followed by TACE is one of the options for controlling advanced T-staged HCCs in patients who are not candidates for curative resection or TACE alone.

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Multiple Resections for Hepatic and Pulmonary Metastases of Colorectal Carcinoma

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Background: Resections are effective for some patients with both hepatic and pulmonary metastases of colorectal cancer, but the best selection criteria for the resections and effective treatment for recurrence after the resections have not been determined.
Methods: A retrospective analysis was performed for 30 consecutive patients who received aggressive multiple resections for both hepatic and pulmonary metastases of colorectal cancer. Recurrences after resections were surgically treated whenever resectable.
Results: For the 30 patients, 45 hepatectomies and 40 pulmonary resections were performed and 17 patients received three or more resections. No mortality was observed. Overall survival after the first metastasectomy for the second organ (liver or lung) was 58% and nine 5-year survivors were observed. Multivariate analyses revealed that primary colon cancer, stage IV in TNM classification and maximum size of hepatic tumor >3 cm at initial hepatectomy were poor prognostic factors, but several long-term survivors were observed even among patients with those factors.
Conclusions: Multiple resections for hepatic and pulmonary metastases of colorectal cancer are safe and effective. No single factor is considered to be a contraindication for the resections. For recurrence after the resections, surgical resection is also recommended if resectable.

Key words: colorectal cancer – hepatic metastasis – pulmonary metastasis – resection

INTRODUCTION

The liver and lung are the most common sites of distant metastases for colorectal carcinoma (1). Hepatic and pulmonary metastases may be detected sequentially or simultaneously in patients with colorectal carcinoma. Efficacy of resections for these two distant metastases has been reported in several studies (2–14). However, the criteria to select patients for those resections are still obscure.

In addition, although recurrence after those resections is one of the major problems of the strategy, further surgical approaches for recurrence after those resections are controversial.

The purpose of this study was to evaluate the efficacy of aggressive multiple resections for hepatic and pulmonary metastases of colorectal carcinoma and to find prognostic factors that might elucidate who would benefit most from hepatic and pulmonary resections for colorectal metastases.

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PATIENTS AND METHODS

Two hundred and sixty-seven patients who had undergone hepatic resection and 98 patients who had undergone pulmonary resection, as the first treatment for colorectal metastasis at the National Cancer Center Hospital East between September 1992 and June 2005 were examined retrospectively. Eight patients had undergone surgical resections for both hepatic and pulmonary metastases as the first treatment for colorectal metastases. Metastases were synchronous with primary colorectal carcinoma in one of the eight patients. In the remaining 259 patients who had undergone hepatic resection as the first treatment for colorectal

metastasis, 83 had the second recurrence in the liver, 29 in the lung, 12 in both liver and lung and 52 in the other organs. Sixteen of the 29 patients with pulmonary recurrence and one of the 12 patients with both hepatic and pulmonary recurrences were treated surgically. Two patients had undergone resections for both hepatic and pulmonary recurrences after more than two hepatic metastasectomies. In the remaining 90 patients who had undergone pulmonary resection as the first treatment for colorectal metastasis, three had the second recurrence in the liver, 27 in the lung, four in both liver and lung and 16 in other organs. All three patients with hepatic recurrence were treated surgically. However, all four patients with both hepatic and pulmonary recurrences underwent systemic chemotherapy as the second treatment.

As a result, 30 patients underwent both hepatic and pulmonary resections for colorectal metastasis. The patients consisted of 19 men and 11 women, ranging in age from 24 to 75 years with a mean of 59 years. Two of the patients had received adjuvant chemotherapy (tegafur/uracil and 5-fluorouracil/leucovorin) after primary colorectal resection and one patient had received preoperative chemoradiation for rectal cancer.

The criteria for hepatectomy were as follows: (1) metastatic lesions are confined to the liver and technically resectable, (2) no extrahepatic metastases except resectable pulmonary metastasis are detected, and (3) liver function is equal to complete resection of all hepatic tumors. The criteria for pulmonary resection were as follows: (1) metastatic lesions are confined to the lung and technically resectable, (2) no extrathoracic metastases except resectable hepatic metastasis are detected, and (3) cardiorespiratory function is equal to complete resection of all pulmonary tumors. The timing of the detection of hepatic and pulmonary metastases or the number of prior resections for metastases did not affect these criteria, so the selection criteria for further resections for recurrences after hepatic and pulmonary resections are the same as above.

At hepatectomy, intraoperative ultrasonography was performed to confirm tumor location and size of the lesions in all patients, and all of the resections were ultrasound-guided procedures. Hepatic resection was performed by the forceps fracture method under inflow occlusion (Pringle's maneuver). At pulmonary resection, hilar or mediastinal lymph node dissection was used to sample lymph nodes of most patients who had a lobectomy.

When hepatic and pulmonary metastases were detected simultaneously, hepatic resection was carried out first, followed by pulmonary resection.

No patient received adjuvant chemotherapy after hepatectomy or pulmonary resection.

After hepatic or pulmonary resection, patients were closely followed with diagnostic imaging [chest X-ray and abdominal computed tomography (CT)] and measurement of serum carcinoembryonic antigen (CEA) levels every 3 months; they also underwent an annual colonoscopy to detect any tumor recurrence. The median follow-up of survivors was 53 months.

Morphological Investigations

The resected specimens of colon or rectum, liver and lung were fixed in 10% phosphate-buffered formalin, cut at intervals of 5 mm and embedded in paraffin. Serial sections of 3-μm thickness were stained with hematoxylin and eosin for morphological examination. Each case was histologically classified according to the histological type, tumor size, location, number of metastases, presence of serosal invasion, nodal status and margin status. Histological diagnosis was performed according to the World Health Organization intestinal tumor classification (15).

Statistical Analysis

The student *t*-test was used to compare data between subgroups by the location of the primary tumor. The Mann–Whitney's U test was used to compare serum CEA levels between subgroups. Analyses of survival rates were performed using the Kaplan–Meier method (16) and differences between the curves were tested using the log-rank test. Factors related to survival were analyzed with the Cox proportional hazards regression model (17). A P value of less than 0.05 was considered to denote significance.

RESULTS

Clinicopathological Features of Primary and Metastatic Tumors

The primary tumors were staged as I ($n = 1$), II ($n = 10$), III ($n = 15$) and IV ($n = 4$) according to TNM classification (Table 1). All patients at stage IV had hepatic metastasis at resection of the primary tumor.

At the initial hepatectomy, the average number of hepatic tumors was 2.1 (range, 1–12), the average maximum size was 3.2 cm (range, 0.3–9 cm) and the average preoperative CEA level was 19.9 ng/ml (range, 0.8–68.5 ng/ml). In all hepatectomies, the average number of hepatic tumors was 2.8 and the average maximum size was 3.3 cm. Lymph node metastasis at the hepatoduodenal ligament was shown in one patient.

Regarding pulmonary metastases, the average number of pulmonary tumors was 1.8 (range, 1–5), the average maximum size was 2.2 cm (range, 0.7–6.7 cm) and the average prethoracotomy CEA level was 12.4 ng/ml (range, 1.0–66.7 ng/ml) at initial pulmonary resection. In all pulmonary resections, the average number of pulmonary tumors was 2.1 and the average maximum size was 2.5 cm. Hilar lymph node metastasis of the lung was shown in two patients.

Surgical Resections for Hepatic and Pulmonary Metastases

Forty-five hepatectomies (30 partial resections, four subsegmentectomies, seven segmentectomies and four lobectomies