殺細胞性抗悪性腫瘍薬

代謝拮抗薬(フッ化ピリミジン・非フッ化ピリミジン)

Metabolic antagonists

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Key words：代謝拮抗薬，ピリミジン拮抗薬，プリン拮抗薬，葉酸拮抗薬

はじめに

代謝拮抗薬は最も古くから癌化学療法の臨床に用いられてきた薬剤である．現在も多くの癌種で重要な基本薬剤として用いられている．なかでもfluorouracil(5-FU)は広く用いられ，最近ではcapecitabineやS-1などprodrugの経口剤も開発されてきている．代謝拮抗薬はピリミジン拮抗薬，プリン拮抗薬，葉酸拮抗薬のサブグループに分けられるが，基本的な作用機序はDNAおよびRNA合成に必要な酵素の阻害によるものであり，癌細胞自体というより，増殖している細胞に対して強い活性を示す．ほとんどの代謝拮抗薬はG1-S期の細胞周期特異的に作用し，毒性は骨髄と消化管粘膜に対するものが主である．

1. ピリミジン拮抗薬

5-FUはラットの正常腸管粘膜より肝癌細胞で効率的にuracilが使われることに基づいて開発され，1957年Heidelbergerにより合成された[1]．その後幾つかのフッ化ピリミジン剤が開発されたが，いずれも広いスペクトルをもち，多くの固形癌に対して有効性を示している．

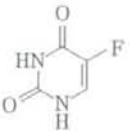

図1 Fluorouracil(5-FU)

a. Fluorouracil(5-fluorouracil: 5-FU)

1) 作用機序と代謝

5-fluoropyrimidine-2,4(1*H*,3*H*)-dioneの化合物であり(**図1**)，uracilと同じ経路で代謝される．5-FU自体は抗腫瘍活性をもたず，uracil transport systemを介して細胞内に入り，thymidine phosphorylase(TP)により5-fluoro-2′-deoxyuridin(FUdR)に変わる．更にthymidine kinase(TK)により5-fluoro-2′-deoxyuridine 5′-monophosphate(FdUMP)などの活性体に変換された後，抗腫瘍効果を発揮する．5-FUの作用機序は主としてDNA阻害とRNA阻害の2つがある(**図2**)．FdUMPはthymidylate synthase(TS)とcovalent ternary complex(三元共有複合体)を形成し，TSの阻害をもたらす[1]．このTS阻害が主な作用機序と考えられる．TSは本来2′-deoxyuridine 5′-monophosphate(dUMP)から2′-deoxyuridine 5′-monophosphate(dTMP)，2′-deoxyuridine 5′-triphosphate(dTTP)などを経てDNAが合成される反

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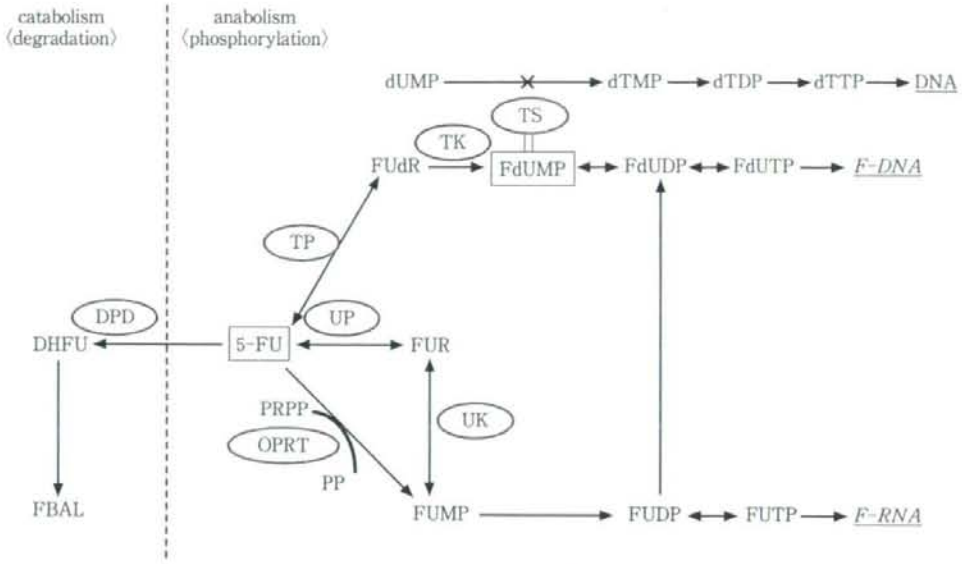

図2 フッ化ピリミジン系薬剤の代謝
略語は本文参照

応を進めるが，FdUMPによりTSが阻害されるとdTTPの生成低下からDNA合成障害や修復障害が生じることになる．また5-FUはuridine phosphorylase(UP)とuridine kinase(UK)による経路とorotic acid phosphoribosyltransferase(OPRT)による経路でfluorouridine monophosphate(FUMP)に代謝される．更にFUMPはfluorouridine diphosphate(FUDP)，fluorouridine triphosphate(FUTP)に変わり，FUTPはfraudulent-RNA(F-RNA)となって正常のRNAのprocessingや機能を阻害するという作用機序もある[1]．

5-FUはほとんどが肝臓などにあるdihydropyrimidine dehydrogenase(DPD)で代謝を受け，5,6-dihydrofluorouracil(DHFU)に変わり，fluoro-β-ureidopropionic acid(FUPA)，fluoro-β-alanine(FBAL)などに不活化され，呼気中と尿中に排泄される．

2) 投与方法

投与法はbolus静脈投与と点滴投与による静脈内注射であるが，半減期が10-20分と短いことから24時間持続点滴が多く用いられる．

3) 毒　性

主な毒性の発現部位は消化管粘膜と骨髄であり，食欲不振，下痢，全身倦怠感，悪心・嘔吐，口内炎など消化器毒性，白血球減少など骨髄毒性のほか，色素沈着，脱毛などが主な副作用である．5-FUの代謝酵素であるDPD酵素欠損の患者では，重篤な毒性が発現する危険がある．またDPD部分欠損は人口の1-3%に認めるとの報告や正常な人口においてもDPD発現に6倍の範囲があることから，毒性の発現は個人差が大きい[2]．

4) Biochemical modulation

5-FUの抗腫瘍効果の増強を狙ったbiochemical modulationとして，methotrexate，leucovorin(5-formyltetrahydrofolate: LV)，interferonなど幾つかの薬剤の併用が試みられている．5-FUの作用機序であるTSとFdUMPの複合体の形成は徐々に解離するため，この複合体の形成と維持のためには細胞内の5,10-methylenetetrahydrofolateが必要となる．これが枯渇するとTS-FdUMPの複合体の減少を招き，抗腫瘍効果の低下をもたらす．LVは細胞内の5,10-methylenetetrahydrofolateを増加させ，

TS阻害を増加させることになる．その結果，5-FUの効果が増強されるという理論で5-FU/LV療法が開発された．大腸癌での基本的な化学療法となっている[3]．

b. 経口フッ化ピリミジン剤

経口フッ化ピリミジン剤は当初，連日経口投与による5-FUの持続点滴と同様の効果と簡便性やQOLの維持を目的に開発された．5-FUのprodrugであるtegafurを含む経口剤としてtegafur/uracil(UFT)とtegafur/gimeracil/oteracil potassium(TS-1)はDPDと拮抗する成分を配合することで抗腫瘍効果の増強が図られている．capecitabineは活性化酵素の臓器分布から腫瘍組織で高濃度となるように工夫された経口剤である．この3剤が実際の臨床上用いられている．

1) Tegafur/uracil(UFT)

tegafurは主に肝の薬物代謝酵素であるcytochrome P450 2A6(CYP2A6)などによる代謝や自然分解により徐々に5-FUに変換される．uracilはDPDの拮抗薬であり，5-FUの異化代謝を妨げることにより5-FUの血中および組織濃度を高めることで効果の増強が図られている．UFTと経口LVの併用療法が用いられ，肺癌，大腸癌などの補助療法で有効性が報告されている[4]．

2) Tegafur/gimeracil/oteracil potassium (TS-1)

TS-1はtegafurとgimeracil(5-chloro-2,4-dihydroxypyridine: CDHP)，oteracil potassium e(Oxo)の3成分を1：0.4：1に配合した経口剤である．CDHPはDPDの可逆的拮抗阻害薬でuracilの約180倍の阻害活性を有し，5-FUの分解を強力に阻害する[5]．一方，Oxoは5-FUのリン酸化酵素であるOPRTを可逆的に拮抗阻害する物質であり，経口投与により消化管における5-FUの活性化を妨げ，消化管毒性の軽減が図られている．

当初胃癌で開発が進められ，第II相試験では40%を超す奏効率が得られた[6]．その後多くの癌で臨床試験が行われ，2008年現在，胃癌，結腸・直腸癌，頭頸部癌，非小細胞肺癌，手術不能または再発乳癌，膵癌，胆道癌に保険適応が承認されている．胃癌において大規模な第III相試験が行われ，術後補助療法や切除不能例で有用性が確認されている[7]．用量規定毒性は骨髄抑制であり，その他主な副作用は発疹，悪心，食欲低下，下痢など消化器症状である．

3) Capeciatbine(Xeloda)

capecitabineは活性化酵素の臓器分布特性を考慮し，腫瘍内で選択的に活性化されるように工夫されている[8]．capeciatbineは消化管で吸収され，肝臓でcarboxylesteraseにより5′-deoxy-5-fluorocytidine(5′-DFCR)に代謝される．次に肝臓や腫瘍組織などに存在するcytidine deaminaseにより5′-deoxy-5-fluorouridine(5′-DFUR)に変換される．更に，腫瘍組織に高レベルで存在するTPにより活性体である5-FUに変換され，抗腫瘍効果を発揮する．TPは乳癌，大腸癌，子宮頸癌，頭頸部癌などで周囲組織より3.5倍近くの高濃度に発現している．腎が主な排泄経路であり，投与後24時間までに投与量の69-80%が尿中へ排泄される．日本では，2008年現在，手術不能または再発乳癌，結腸癌における術後補助化学療法に適応が承認されている．用量規定毒性は悪心，嘔吐，下痢，手足皮膚反応である．

乳癌ではanthracycline系薬剤抵抗性乳癌においてdocetaxel単独とdocetaxel+capecitabine併用の第III相試験により，併用群で良好な生存期間が得られている[9]．大腸癌ではStage III結腸癌の術後補助化学療法および転移性大腸癌の初回化学療法においてcapecitabineと5-FU/LVの第III相試験が行われ，capecitabineの非劣性が示されている[10,11]．

c. Cytidine類縁化合物

1) Cytarabine(Cylocide)

cytarabine(1-β-D-arabinofuranosyl-cytosine: Ara-C)は2′-deoxycytidineのdeoxyriboseが5炭糖であるarabinose(Ara)に置換されたnucleosideである(図3)．Ara-Cはnucleoside transporterを介して細胞内に入る．細胞内でdeoxycytidine kinase(dCK)の酵素によりAra-cytidine monophosphate(Ara-CMP)，Ara-cytidine diphosphate(Ara-CDP)，ara-cytidine tri-

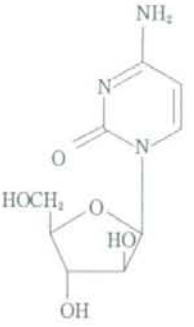

図3 Cytarabine

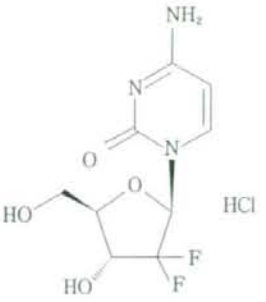

図4 Gemcitabine

phosphate(Ara-CTP)へと代謝され，抗腫瘍効果を発揮する．Ara-CTPはDNA polymeraseによるDNA合成時，本来のDNA合成の基質であるdeoxycytidine triphosphate(dCTP)と競合阻害してDNAの合成を阻害する．Ara-CはS期で最も効果を示す細胞周期依存性であり，持続点滴での投与が必要である．急性白血病や悪性リンパ腫が適応となり，大量投与によりnucleoside細胞膜透過能の低下を克服する細胞外Ara-C濃度を得る，細胞内Ara-CTP濃度を高めるなどからcytarabine大量療法が行われる．Ara-Cは90％以上が肝，血液中でcytidine deaminase(CDA)とdeoxycytidylate deaminaseによりuracil arabinoside(Ara-U)，arabinouracil 3′-phosphate(Ara-UMP)の不活体に代謝され，ほとんどが尿から排泄される．

用量規定毒性は骨髄抑制であり，ショック，呼吸困難，全身潮紅，血管浮腫，じんま疹などのアナフィラキシー様症状，シタラビン症候群(発熱，筋肉痛，骨痛，ときに斑状丘疹性皮疹，胸痛，結膜炎および倦怠感)があらわれることがある．

2) Enocitabine(Sunrabin)

enocitabine(behenoyl Ara-C: BHAC)はAra-Cのprodrugで，Ara-Cの代謝酵素CDAに抵抗性を示す．肝でdeacylationを受けて徐々にAra-Cに変わり，抗腫瘍効果を発揮する．慢性白血病の急性転化を含む急性白血病に対して用いられる．

3) Gemcitabine(Gemzar)

gemcitabine(2′,2′-difluorodeoxycytidine: dFdC)はdeoxycytidineの糖鎖deoxyriboseの2′位の水素がフッ素に置換された化合物である(**図4**)．Ara-Cと構造的に類似する．Ara-Cと同様，nucleoside transporter systemにより細胞内に入り，dCKによりリン酸化され，dFdC monophosphate(dFdCMP)に変わる．続いてnucleoside monophosphateとdiphosphate kinaseにより5′-diphosphate(dFdCDP)，5′-triphosphate(dFdCTP)に代謝され，活性体となり，Ara-Cと同様にdCTPと競合してDNA合成を阻害する(**図5**)．dFdCTPはdCMP deaminase(dFdCMPからdFdUMPへの不活性化の酵素)を抑制することによるself-potentiation(自己増強)と半減期の著明な延長を認める．またdFdCDPによるribonucleotide reductaseの抑制，dCTP濃度低下がdCKの活性を促して，gemcitabineからdFdCMPへの代謝が促進されるなど，Ara-Cにはない特性を認め，活性体である三リン酸化物(dFdCTP)が細胞内で高濃度に長時間維持されることにより，固形癌に対して強い殺細胞作用を示すと考えられている．gemcitabineのほとんどがCDA，deoxycytidine deaminase(dCDA)によりdifluorodeoxyuridine(dFdU)の不活体に代謝され，尿から排泄される．

用量規定毒性は骨髄抑制であり，その他食欲不振，悪心，嘔吐などの消化器症状，肝機能障害，発熱などを認める．一般に毒性は軽度であるが，頻度は低いものの間質性肺炎(1.4％)など重篤な副作用も認められる．

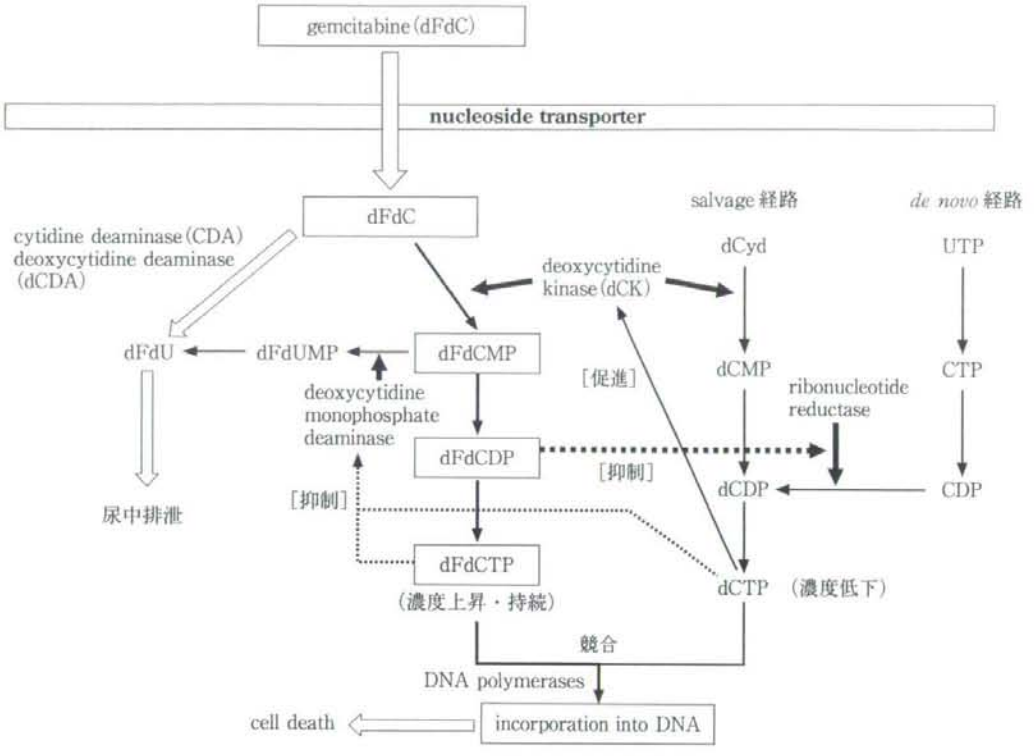

図5 Gemcitabineの代謝経路

2008年現在，非小細胞肺癌，膵癌，胆道癌に適応が承認されている．非小細胞肺癌ではcisplatinとの併用が標準治療の一つとして用いられている．膵癌では米国，カナダで行われた5-FUとの無作為化比較試験にて，gemcitabin群で疼痛やperformance status，体重減少など症状緩和効果とともに生存期間でも有意差をもって有用性が確認された[12]．以降gemcitabine単独治療が切除不能進行膵癌の標準治療として位置付けられている．術後補助療法においても比較試験が行われ，無治療群に比べgemcitabine群で無再発生存期間と全生存期間について有用性が確認された[13]．胆道癌では日本を含め幾つかの第II相試験が行われ，更に英国と日本ではgemcitabine単独とgemcitabine+cisplatinの比較試験が実施されている．

2. プリン拮抗薬

1950年代前半に抗癌剤として合成され，主に急性白血病，慢性骨髄性白血病に用いられている．purine合成とpurine相互転化反応の阻害による核酸合成阻害薬である．

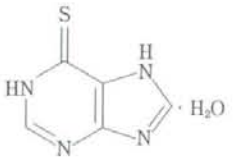

図6 Mercaptopurine

a. Mercaptopurine(6-MP, Leukerin)

6-MP(図6)は細胞内に取り込まれ，hypoxanrhin-guanine phosphoribosyl transferase (HGPRT)によりthioinosine monophosphate (TIMP)に変換される．TIMPは主にinosinic acidからadenylic acidおよびguanylic acidへの合成を阻害し，抗腫瘍効果を発揮する．経口剤であり，16-50%が吸収され，個人差が大きい．血中濃度のピークは約2時間であり，xanthine

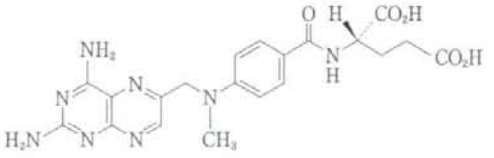

図7 Methotrexate

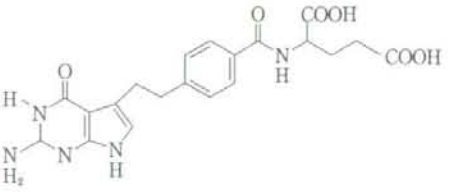

図8 Pemetrexed

oxidaseにより不活化され，尿中に排泄される．主な毒性は骨髄抑制，肝機能障害，消化器毒性などである．

b. その他

fludrabine(Fludara)はDNAポリメラーゼおよびRNAポリメラーゼを阻害し，DNAの合成・修復ならびにRNA合成を阻害することにより増殖細胞および静止細胞のいずれにも抗腫瘍効果を発揮する．再発または難治性の低悪性度B細胞性非ホジキンリンパ腫およびマントル細胞リンパ腫，貧血または血小板減少症を伴う慢性リンパ性白血病に適応が得られている．

nelarabineは9-β-D-arabinofuranosyl guanin(ara-G)のprodrugで，体内でT細胞に高い選択性のあるara-Gへ変換され，その活性体であるara-GTPが，DNA合成を阻害することで抗悪性腫瘍効果を発揮する．再発または難治性T細胞急性リンパ性白血病およびT細胞リンパ芽球性リンパ腫に適応が得られている．

3. 葉酸拮抗薬

葉酸は，生体内でdihydrofolate(FH_2)からtetrahydrofolate(FH_4)に変わり，nucleoside合成に関係する補酵素である．葉酸拮抗抗癌剤としてaminopterinが最初に開発され，1940年代小児急性白血病に用いられた．その後，aminopterinはmethotrexate(MTX)に変わり，現在も白血病や胃癌などの固形癌に用いられている．

a. Methotrexate

MTXは葉酸の4-amino,10-methyl異性体である(図7)．dihydrofolate reductase(DHFR)は葉酸がFH_2からFH_4に変わる反応の還元酵素であり，MTXはDHFRと強固に結合して還元反応を阻害する．この結果，FH_2プールが拡大し，TSなどの阻害につながるなどによりpurine，pyrimidyne nucleosideの枯渇，DNA合成と修復の阻害などの抗腫瘍効果を発揮する．主な副作用は骨髄抑制，肝機能障害である．主な適応は次のとおりである．

- MTX通常療法：白血病，絨毛性疾患
- CMF療法(シクロホスファミドおよびフルオロウラシルとの併用)：乳癌
- MTX・leucovorin救援療法：肉腫(骨肉腫，軟部肉腫など)，急性白血病の中枢神経系および睾丸への浸潤に対する寛解，悪性リンパ腫の中枢神経系への浸潤に対する寛解に適応．
- MTX・5-FU交代療法：胃癌に対する5-FUの抗腫瘍効果の増強を狙ったbiochemical modulationの一つ．MTXを前投与することによりpurine生合成が阻害され，その結果，細胞内のphosphoribosyl pyrophosphate(PRPP)が増加し，ORPTの働きが増すことで，5-FUからFUMP，FUTPへの変換が促進され，RNA障害が増強される．
- M-VAC療法：binblastin，doxorubicin，cisplatinとの併用で，尿路上皮癌に適応．

b. Pemetrexed(Alimta)

pemetrexedはpyrrolopyrimidineを基本骨格とした複数の葉酸代謝酵素を阻害する薬剤である(図8)．pemetrexedは主にreduced folate carrier(RFC)systemにより細胞内に取り込まれ，速やかにpolyglutamation(ポリグルタミン酸化)を受ける．pemetrexedのポリグルタミン酸塩は，複数の主要な葉酸代謝酵素であるTS，DHFR，glycinamide ribonucleotide formyltransferase(GARFT)，aminoimidazole carboxamide formyltransferaseを阻害し，細胞内のnucleotide poor balanceを崩すことによりアポトーシスを誘導する．細胞周期のG1-S期で殺細胞性

活性を示す．pemetrexedは複数の酵素を阻害することから，ほかの葉酸代謝拮抗薬に耐性をもつ細胞に対しても有効性が期待できることが示唆されている[14]．半減期は3.1時間で，投与後24時間までに約90％が尿中に排泄される．したがって，腎機能低下を認める患者での使用には注意を払う必要がある．用量規定毒性は骨髄抑制，粘膜炎，皮疹であり，その他の主な副作用は肝機能障害，悪心，嘔吐，食欲低下など消化器毒性，易疲労感である．

欧米では，1999年，悪性胸膜中皮腫に対しpemetrexedとcisplatin併用とcisplatin単独との第III相試験が実施され，全生存期間中央値は併用群12.1カ月，cisplatin単独群9.3カ月と併用群での有効性が確認された(p=0.02)[15]．また2007年，gemcitabine/cisplatin併用とpemetrexed/cisplatin併用の第III相試験でpemetrexed/cisplatin併用の非劣性が報告されている．日本では2008年5月現在，cisplatinとの併用で悪性胸膜中皮腫に適応が承認されている．

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Predictive Factors of Outcome and Tumor Response to Systemic Chemotherapy in Patients with Metastatic Hepatocellular Carcinoma

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Objective: Systemic chemotherapy is an important treatment modality for metastatic hepatocellular carcinoma (HCC); however, the predictive factors of outcome and tumor response have not been fully investigated. The aim of this study was to identify factors that could be used to predict outcome and tumor response to systemic chemotherapy in patients with metastatic HCC.
Methods: We retrospectively examined 82 consecutive patients with metastatic HCC undergoing systemic chemotherapy to investigate factors associated with outcome and tumor response. The patients underwent 5-fluorouracil, mitoxantrone and cisplatin (FMP) therapy.
Results: The overall objective response rate was 22% (95% confidence interval, 14–32), and the median survival time and 1-year survival for all patients were 11.2 months and 43.5%, respectively. Multivariate analysis demonstrated that the absence of radiologically active intrahepatic disease ($P = 0.02$) and ascites ($P = 0.002$) was independent favorable prognostic factors. Although multivariate analysis revealed no significant predictive factors of tumor response, the response rates in patients without radiologically active intrahepatic disease (response rate, 46%) tended to be higher than those in patients with active intrahepatic disease (response rate, 17%) ($P = 0.05$).
Conclusion: Patients with metastatic HCC, who had sufficient hepatic function and no radiologically active intrahepatic disease, might be good candidates for systemic chemotherapy.

Key words: hepatocellular carcinoma – prognostic factor – tumor response – chemotherapy – metastasis

INTRODUCTION

The prognosis of patients with advanced hepatocellular carcinoma (HCC) remains poor, particularly for those with extrahepatic metastases (1). For patients with extrahepatic disease, systemic chemotherapy is one of the most important treatment modalities (2–6), but it has only limited value in clinical practice. Various clinical trials conducted after 1980s using different single agents reported overall response rates of 0–20%. Combination chemotherapy with cytotoxic agents yields higher response rates (2–7); however, a randomized controlled study comparing a promising combination therapy with a single agent failed to show any overall survival advantage (8). Recently, attention has focussed on molecularly targeted agents for the treatment of advanced HCC, because they have been reported to offer some degree of success for the treatment of challenging cancers like renal cell carcinoma (6,9,10). Among them, sorafenib, which is an oral multikinase inhibitor targeting Raf kinase and receptor tyrosine kinases, has been reported to confer an overall survival advantage of 12 weeks, with manageable toxicity, in comparison with placebo in a Phase III trial (11). These encouraging results suggest that sorafenib promise as a standard treatment for patients with advanced HCC. Recently, combination therapy using sorafenib with cytotoxic agents or other newly developed molecularly targeted agents have been tested for the activity against HCC (6,10).

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Analysis of predictive factors of outcome and tumor response can suggest the appropriate candidates for systemic chemotherapy. Although those in patients with advanced HCC, not limited to metastatic disease, have often been reported (12–18), these factors have not been investigated in patients with metastatic HCC receiving systemic chemotherapy. Since there may be some differences in predictive factors of outcome and tumor response between the patients with metastatic HCC alone and those with advanced HCC partially, including metastatic disease, the present study was conducted to evaluate a number of variables that may affect survival and tumor response in patients with metastatic HCC alone treated by systemic chemotherapy. To our knowledge, this is the first report concerning predictive factors of outcome and tumor response in patients with metastatic HCC alone receiving systemic chemotherapy.

PATIENTS AND METHODS

Patients

The study subjects were 82 consecutive patients with metastatic HCC for whom surgical resection was not indicated, and who underwent continuous infusion of 5-fluorouracil, mitoxantrone and cisplatin (FMP therapy) between September 1993 and January 2005 at the National Cancer Center Hospital, Tokyo, Japan (Table 1). The FMP therapy has been reported to show promising anti-tumor activity (response rate: 27%, median survival time: 11.6 months) with tolerable toxicity in a Phase II trial (19). HCC was diagnosed on the basis of histological examination or distinctive findings of computed tomography (CT) and/or angiography, along with the elevated levels of serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA II). Pretreatment evaluation included a complete medical history and a physical examination. The laboratory procedures included a complete differential blood count, biochemistry tests, viral markers including serum hepatitis B surface antigen and serum hepatitis C antibody, urinalysis and tumor markers including serum levels of AFP and PIVKA II. All patients underwent electrocardiography, chest radiography and CT/magnetic resonance imaging within 4 weeks before chemotherapy. Written informed consent was obtained from all patients before treatment.

Table 1. Patient characteristics

	Number of patients (%)
Age (years)	
Median (range)	61 (34–74)
Gender	
Male	76 (93)
Female	6 (7)
Performance status	
0	72 (88)
1–2	10 (12)
History of blood transfusion	
Present	18 (22)
Alcohol abuse[a]	
Present	15 (18)
Smoking habit[b]	
Present	39 (48)
Hepatitis B surface antigen	
Positive	33 (40)
Hepatitis C virus antibody	
Positive	43 (52)
Prior treatment	
Hepatic resection	53 (65)
Local ablation	18 (22)
Transcatheter arterial chemoembolization	50 (61)
None	13 (16)
Organs affected by metastases	
Lung	55 (67)
Lymph nodes	38 (46)
Bone	11 (13)
Adrenal gland	5 (6)
Child-Pugh class	
A	65 (79)
B	17 (21)
Radiologically active intrahepatic disease	
Absent	13 (16)
Portal vein tumor thrombosis	
Present	14 (17)
Alpha-fetoprotein (ng/dl)	
Median (range)	261 (3–959 300)
PIVKA II (mAU/ml)	
Median (range)	860 (10–418 000)

PIVKA II, protein induced by vitamin K absence or antagonist-II. [a]Alcohol intake of $\geq$80 g/day $\times$ 5 years, [b]Smoking habit of $\geq$20 cigarettes/day for $\geq$10 years.

Treatment Schedule

All patients received systemic chemotherapy using the FMP regimen as follows: 5-fluorouracil, mitoxantrone and cisplatin were given as a continuous intravenous infusion at a dose of 450 mg/m^2 on Days 1–5, 6 mg/m^2 on Day 1 and 80 mg/m^2 over a 2-h period on Day 1, respectively, with standard hydration. If there was no evidence of tumor progression or unacceptable toxicity with dose adjustments based on the toxic effects observed, the treatment was repeated every 4 weeks until a maximum of six courses were achieved. The patients who were refractory to this regimen

were allowed to undergo other anticancer treatments at their physician's discretion.

Factors Analyzed

Pretreatment clinical variables were evaluated for their relationship to the survival and tumor response by univariate and multivariate analyses. The pretreatment variables were chosen by considering possible effects on the prognosis and tumor response as indicated by previous investigations (2–6,12–18) or suggested from our own clinical experience. Each variable, which was classified as host- or tumor-related, was divided into two subgroups in accordance with clinically meaningful values as given in Table 2. No radiologically active intrahepatic disease was defined as complete tumor necrosis and no residual lesion in the entire liver as a result of prior local therapy on contrast-enhanced CT or magnetic resonance imaging before FMP therapy.

Overall survival was measured from the date of initial treatment to the date of death or last follow-up. The objective tumor response was assessed by CT or magnetic resonance imaging every 4 weeks after the start of FMP therapy. Response was evaluated according to the World Health Organization guidelines. The best overall response was recorded for each patient. Bone metastases were not regarded as measurable lesions.

Statistical Analyses

Survival curves were calculated by the Kaplan–Meier method, and the differences in survival were evaluated by log-rank test. The Cox proportional hazard model was used to determine the most significant variables related to survival. Differences in response rate were evaluated by the chi-squared test as univariate analyses. The logistic regression model was used to determine the most significant variables related to tumor response. In the multivariate analyses, all variables considered in univariate analysis were entered, and variable selection was not conducted. Statistical analyses were performed using SPSS 11.0J (SPSS Inc. Chicago, IL, USA). All P values presented in this report are of the two-tailed type. Differences at $P \leq 0.05$ were considered significant.

RESULTS

Patient Characteristics

The characteristics of all 82 patients are given in Table 1. The diagnosis of HCC was made on the basis of either histological examination (71 patients, 87%) or distinctive findings of CT and/or angiography with elevated serum levels of AFP or PIVKA II (11 patients, 13%). Prior treatments included hepatic resection in 53 patients (65%), local ablative therapy in 18 (22%), transcatheter arterial chemoembolization in 50 (61%) and no treatment in 13 (16%). Thirteen patients (16%) had been judged as having no active intrahepatic disease radiologically by two radiologists, and their prior treatments for the primary tumor had been hepatic resection in 12 patients, radiofrequency ablation therapy in one, percutaneous ethanol injection in one and transcatheter arterial chemoembolization in two. The median period between the latest prior treatment and the start of FMP therapy was 4.1 (range: 1.1–51.7) months. The median number of courses of FMP therapy was 2 (range: 1–6).

Survival and Tumor Response

The median survival time, 1-year survival proportion and median progression-free survival in all 82 patients were 11.2 months, 43.5%, 3.2 months, respectively (Fig. 1). At the time of analysis, 70 patients had died, and the causes of death were tumor progression and/or hepatic decompensation (65 patients), rupture of esophageal varices (one patient), cerebral bleeding from brain metastasis (three patients) and treatment-related death (one patient).

Eighty-one patients were evaluable for response; the remaining one patient could not be evaluated because of treatment-related death on Day 22 of the first course of FMP therapy. Although no patient achieved a complete response, 18 patients achieved a partial response, giving an overall response rate of 22% (95% confidence interval, 14–32). Forty-two patients (51%) showed no change and the remaining 21 patients (26%) had progressive disease. The median survivals of the patients with partial response, no change and progressive disease were 22.3, 11.9 and 5.5 months, respectively ($P < 0.01$). After this chemotherapy, two partial responders underwent surgical resection for residual HCC lesions in the lung and liver, respectively. These resections were successful and both patients achieved complete clinical remission after surgery, and one of both has survived with no recurrence over 7.5 years although the remaining had died of hepatic failure 3 months after resection.

Prognostic Factors

Median survival times, hazard ratios and P values of survival time for univariate analysis are given in Table 2. Among host-related factors, absence of ascites and an alkaline phosphatase level of $\leq$333 U/l were significantly associated with longer survival times. Among tumor-related factors, absence of lymph node metastasis and active intrahepatic disease was significantly associated with longer survival times. The results of multivariate analysis are given in Table 3. Absence of active intrahepatic disease and ascites was shown by multivariate analysis to be significantly favorable prognostic factors. The overall survival of patients without active intrahepatic disease (median: 22.3 months) was significantly better than that of patients with active intrahepatic disease (median: 10.6 months) (Fig. 2). The overall survival of

Table 2. Predictive factors of outcome and tumor response to FMP therapy for metastatic HCC

	Number of patients	Median survival survival (months)	Hazard ratio (95% CI)	*P* value*	Response (%)	*P* value
Host-related variables						
Age (years)						
>65	21	9.1			14.2	
≤65	61	12.1	0.69 (0.40–1.19)	0.18	24.5	0.50
Gender						
Male	76	11.7			21.0	
Female	6	7.3	2.15 (0.91–5.09)	0.07	33.3	0.85
Performance status						
0	72	11.2			22.7	
1–2	10	6.9	0.93 (0.46–1.89)	0.84	20.0	0.99
Alcohol abuse[a]						
Present	15	12.1			20.0	
Absent	67	10.5	1.06 (0.59–1.90)	0.86	22.4	0.99
Smoking habit[b]						
Present	39	12.1			17.9	
Absent	43	9.8	1.30 (0.81–2.10)	0.27	25.6	0.57
Blood transfusion						
Present	18	14.8			27.8	
Absent	64	10.0	1.66 (0.92–3.00)	0.09	20.3	0.72
Hepatitis B surface antigen						
Negative	49	11.8			20.4	
Positive	33	9.8	1.17 (0.72–1.91)	0.52	24.2	0.89
Hepatitis C virus antibody						
Negative	39	10.5			25.6	
Positive	43	12.1	0.84 (0.52–1.35)	0.47	18.6	0.62
Ascites						
Present	4	2.0			25.0	
Absent	78	11.7	0.17 (0.06–0.48)	0.0002	21.8	0.99
White blood cells $\times 10^3$ (/mm^3)						
≥4.0	67	11.5			20.9	
<4.0	15	9.1	1.21 (0.64–2.26)	0.56	26.7	0.89
Hemoglobin (g/dl)						
≥11	77	11.2			20.8	
<11	5	9.3	1.07 (0.39–2.96)	0.90	40.0	0.65
Platelets $\times 10^4$ (/mm^3)						
≥10	69	11.5			24.6	
<10	13	9.9	1.17 (0.63–2.20)	0.62	7.7	0.32
Total bilirubin (mg/dl)						
>1.0	18	10.0			11.1	
≤1.0	64	11.5	0.73 (0.42–1.28)	0.27	25.0	0.35
Albumin (g/dl)						
>3.5	52	10.6			23.1	
≤3.5	30	11.7	0.99 (0.60–1.62)	0.96	20.0	0.96

Continued

Table 2. *Continued*

	Number of patients	Median survival survival (months)	Hazard ratio (95% CI)	*P* value*	Response (%)	*P* value
Aspartate aminotransferase (U/l)						
>82	18	8.1			11.1	
≤82	64	11.7	0.76 (0.44–1.34)	0.35	25.0	0.35
Alanine aminotransferase (U/l)						
>70	16	11.5			0.0	
≤70	66	10.6	1.19 (0.65–2.19)	0.57	27.3	0.13
Alkaline phosphatase (U/l)						
>333	32	9.4			12.5	
≤333	50	10.1	0.59 (0.36–0.97)	0.03	28.0	0.17
Prothrombin time (%)						
>80	31	11.7			22.5	
<80	44	10.0	0.81 (0.49–1.35)	0.42	20.5	0.99
Prior treatments						
Absent	13	11.7			33.3	
Present	69	11.2	0.78 (0.41–1.48)	0.44	20.0	0.51
Tumor-related variables						
Metastatic site						
Lung						
Absent	27	9.9			18.5	
Present	55	11.4	0.78 (0.47–1.30)	0.34	23.6	0.81
Lymph node						
Absent	44	12.1			25.0	
Present	38	9.8	1.71 (1.05–2.77)	0.03	18.4	0.65
Bone						
Absent	71	11.1			20.2	
Present	11	14.7	0.77 (0.38–1.55)	0.46	36.4	0.40
Adrenal gland						
Absent	77	10.6			23.4	
Present	5	11.8	1.60 (0.64–4.04)	0.31	0.0	0.51
Radiologically active intrahepatic disease						
Absent	13	22.3			46.1	
Present	69	10.6	2.43 (1.17–5.03)	0.01	17.4	0.05
Portal vein tumor thrombosis						
Absent	68	11.7			22.1	
Present	14	10.0	1.49 (0.80–2.80)	0.21	21.4	0.99
Alpha-fetoprotein (ng/dl)						
>1000	32	8.1			18.8	
<1000	50	12.1	0.76 (0.47–1.24)	0.27	24.0	0.77
PIVKA II (mAU/ml)						
>1000	38	11.7			26.3	
<1000	40	11.2	1.24 (0.76–2.02)	0.40	17.5	0.94

HCC, hepatocellular carcinoma; FMP therapy, combination therapy of 5-fluorouracil, mitoxantrone and cisplatin; CI, confidence interval, –; reference category. *Log-rank test. [a]Alcohol intake of ≥80 g/day × 5 years, [b]Smoking habit of ≥20 cigarettes/day for ≥10 years.

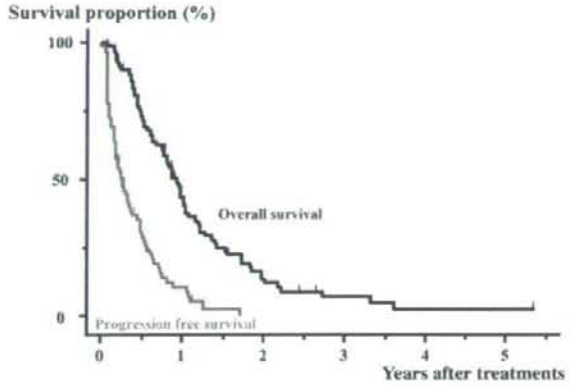

Figure 1. Overall survival and progression free survival curves of 82 patients who received 5-fluorouracil, mitoxantrone and cisplatin (FMP) therapy for metastatic hepatocellular carcinoma (HCC).

Table 3. Significant prognostic factors determined by multivariate analysis with the Cox proportional hazard model

Variable	Hazard ratio (95% CI)	*P* value
Radiologically active intrahepatic disease (−)	0.42 (0.21–0.89)	0.02
Ascites (−)	0.195 (0.07–0.54)	0.002

patients without ascites (median: 11.7 months) was significantly better than that of patients with ascites (median: 2.0 months) (Fig. 3).

Predictive Factors of Tumor Response

The response rates of the two subgroups for each variable are given in Table 2. Univariate and multivariate analyses revealed no significant predictive factors of tumor response. However, the response rates in patients without active intrahepatic disease in the liver (response rate, 46%) tended to be higher than those in patients with active intrahepatic disease (response rate, 17%) ($P = 0.05$).

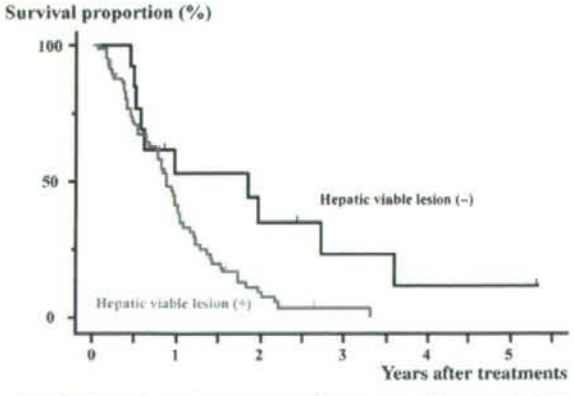

Figure 2. Comparison of overall survival in patients with metastatic HCC receiving FMP therapy with and without radiologically active intrahepatic disease.

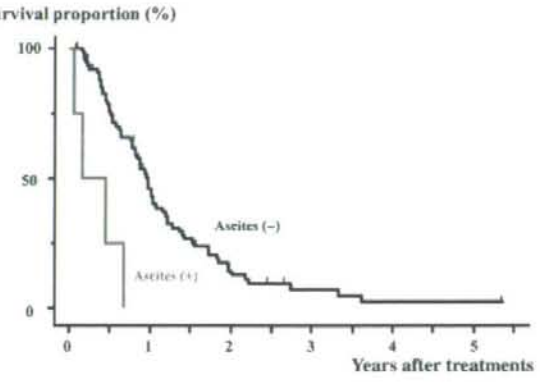

Figure 3. Comparison of overall survival in patients with metastatic HCC with and without ascites receiving FMP therapy.

DISCUSSION

Systemic chemotherapy for metastatic HCC is an important treatment modality (2–6), especially in view of the verified survival benefit of sorafenib (10,11). In this Phase III trial comparing sorafenib with placebo for advanced HCC (11), the rate of response to sorafenib was low (complete response: 0%, partial response: 2.3%), but the time-to-progression and overall survival were significantly longer for sorafenib than for placebo (median time-to-progression: 24.0 versus 12.3 weeks, hazard ratio 0.69, $P = 0.000007$, overall median survival: 46.3 versus 34.4 weeks, hazard ratio 0.58, $P = 0.00058$). Sorafenib is the first agent that has been proven to confer a survival benefit, and to show promise as a standard treatment, for patients with advanced HCC (10). To improve the treatment efficacy, development of further regimens of systemic chemotherapy, such as combination therapy comprising sorafenib and cytotoxic agents or other molecularly targeted agents, remains challenging.

In this study, to clarify the appropriate candidates for systemic chemotherapy, analysis of predictive outcomes and tumor response was conducted in patients with metastatic HCC receiving systemic chemotherapy. The study subjects were patients with metastatic HCC receiving FMP therapy, which has been shown in a Phase II trial to have promising anti-tumor activity (response rate: 27%, median survival time: 11.6 months) with tolerable toxicity for metastatic HCC (19). This regimen consists of three kinds of cytotoxic agents, not molecularly targeted agents like sorafenib. There were some differences between conventional cytotoxic chemotherapy and molecularly targeted agents. First, the tumor responses to conventional cytotoxic chemotherapy were greater than for molecularly targeted agents. Secondly, the

toxicities of conventional cytotoxic chemotherapy, especially hematological and hepatic toxicities, were more severe and difficult to manage than those of molecularly targeted agents. Thirdly, some conventional cytotoxic regimens need to be administered on an inpatient basis with standard hydration. However, many molecularly targeted agents are orally active and can be taken by patients on an outpatient basis. Therefore, there may be some differences in predictive outcome and tumor response between combination therapy comprising cytotoxic agents alone and therapy that includes molecularly targeted agents. Recently, trials of combinations of cytotoxic and molecularly targeted agents have been reported increasingly (10), and it would be worthwhile to analyze the predictive outcomes and tumor response in these subjects.

Multivariate analysis of prognostic factors in this study showed that absence of ascites and radiologically active intrahepatic disease was independent favorable factors. Presence of ascites is one of the most important factors to consider when evaluating hepatic reserve, being included in the Okuda staging system (20) and Child-Pugh classification (21) and has been shown to be a prognostic factor in previous studies of patients with advanced HCC (3,12,18). Although patients with massive or moderate ascites were not included in the present study, the outcome for patients with even a small amount of ascites was extremely poor, with a median survival of only 2.0 months. In such patients with impaired hepatic reserve, the toxicity of chemotherapy might outweigh its benefits.

There were some possible reasons why patients without active intrahepatic disease showed better survival than those with active intrahepatic disease. First, those without active intrahepatic disease might have a lower risk of hepatic failure due to progression of the intrahepatic tumor compared with those with active intrahepatic disease. Second, they might have a smaller tumor burden than those with active intrahepatic disease. A smaller tumor burden has been reported to be a favorable, independent prognostic indicator for advanced HCC (3,12,18). Finally, they might obtain a better tumor shrinkage effect of FMP therapy compared with patients with active intrahepatic disease, and this in turn might result in longer survival. Therefore, we also analyzed the predictive factors of tumor response to identify patients who might obtain a tumor response to FMP therapy. In patients receiving conventional cytotoxic agents/regimens like FMP therapy, it is important to predict the tumor response, because patients who achieve tumor shrinkage may show prolonged survival and improvement of clinical symptoms, such as tumor-related pain, and their general condition.

Among the variables investigated, the response rates in patients without active intrahepatic disease tended to be higher than those in patients with active intrahepatic disease, although the differences did not reach significance in univariate and multivariate analyses. The response rates in the patients with active intrahepatic disease were also analyzed, but no specific findings were obtained (data not shown). The precise reasons why patients without active intrahepatic disease obtained a better tumor response than those with such lesions remain unknown. There were no differences in patient characteristics, such as performance status, hepatic function, tumor burden outside the liver and tumor markers, between patients with and without active intrahepatic disease. The limited tumor heterogeneity in this population might have been a factor: heterogeneity of HCC has been reported to be closely related to chemoresistance (2–6,10,22). Yang et al. (18) have also reported a better response in patients with distant metastases than in those without, the difference perhaps suggesting heterogeneity of intrahepatic HCC. The limited heterogeneity in patients without active intrahepatic disease might result in a better response to FMP therapy. From these analyses of predictive factors of outcome and tumor response in patients with metastatic HCC receiving systemic chemotherapy, the best candidates were considered to be patients without ascites and active intrahepatic disease, although it may be considered that the results merely reflect the patients' conditions classified according to outcome. In such patients, FMP therapy resulted in favorable survival (median: 22.3 months) and tumor response (46%), although such patients comprised a very small population (only 16% in this study). These factors need to be considered in future clinical trials, including randomized trials, for patients with advanced HCC.

In conclusion, patients with metastatic HCC who have sufficient hepatic function and no active intrahepatic disease might be good candidates for systemic chemotherapy. This analysis may be helpful for predicting life expectancy and tumor response, determining treatment strategies and designing future clinical trials, including randomized trials for patients with advanced HCC.

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Conflict of interest statement

None declared.

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Identification of a Novel Tumor-Associated Antigen, Cadherin 3/P-Cadherin, as a Possible Target for Immunotherapy of Pancreatic, Gastric, and Colorectal Cancers

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Abstract

Purpose: To establish cancer immunotherapy, it is important to identify the tumor-associated antigens (TAA) that are strongly expressed in the tumor cells but not in the normal cells. In this study, to establish an effective anticancer immunotherapy, we tried to identify the useful TAA of pancreatic cancer.

Experimental Design: Based on a previous genome-wide cDNA microarray analysis of pancreatic cancer, we focused on cadherin 3 (CDH3)/P-cadherin as a novel candidate TAA for anticancer immunotherapy. To identify the HLA-A2 (*A*0201*)–restricted CTL epitopes of CDH3, we used HLA-A2.1 (HHD) transgenic mice (Tgm). Furthermore, we examined the cytotoxicity against the tumor cells *in vitro* and *in vivo* of CTLs specific to CDH3 induced from HLA-A2–positive healthy donors and cancer patients.

Results: *CDH3* was overexpressed in the majority of pancreatic cancer and various other malignancies, including gastric and colorectal cancers, but not in their noncancerous counterparts or in many normal adult tissues. In the experiment using HLA-A2.1 Tgm, we found that the CDH3-$4_{655-663}$ (FILPVLGAV) and CDH3-$7_{757-765}$ (FIIENLKAA) peptides could induce HLA-A2–restricted CTLs in Tgm. In addition, peptides-reactive CTLs were successfully induced from peripheral blood mononuclear cells by *in vitro* stimulation with these two peptides in HLA-A2–positive healthy donors and cancer patients, and these CTLs exhibited cytotoxicity specific to cancer cells expressing both CDH3 and HLA-A2. Furthermore, the adoptive transfer of the CDH3-specific CTLs could inhibit the tumor growth of human cancer cells engrafted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusions: These results suggest that CDH3 is a novel TAA useful for immunotherapy against a broad spectrum of cancers, including pancreatic cancer.

Pancreatic cancer has a poor prognosis, with an overall 5-year survival rate of ~5% (1). A surgical resection remains the only option for a long-term survival, but patients with resectable pancreatic cancer are in the minority (9-22%; refs. 2–4). Even in these patients, however, the 5-year survival rate remains ~20% in spite of surgery with a curative intent (5, 6). Up to 80% of patients present with locally advanced or metastatic disease, and their median survival ranges from 6 to 9 months (7). It is generally thought that the presence of few signs or symptoms in the early stage, lack of an effective screening test, high rate of relapse, and poor response to current therapies contribute to the poor prognosis of this malignancy. Hence, the development of novel therapeutic modalities is an issue of great importance, and immunotherapy may be a potential treatment for pancreatic cancer.

To establish an effective antitumor immunotherapy, the identification of tumor-associated antigens (TAA) plays a key role. In the past, many TAAs in various malignancies have been identified using the method of cDNA expression cloning (8–10) and a serologic analysis of the recombinant cDNA expression library (11–15). Recently, cDNA microarray technologies have been developed, and the analyses of the gene expression profiles of cancer and normal cells have provided us an effective approach for the identification of the TAAs (16–21). As a result, we have identified the proliferation potential-related protein (16) and glypican-3 (20, 21) as ideal

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Translational Relevance

Efforts to find novel therapeutic modalities for pancreatic cancer patients have to be increased to improve poor patient prognosis, and immunotherapy could be a potentially effective treatment modality. In the current study, we identified a novel tumor-associated antigen, cadherin 3 (CDH3)/P-cadherin, which was overexpressed in various malignancies, including pancreatic cancer, whereas it was not expressed in most normal organs based on genome-wide cDNA microarray analyses. We identified two HLA-A2–restricted epitopes that could induce CDH3-reactive CTLs in HLA-A2.1 (HHD) transgenic mice. In addition, CDH3-reactive CTLs were successfully induced from the peripheral blood mononuclear cells of healthy donors and cancer patients, and we showed the efficacy of the anticancer effect of CDH3-reactive CTLs *in vitro* and *in vivo*. These results suggested that CDH3 is a good candidate of immunotherapeutic target for not only pancreatic cancer but also various malignancies overexpressing CDH3. Based on the findings of the current study, we are now planning to take this study forward to the next stage, a phase I clinical trial of CDH3 peptide-based immunotherapy of broad-spectrum malignancies including pancreatic cancer. The final goal of this immunotherapy is established on an adjuvant immunotherapeutic regimen for various malignancies in combination with other therapies.

TAAs, useful for the tumor immunotherapy of esophageal cancer and hepatocellular carcinoma, respectively, and we have started a clinical trial of glypican-3 peptide-based immunotherapy of hepatocellular carcinoma. In this study, we analyzed the gene expression profiles of pancreatic cancer using the genome-wide cDNA microarray consisting of 27,648 genes, and we focused on cadherin 3 (CDH3)/P-cadherin as a novel promising target for the anticancer immunotherapy of pancreatic cancer and various malignancies, including gastric and colorectal cancers.

CDH3 was first identified in mouse placenta (22). CDH3 is a classical cadherin, a member of the cell-cell adhesion molecules that mediate intracellular adhesion by Ca^{2+}-dependent homophilic interactions (23). The intracellular domain interacts with the catenins, which connect the cadherins to the actin cytoskeleton (24). These molecules play an important role in not only mediating cellular adhesion but also in its signal transduction activity that influences several important biological processes, such as tissue development, cell migration, cell scattering, and tumorigenesis (23). In humans, the expression of CDH3 is restricted to the basal layers of the stratified epithelia, indicating a role in cell growth and differentiation (25). A few studies have reported the up-regulation of CDH3 as a factor in the aggressive biological behavior and poor prognosis in breast (26–28) and endometrial cancers (29).

We herein identified the human CDH3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these epitope peptides could induce tumor-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of healthy donors and cancer patients.

Materials and Methods

cDNA microarray analysis. A profiling of the gene expression by a cDNA microarray analysis was done, as described previously (30). The tissue samples from pancreatic cancers and adjacent noncancerous normal pancreatic tissues were obtained from surgical specimens, and all patients provided their written informed consent to participate in this study.

Mice. HLA-A2.1 (HHD) Tgm; H-2D$^{b-/-}$ β2m$^{-/-}$ double knockout mice introduced with a human β2m-HLA-A2.1 (α1, α2)-H-2D^{b} (α3 transmembrane cytoplasmic; HHD) monochain construct gene were generated in the Département SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur (31, 32) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 wk of age were purchased from Charles River Japan. The mice were maintained at the Center for Animal Resources and Development of Kumamoto University, and they were handled in accordance with the animal care guidelines of Kumamoto University.

Cell lines and HLA expression. The human pancreatic cancer cell line PANC1, oral squamous cancer cell line HSC3, and a TAP-deficient and HLA-A2 (*A*0201*)–positive cell line T2 were purchased from Riken Cell Bank. The human pancreatic cancer cell line PK8 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University. The human colon cancer cell line HCT116 was kindly provided by Dr. B. Vogelstein, Johns Hopkins University. The human liver cancer cell line SKHep1 was kindly provided by Dr. Kyogo Ito, Kurume University. The expression of HLA-A2 was examined using flow cytometry with an anti–HLA-A2 monoclonal antibody (mAb), BB7.2 (One Lambda, Inc.), to select the HLA-A2–positive blood donors and target cell lines for the cytotoxicity assays. These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS in a 5% CO_2 atmosphere at 37°C.

Patients, blood samples, and tumor tissues. The clinical research using PBMCs from the donors was approved by the Institutional Review Board of Kumamoto University. The blood samples and the cancer and adjacent noncancerous tissues were obtained during routine diagnostic procedures after obtaining formal written informed consents by the patients in Kumamoto University Hospital. We also obtained blood samples from healthy donors after receiving their written informed consent. All samples were anonymized, numbered at random, and stored at -80°C until use. All patients and healthy donors were of Japanese nationality.

Reverse transcription-PCR and Northern blot analysis. The reverse transcription–PCR (RT-PCR) analysis of the normal and cancer tissues and cell lines was done as described previously (33). The primer sequences were as follows: *CDH3*, sense 5′-GTCCCTTCCCCCAGAGACTGAA-3′ and antisense 5′-CCTCAAAATCCAAACCCTTCC-3′ and *β-actin*, sense 5′-CATCCACGAAACTACCTTCAACT-3′ and antisense 5′-TCTCCTTAGAGAGAAGTGGGGTG-3′. After normalization by *β-actin* mRNA as a control, we compared the expression of *CDH3* mRNA in the tissues and cell lines. A Northern blot analysis was done as described previously by using a *CDH3* gene-specific cDNA probe (365–1198 bp; ref. 34).

Immunohistochemical staining. Immunohistochemical examinations of CDH3 protein were done as described previously (15, 16). The primary antibody used in this study, anti-CDH3 mAb, was purchased from BD Transduction Laboratories.

Lentiviral gene transfer. A lentiviral vector-mediated gene transfer was done as described (35). Briefly, 17 μg of CSII-CMV-RfA and CSIIEF-RfA self-inactivating vectors (36) carrying *CDH3* cDNAs and 10 μg of pCMV-VSV-G-RSV-Rev and pCAG-HIVgp were transfected into the 293T cells grown in the 10-cm culture dish using Lipofectamine 2000 reagent (Invitrogen Corporation). After 60 h of transfection, the medium was recovered and the viral particles were pelletted by ultracentrifugation (50,000 × *g*, 2 h). The pellet was suspended in 50 μL of RPMI 1640, and 10 μL of viral suspension were added to 5×10^4 PANC1 cells or

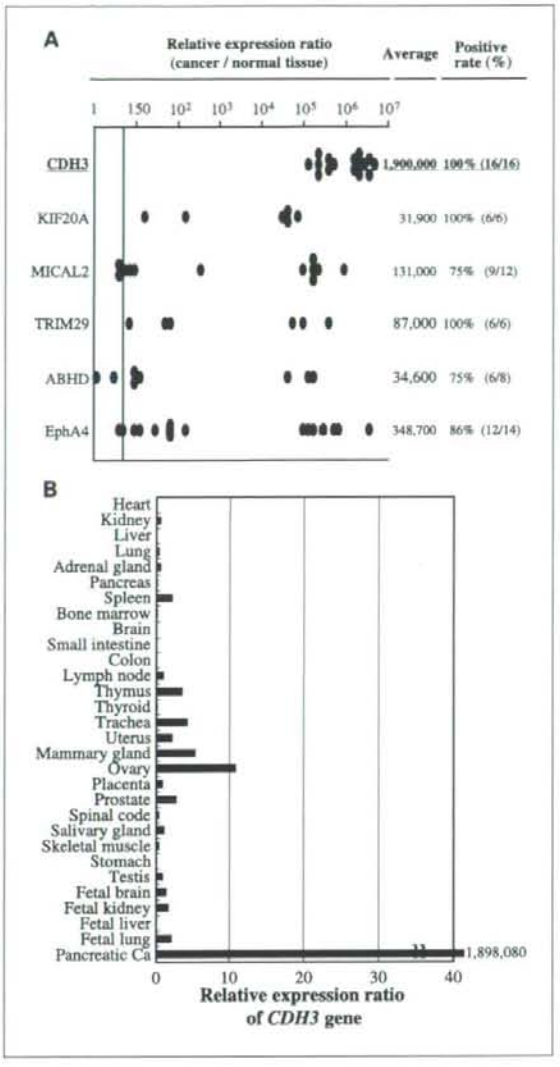

Fig. 1. Markedly and frequently enhanced expression of *CDH3* mRNA in pancreatic cancer tissues based on a cDNA microarray analysis. *A*, a list of up-regulated genes in pancreatic cancer cells. These genes were overexpressed in cancer cells compared with their normal counterparts. The expression of *CDH3* mRNA in pancreatic cancer cells was markedly enhanced in all of 16 pancreatic cancer patients. *B*, the relative expression ratio of *CDH3* gene in normal tissues based on a cDNA microarray analysis. *CDH3* gene was faintly expressed only in the ovary and mammary gland.

SKHep1 cells per well in a flat-bottomed 96-well plate. The expression of the transfected *CDH3* gene was confirmed by a Western blot analysis.

Induction of CDH3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human CDH3-derived peptides (purity, >95%), carrying binding motifs for HLA-A2 (*A***0201*)-encoded molecules, were selected using the BIMAS software program (BioInformatics and Molecular Analysis Section, Center for Information Technology), and 18 peptides were synthesized (American Peptide Company; Supplementary Table S1). The immunizations of the mice with peptides were done as previously described (34). The frequency of cells producing IFN-γ/1 × 10^5 $CD4^+$ spleen cells upon stimulation with syngeneic bone marrow–derived dendritic cells (BM-DC; 1 × 10^4 per well) pulsed with or without each peptide was analyzed by an enzyme-linked immunospot (ELISPOT) assay as previously described (21).

Induction of CDH3-reactive human CTLs. We isolated the PBMCs from the heparinized blood of HLA-A2–positive Japanese patients with pancreatic, gastric, and colorectal cancers or healthy donors, by means of Ficoll-Conray density gradient centrifugation, and the peripheral monocyte-derived dendritic cells (DC) were generated as described previously (16, 21). The DCs were pulsed with 20 μg/mL of the candidate peptides in the presence of 4 μg/mL β2-microglobulin (Sigma-Aldrich) for 2 h at 37°C in AIM-V (Invitrogen) containing 2% heat-inactivated autologous plasma. The cells were then irradiated (40 Gy) and incubated with the $CD8^+$ T cells. These cultures were set up in 24-well plates; each well contained 1 × 10^5 peptide-pulsed DCs, 2 × 10^6 $CD8^+$ T cells, and 5 ng/mL human recombinant interleukin-7 (Wako, Osaka, Japan), in 2 mL AIM-V with 2% autologous plasma. After 2 d, these cultures were supplemented with human recombinant interleukin-2 (PeproTec, Inc.) to a final concentration of 20 IU/mL. Two additional weekly stimulations with peptide-loaded autologous DCs, using the same procedure, were carried out on days 7 and 14. Six days after the last stimulation, the antigen-specific responses of the induced CTLs were investigated by a ^{51}Cr release assay and an IFN-γ ELISPOT assay.

CTL responses against cancer cell lines. The CTLs were cocultured with each of the cancer cells or the peptide-pulsed T2 cells as a target cell (5 × 10^3 per well) at the indicated effector/target ratio, and a standard ^{51}Cr release assay was done as described previously (21). The blocking of HLA class I or HLA-DR was done as described previously (21). In brief, before the coculture of the CTLs with a cancer cell line in the ^{51}Cr release assay or ELISPOT assay, the target cancer cells were incubated for 1 h with 10 μg/mL anti–class I mAb, W6/32, or 10 μg/mL anti–HLA-DR mAb, H-DR-1 and then the effects of the mAbs on either the cytotoxic activity or the production of IFN-γ by the CTLs were examined as reported previously (37).

An adoptive immunotherapy model. An experimental adoptive immunotherapy was done as described previously (21). Briefly, we s.c. inoculated HCT116 cells (4 × 10^6) positive for both HLA-A2 and endogenous CDH3 at the right flank of the NOD/SCID mice. When the tumor size became 25 mm^2 on day 7 after the tumor inoculation into the mice, the CDH3 peptide $4_{655\text{-}663}$ or $8_{757\text{-}763}$-specific CTL lines and irrelevant HLA-A2–restricted HIV peptide (SLYNTYATL) stimulated $CD8^+$ T-cell line (4 × 10^6), derived from five healthy donors, were suspended in 100 μL of PBS and injected i.v. The T cells were injected i.v. one more time on day 14. The size of the tumors was measured twice a week, and the tumor size was evaluated by measuring two perpendicular diameters using calipers.

Statistical analysis. The two-tailed Student's *t* test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay and in the tumor size between the treatment groups. A value of $P < 0.05$ was considered to be significant. The statistical analysis was done using a commercial statistical software package (SPSS for Windows, version 11.0).

Results

Identification of CDH3 gene up-regulated in pancreatic cancer and various malignancies based on a cDNA microarray. Using a genome-wide cDNA microarray containing 27,648 genes, we had previously examined the gene expression profiles of 18 pancreatic cancer tissues and their adjacent normal counterparts. After the analysis, we chose six genes of which the relative expression ratio was more than five times higher in pancreatic cancer tissues compared with their normal counterparts (Fig. 1A). We analyzed the expression of these genes using a cDNA microarray analysis in 29 kinds of normal tissues,

Table 1. Expression of *CDH3* gene in pancreatic cancer and various malignancies investigated by cDNA microarray analyses

	N	Positive rate* (%)	Average of relative expression ratio
Pancreatic cancer	16/16	100	1,900,000
Testicular cancer	10/10	100	396,000
Soft tissue tumor	21/21	100	248,000
Cholangiocellular carcinoma	19/19	100	3,600
Non-small cell lung cancer	35/37	95	73,000
Colorectal cancer	31/34	91	84,000
Cervical cancer	14/19	74	1,500
Gastric cancer	20/28	71	35,000
Urinary bladder cancer	24/34	71	30
Small cell lung cancer	3/14	21	7
Breast cancer	5/81	6	1
Prostate cancer	2/57	4	1,500
Renal cell carcinoma	0/20	0	0
Esophageal cancer	0/19	0	2

NOTE: Data are obtained from our previous studies (30, 38–41).
*The relative expression ratio (cancer/normal tissue) of >5 was considered to be positive.

including four embryonic tissues (Fig. 1B; refs. 16, 21). Consequently, we focused on CDH3 as a novel TAA of pancreatic cancer. The expression of the *CDH3* gene in pancreatic cancer tissues was markedly enhanced in all of the 16 patients tested (average of the relative expression ratio, 1,900,000; range, 94,900–4,890,000). In addition, the *CDH3* gene was faintly expressed only in ovary and mammary gland (Fig. 1B). The expression level of the *CDH3* gene was also enhanced in the majority of various malignancies, including gastric and colorectal cancers, based on the previous cDNA microarray analyses (Table 1; refs. 30, 38–41).

Expression of CDH3 mRNA and protein in normal organs, cancer cell lines, and pancreatic, gastric, and colorectal cancer tissues. The expression of the *CDH3* gene in normal tissues at the mRNA level was analyzed using RT-PCR and Northern blot analysis. A semiquantitative RT-PCR analysis of *CDH3* in the normal tissues revealed that it was faintly expressed only in thymus and fetal brain (Fig. 2A, *left*). A Northern blot analysis in normal organs using *CDH3* cDNA as a probe revealed that it was not expressed in all nine vital organs (Fig. 2A, *right*). The expression of the *CDH3* gene was detected in the various cancer cell lines using an RT-PCR analysis (Fig. 2B).

Subsequently, we analyzed the expression of the *CDH3* gene using an RT-PCR analysis in the cancer tissues and their adjacent normal counterparts, which were surgically resected. The expression of the *CDH3* gene was detected in six of eight pancreatic cancer tissues, but little expression was detected in their normal counterparts (Fig. 2C). In addition, its expression was detected in the metastatic foci of the skin and peritoneum. Furthermore, the expression of the *CDH3* gene was also detected in two of four gastric cancer tissues and six of seven colorectal cancer tissues.

To confirm the tumor-associated overexpression of CDH3 protein, we then examined many paraffin-embedded normal tissue specimens, as well as pancreatic, gastric, and colorectal cancer tissue specimens, by immunohistochemical analyses. CDH3 was not stained in the normal brain, lung, liver, kidney, spleen, stomach, small intestine, colon, pancreas, skin, and testis (Supplementary Fig. S1). In this study, we investigated 21 samples of pancreatic cancer, and strong staining of CDH3 was mainly observed at the plasma membrane of cancer cells in 15 cases, whereas very weak staining was observed in aciner cells and normal ductal epithelium of their normal adjacent pancreatic tissues (Fig. 2D, *a-d*). In addition, similar strong staining was observed in the metastatic foci of the skin and peritoneum (Fig. 2D, *e* and *f*). Furthermore, strong staining was also observed in gastric and colorectal cancer tissues (Fig. 2D, *g* and *h*). No staining was detected in the tissue specimens of all three independent tumor-forming pancreatitis tested (Fig. 2D, *i*).

Identification of CDH3-derived and HLA-A2–restricted mouse CTL epitopes using HLA-A2.1 (HHD) Tgm. To identify the CDH3-derived and HLA-A2–restricted CTL epitopes, we selected a total of 18 different candidate 9 or 10 amino acid peptides that were expected to have a higher binding affinity to HLA-A2 (*A*0201*), the most common *HLA* allelic product worldwide, by the HLA Peptide Binding Predictions in the NIH BIMAS (Supplementary Table S1). To test which could induce peptide-reactive CTLs, the $CD4^-$ spleen cells from HLA-A2.1 (HHD) Tgm, immunized i.p. twice with BM-DCs pulsed with the mixture of these 18 peptides, were again stimulated *in vitro* with BM-DCs pulsed with each peptide. We found that the $CD4^-$ spleen cells, stimulated with CDH3-$4_{655\text{-}663}$ (FILPVLGAV) and CDH3-$7_{757\text{-}765}$ (FIIENLKAA) peptide, produced a large amount of IFN-γ in a peptide-specific manner in an ELISPOT assay (Fig. 3A and B). These $CD4^-$ spleen cells (2×10^4) showed 283.7 ± 40.0 spot counts per well in response to the BM-DCs pulsed with the CDH3-$4_{655\text{-}663}$ peptide, whereas they showed 48.7 ± 11.9 spot counts per well in the presence of the BM-DCs without peptide loading ($P < 0.05$). Likewise, the $CD4^-$ spleen cells stimulated with BM-DCs pulsed with CDH3-$7_{757\text{-}765}$ peptide showed 79.3 ± 3.2 spot counts per well, whereas they showed 42.7 ± 2.5 spot counts per well in the presence of BM-DCs without peptide loading ($P < 0.05$). These results suggest that the CDH3-$4_{655\text{-}663}$ and CDH3-$7_{757\text{-}765}$ peptides could be the HLA-A2–restricted CTL epitope peptides in the HLA-A2.1 (HHD) Tgm, and we also expected these peptides to be epitopes for human CTLs.

Induction of CDH3-reactive CTLs from PBMCs of HLA-A2–positive healthy donors and cancer patients. We attempted to generate CDH3-specific CTLs from the PBMCs of healthy donors and various cancer patients positive for HLA-A2 by the stimulation with the CDH3-$4_{655\text{-}663}$ and CDH3-$7_{757\text{-}765}$ peptides. The $CD8^+$ T cells sorted from the PBMCs were incubated with the autologous monocyte-derived DCs pulsed with each peptide. After three stimulations, the cytotoxic activity against the peptide-pulsed T2 cells was examined by a ^{51}Cr release assay (Fig. 4A) and an IFN-γ ELISPOT assay (data not shown). The CTLs induced from the PBMCs of a healthy donor exhibited cytotoxic activity to the T2 cells pulsed with CDH3-$4_{655\text{-}663}$ or CDH3-$7_{757\text{-}765}$ peptide, but not to the T2 cells without peptide loading. Similar responses were observed in other donors (data not shown). These results indicate that these CTLs had a peptide-specific cytotoxicity.

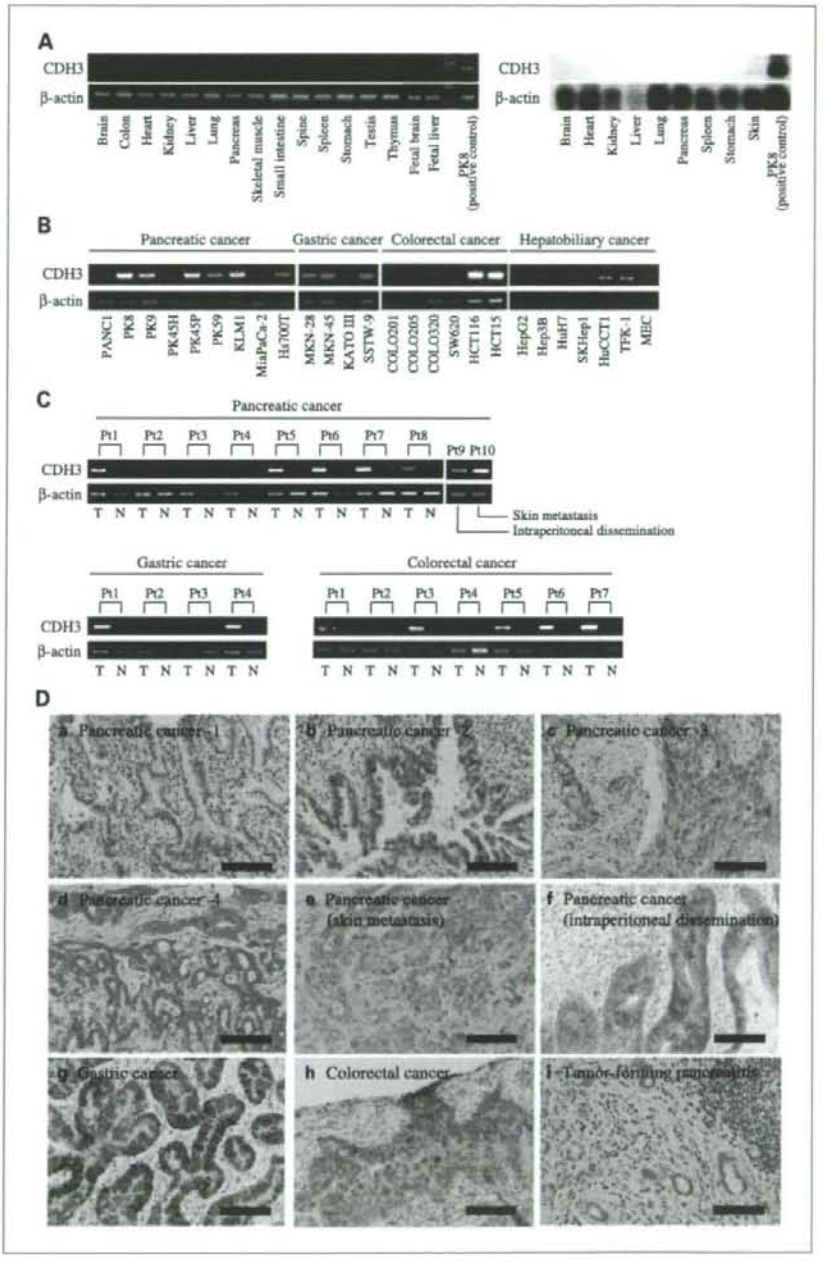

Fig. 2. The analyses of *CDH3* mRNA expressed in human normal tissues, cancer cell lines, and cancer tissues. *A*, expression of *CDH3* mRNA was investigated in various normal tissues by using RT-PCR (*left*) and Northern blot analysis (*right*). *CDH3* mRNA was faintly expressed only in thymus and fetal brain. *B*, RT-PCR analysis of the *CDH3* expression in various cancer cell lines. *C*, RT-PCR analysis of the *CDH3* expression in pancreatic, gastric, and colorectal tumor tissues (*T*) and their normal counterparts (*N*). The expression of the *CDH3* gene was detected in six of eight pancreatic cancer tissues, two of four gastric cancer tissues, and six of seven colorectal cancer tissues. In contrast, little expression was detected in their normal counterparts. *D*, immunohistochemical analyses of CDH3 protein in pancreatic, gastric, and colorectal cancer tissues. Positive staining signals are seen as brown. Scale bars, 100 μm.