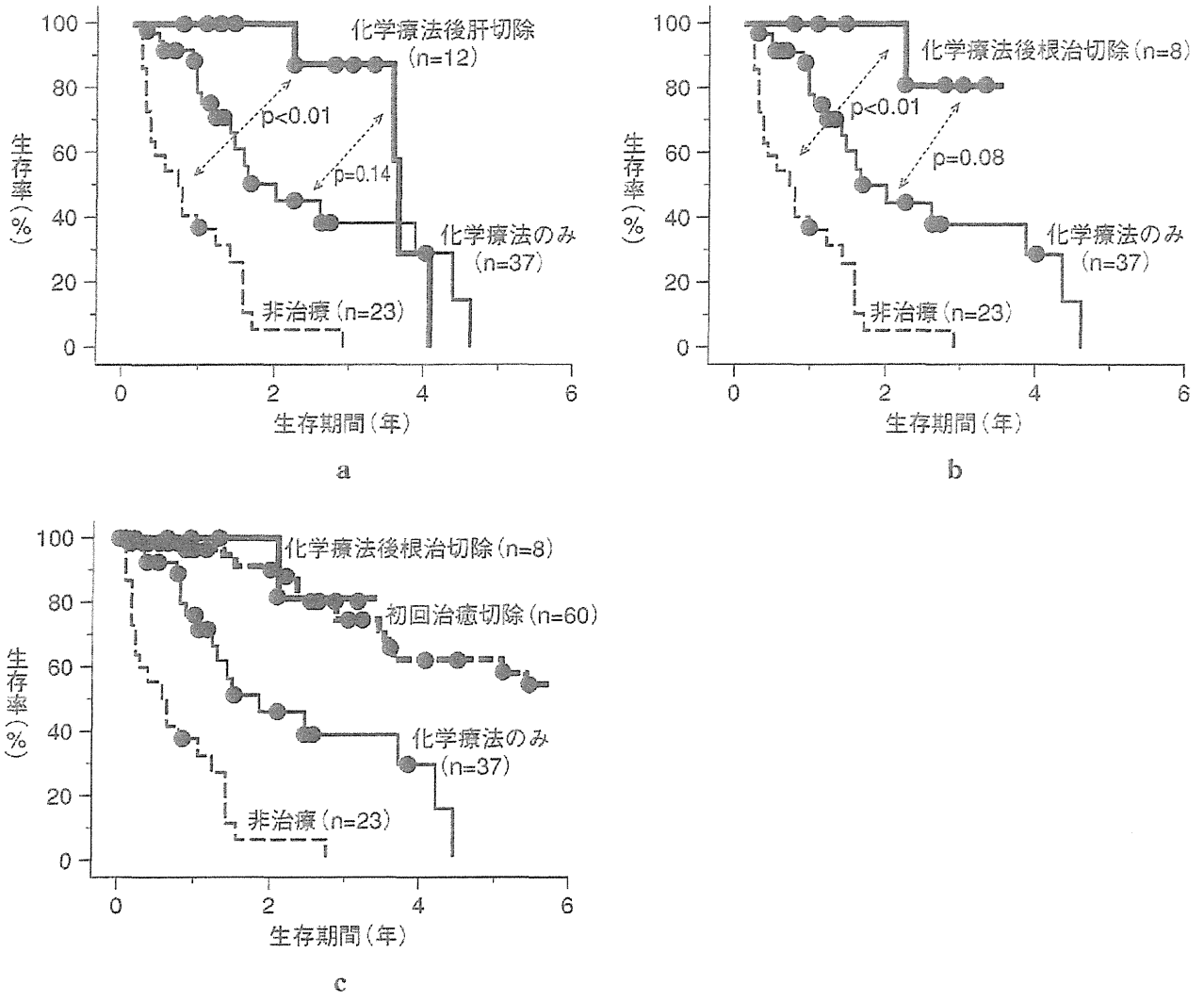

図2　治療法別生存率の比較
a：化学療法後肝切除12例の予後
b：化学療法後根治切除8例の予後
c：化学療法後根治切除8例と初回治癒切除例の予後

3 結果のまとめ

1. 現行の化学療法導入後は肝切除率が31%に増加した.

2. 化学療法後根治切除症例の予後は初回治癒切除症例とほぼ同等であった.

3. bevacizumab併用例では，肝の組織学的な類洞拡張は軽度で，血清ヒアルロン酸は低値の傾向であった.

4. ラット肝切除モデルを用いた基礎的検討でも，bevacizumabの投与は肝再生を抑制せず，むしろ肝傷害を軽減した.

4 考　察

当科では，2004年に切除不能の進行大腸癌肝転移例に対してFOLFOXやFOLFIRIを中心とする新規化学療法を開始後31%の肝切除率を得ている．中でも肝転移のみをターゲットとしてBevacizumab併用FOLFOXILI療法を施行した5例の肝切除導入率は100%で，Bevacizumab併用mFOLFOX6でSDであった症例も肝切除へconversionできた．また現在のところこれら5例の全例が生存中であり良好な成績を得ている．FOLFOXILI療法に関しては，第2相試験で既にその安全性と有効性が報告されている[4,5]．さら

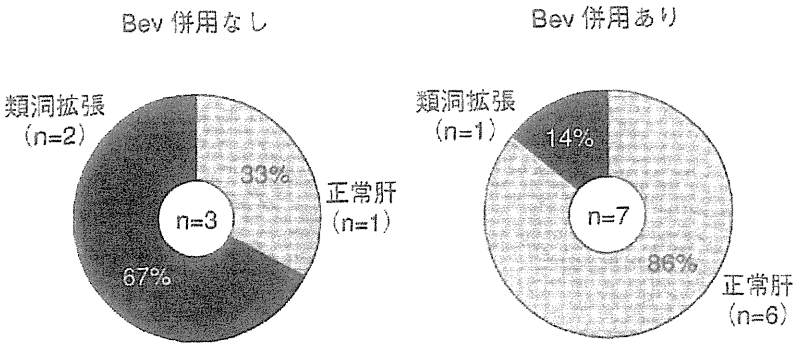

図3　Bevacizumab の化学療法後肝傷害への影響（病理診断）
オキサリプラチンを基本とした化学療法施行10例の病理診断

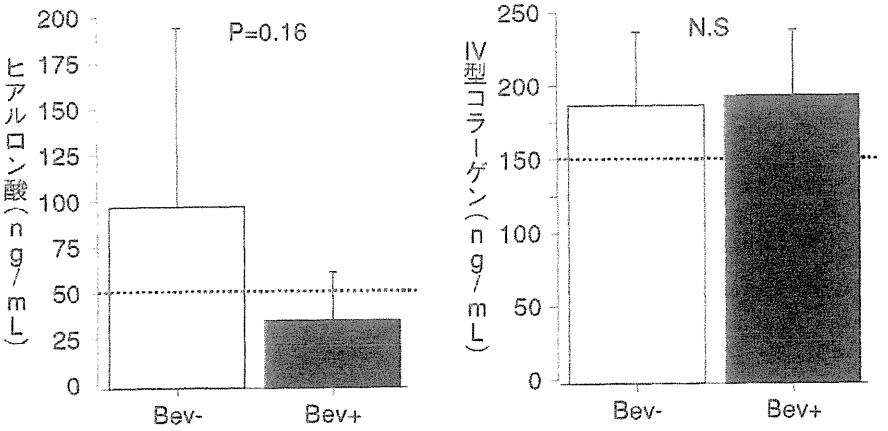

図4　Bevacizumab の化学療法後肝傷害への影響（血液検査）
Bev－：bevacizumab 併用なし，Bev＋：bevacizumab 併用あり．

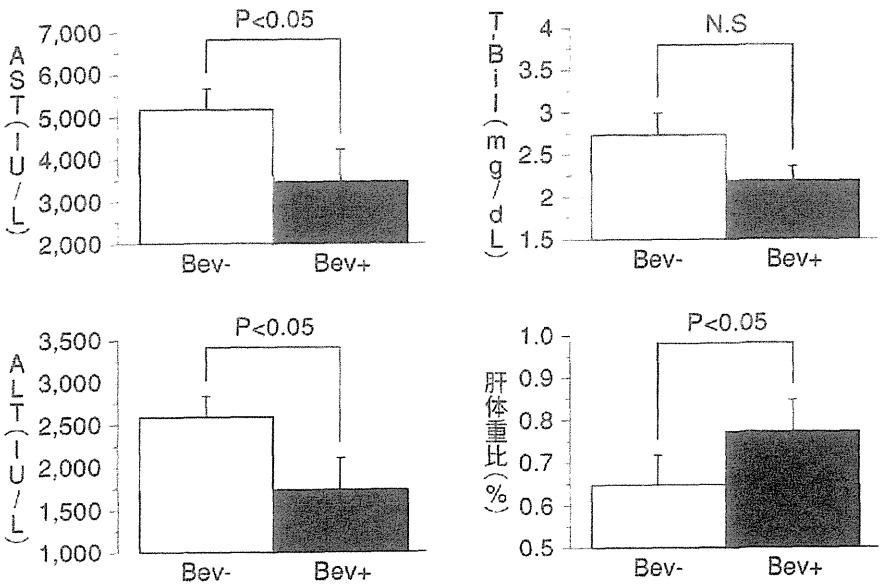

図5　ラット90%肝切除モデルにおける基礎的検討
Bevacizumab 投与の有無による肝機能および肝体重比の比較（24時間後）
Bev－：Bevacizumab 投与なし，Bev＋：Bevacizumab 投与あり．

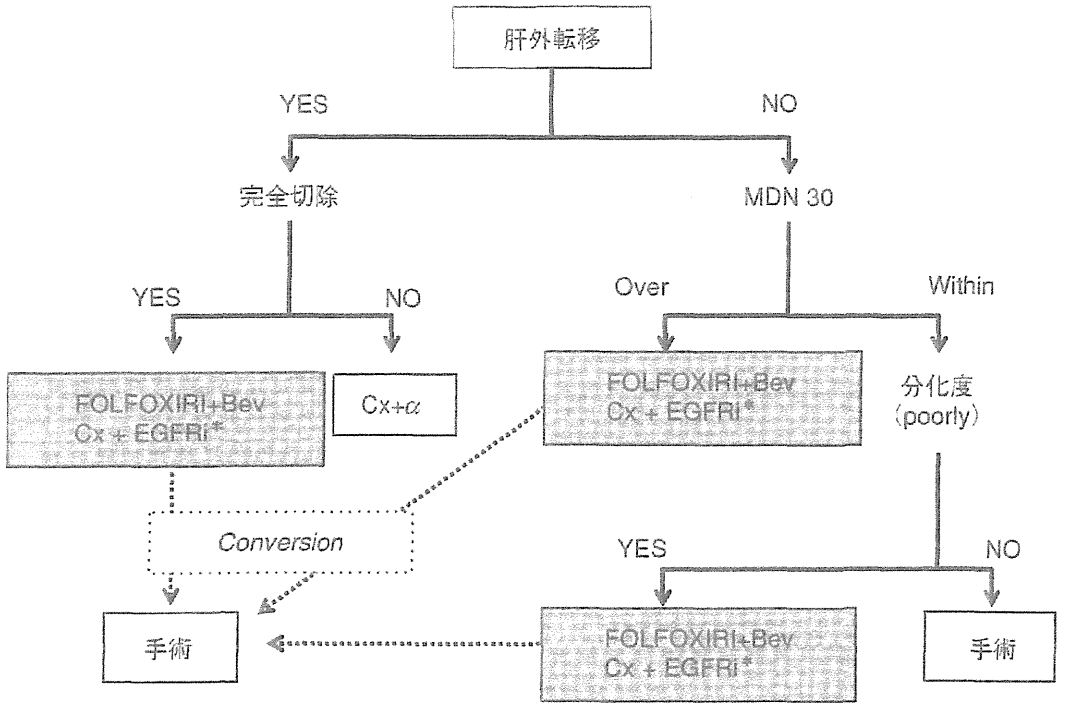

図6　大腸癌肝転移に対する治療戦略
MDN 最大腫瘍径＋腫瘍個数，Cx：chemotherapy, EGFRi：epidermal growth factor receptor inhibitor, *K-ras wild type

にFOLFIRIとのランダム比較試験にてその優位性も報告されている[6]．その後FOLFOXILIとBevacizumabの併用に関しても第2相試験が行われ，FOLFOXILI単独でみられなかったような新たな副作用の出現は認めないことや有効性(Response rate: 77%, Disease control rate: 100%）が最近報告された[7]．また，切除不能大腸癌肝転移196例に対してFOLFOXILI療法を行った結果37例（19%）にR0切除が可能となり，これら37例の予後は非切除例と比較し有意に良好であったとの報告もある[8]．当科での経験ではさらに良い結果も伺われるが，症例数が5例と少なく観察期間も3年以内であり，今後の症例数の蓄積と観察期間の延長が必要である．

Bevacizumabの併用が化学療法による肝傷害に与える影響に関しては，Riberoらが，むしろ肝傷害を軽減すると報告している[9]．彼らは，大腸癌肝転移105例に対して5-FUとオキサリプラチンによる化学療法を行い，62例のBevacizumab併用例と43例の非併用例を比較した．類洞拡張の頻度は，全体では併用例27.4%，非併用例53.5%であり，Grade 2～3の高度傷害例に限っても併用例8.1%，非併用例27.9%と有意に併用例で低率であった．Bevacizumabの併用が類洞拡張の頻度を低下させる機序は現在なお不明であるが，血管内皮細胞から過剰産生された活性酸素やmatrix metalloproteinase（MMP）-2やMMP-9の抑制が示唆されている[9]．さらに最近，術前化学療法を行った肝切除例の検討で，腫瘍の壊死はBevacizumab併用により増強したが術後肝傷害は増強しなかったとの報告もある[10]．われわれの検討でも，Bevacizumab併用例で血中ヒアルロン酸濃度が低い傾向を認め，内皮障害が軽減されている可能性が考えられた．しかしながら，今後も基礎的研究も含め更なる機序解明のための検討が必要である．

以上より現時点における大腸癌肝転移に対する戦略として，まず，肝外転移がない症例では，腫瘍個数と腫瘍径による予後因子であるMDNが30以下であり低分化型でなければ手術を選択する．それ以外の症例では強力な化学療法を行い手術へのconversionを目指す．一方，肝外転移陽性例では，特に完全切除の可能性のある症例ではまず，同様に強力な化学療法を行い，手術への

conversionを行うことで根治を目指すという方針が妥当と考えている.

結　語

切除不能進行大腸癌肝転移症例といえども強力な化学療法（Bevacizumab併用FOLFOXILI療法）と積極的な肝切除により肉眼的根治を目指すことで良好な予後が期待される．また，Bevacizumabを併用することでオキサリプラチンを基本とした化学療法による肝傷害を軽減する可能性がある.

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原　著（第26回徳島医学会賞受賞論文）

肉眼的門脈侵襲陽性肝癌切除後のSystemic IFN＋Low dose FPの有用性
－理論的根拠と臨床的効果－

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肉眼的脈管侵襲陽性肝細胞癌（肝癌）に対するIFNα併用Low dose FP全身化学療法（IFP療法）の有用性につき基礎的および臨床的に検討した。基礎的検討としてMH134マウス肝癌細胞株を用いてPeg-IFNαの増殖・浸潤・転移抑制効果を検討し，臨床的検討として二次分枝以上の門脈内腫瘍栓を有する（Vp≥2）進行肝癌症例30例をIFP施行群14例，非施行群16例に分けレトロスペクティブに生存率，再発形式などを検討した。基礎的検討では，MH134細胞はPeg-IFNα投与により増殖・浸潤能が抑制され（*in vitro*），皮下腫瘍モデルにおいて増殖を抑制した。癌細胞脾注による肝転移モデルにおいてPeg-IFNαは肝転移個数減少（投与群6個vs.対照群19個）および転移巣におけるmicrovessel densityが減少した（*in vivo*）。臨床的効果の検討では，累積および無再発生存率において，IFP施行群は非施行群と比較し，有意に予後良好であった。無再発生存率においてもIFP施行群が非施行群と比較し，有意に良好であった。Vp≥3（門脈一次分子内に腫瘍栓）症例で特に差を認めた。再発パターンは，非施行群はほとんど全て残肝多発，遠隔転移といった制御不能再発であったが，IFP施行群は再発9例中4例が残肝単発再発であり局所治療にて制御可能であった。以上より，Vp≥2肝癌術後のIFP療法は予後を改善する有用な治療法であると考えられた。

はじめに

肝細胞癌（肝癌）に対して根治的治療として肝切除を施行しても術後の再発率は2年で50－60%，5年で80%と高く，予後不良な癌の1つである。特に脈管侵襲を伴うものは再発の危険性が高い[1-3]。また，門脈二次分枝より中枢側に腫瘍栓を有する（Vp≥2）肉眼的門脈侵襲陽性肝癌は積極的に肝切除を施行しても術後早期に制御不能な再発をきたすことが多く非常に予後不良である。しかし，現行の肝癌診療ガイドライン[4]やコンセンサスミーティングが提唱する治療アルゴリズム[5]には進行肝癌に対する術後補助療法に関する規定はない。われわれは以前より肉眼的門脈侵襲陽性肝癌術後の再発防止を目的としたSystemic IFNα＋Low dose FP（IFP）療法を行ってきた。今回，根拠となる基礎的研究を含め，IFP療法が肉眼的門脈侵襲陽性肝癌術後の補助療法として有用であるという知見を報告する。

材料・対象と方法

（1）基礎的検討

＜*in vitro*＞

MH134マウス肝癌細胞に対するIFNαの腫瘍細胞増殖抑制効果についてMTT Assay（Chemicon International Inc.）を用いて，また浸潤抑制効果の検討はCell Invasion Assay（Cultrex® 96 Well BME Cell Invasion Assay）を

用いて行った。さらに血管新生因子の発現をRT-PCRを用いて検討した。IFNαはPeg-IFNα（Pegylated IFNα2b, Schering-Plaugh K. K. Osaka, Japan）を使用した。

＜*in vivo*＞

6週齢の雄性マウス（C3H/HeN Crj）にMH134細胞を1×10^5個注入し皮下腫瘍モデルを作製し，接種後1週間で腫瘍径を測定する。Peg-IFNαはMH134接種24時間前に皮下投与し，皮下腫瘍モデルにおけるPeg-IFNαの腫瘍増殖抑制効果につき検討した。

さらにMH134細胞1×10^5個を脾下極に注入して脾注肝転移モデルを作製した。Peg-IFNαはMH134脾注24時間前に皮下投与し，Peg-IFNαの肝転移抑制効果を検討した。

（2）臨床的検討

1992年11月から2010年3月までの期間に肝切除を施行した肝癌のうち，二次分枝より中枢側に門脈内腫瘍栓を有する（Vp≧2）進行肝癌症例30例を対象として，IFP療法施行群14例，非施行群16例に分け，レトロスペクティブに予後および再発形式を含む臨床病理学的検討を行った。また門脈一次分枝から本幹にかけて腫瘍栓を有するVp3-4の症例についても検討した。

IFP療法は図1に示すプロトコールで術後できるだけ早期から行い，投与期間は4週間としている。

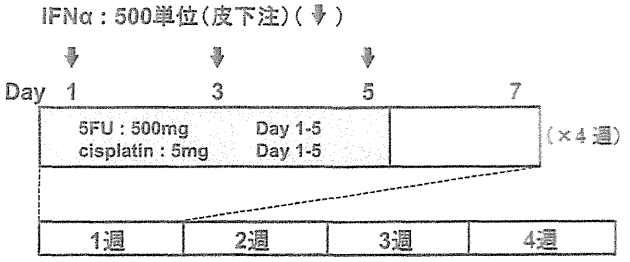

図1．IFP療法のプロトコール
IFNαは500万単位を週3回皮下投与，FP投与は5投2休で経静脈投与する（4週間投与）。

結　果

（1）基礎的検討

＜*in vitro*＞

MTT Assayを用いた検討では，MH134細胞の増殖能はPegIFNαにより濃度依存性に抑制された。Cell Invasion Assayを用いた腫瘍浸潤能の評価では，MH134細胞の浸潤能はPegIFNαにより濃度依存性に抑制された（図2，文献17より引用）。

＜*in vivo*＞

皮下腫瘍モデルにおいて，PegIFNα投与群は対照群と比較し腫瘍径が有意に小さかった（図3，文献17より引用）。またMH134細胞脾注による肝転移モデルでは，PegIFNα投与により肝転移個数は有意に抑制された（PegIFNα投与群6個 vs. 対照群19個，図4，文献17より引用）。

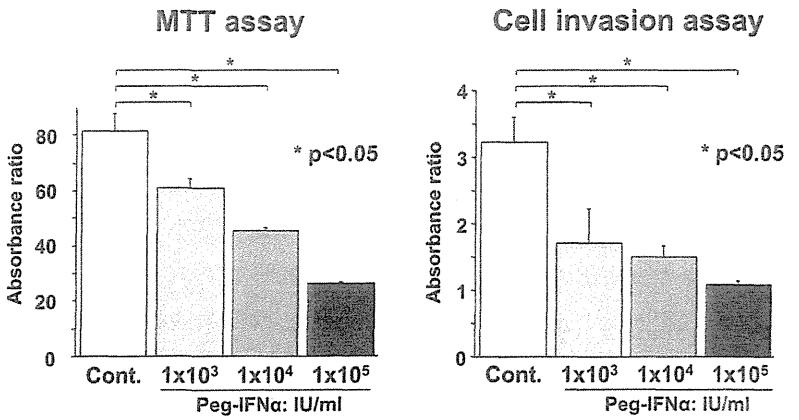

図2．MH134に対するPegIFNαの増殖・浸潤抑制効果
MH134細胞はPegIFNαにより濃度依存性に増殖，浸潤能が抑制された。

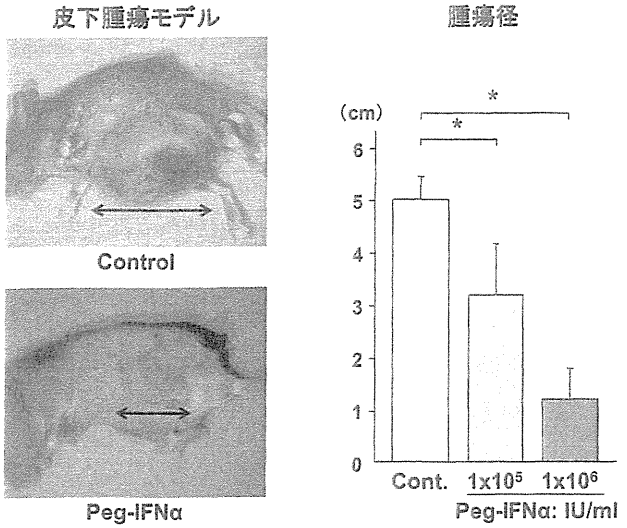

図3．皮下腫瘍モデルにおけるPegIFNαの抗腫瘍効果
皮下腫瘍モデルにおいて，PegIFNα投与群は対照群と比較し腫瘍径が有意に小さかった。

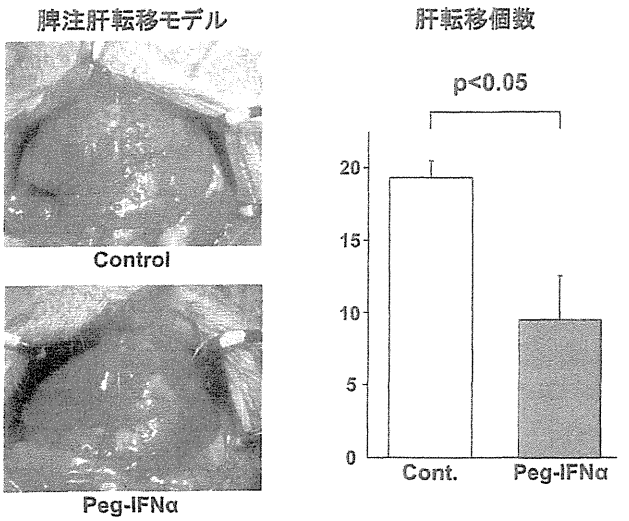

図4．脾注肝転移モデルにおけるPegIFNαの転移抑制効果
PegIFNα投与により肝転移個数は有意に減少した（投与群6個 vs. 対照群19個）。

（2）臨床的検討

IFP施行群において，問題となる有害事象は認めなかった。患者背景では年齢，性別，肝炎ウイルス，肝機能，腫瘍因子において両群間で有意差は認めなかった（表1）。

IFP群の累積生存率は1年100%，3年65%，5年54%であり，非施行群の1年38%，3年25%，5年19%と比較し有意に良好であった。無再発生存率でもIFP施行群が非施行群と比較し有意に良好であったが，IFP群においても術後早期に再発をきたす症例が多かった（図5，IFP群：1年36%，3年36%，非施行群：1年25%，3年19%）。

表1．臨床病理学的背景因子の比較

因子	IFP群 (n=14)	非施行群 (n=16)
年齢（歳）	56.0	57.5
男性/女性	12/2	12/4
HBV/HCV/NBNC	6/5/3	6/5/5
ICGR15（%）	12.3	13.5
T-bil（mg/dl）	0.9	0.7
Alb（g/dl）	3.95	3.74
AFP（ng/ml）	2781	711
PIVKA-II（mAU/ml）	4050	5110
Child-Pugh（A/B）	14/0	15/1
腫瘍径（cm）	8.6	8.2
分化度（高/中/低）	0/11/3	1/10/5

全ての項目において有意差なし

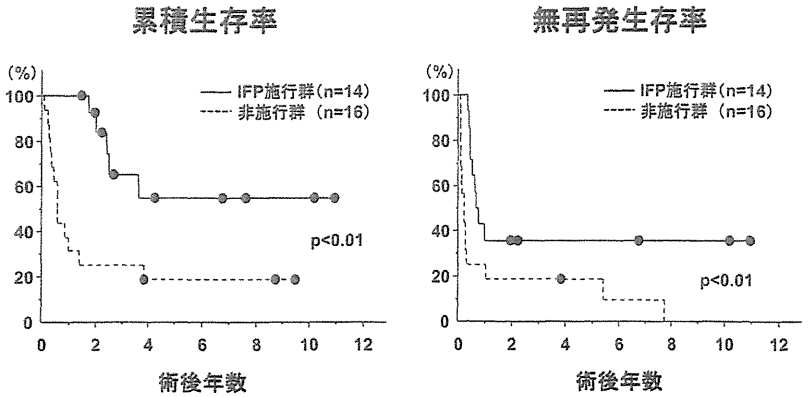

図5．Vp≧2症例における累積・無再発生存率
IFP群の累積生存率は非施行群と比較し有意に良好であった。無再発生存率でもIFP施行群が非施行群と比較し有意に良好であった。

再発パターンをみるとIFP非施行群ではほとんど全て残肝多発，遠隔転移再発といった制御不能な再発であったのに対して，IFP施行群では再発9例中4例は残肝単発での再発であり，再発時に局所治療での病勢コントロールが可能であった（図6）。同様にVp3-4症例で

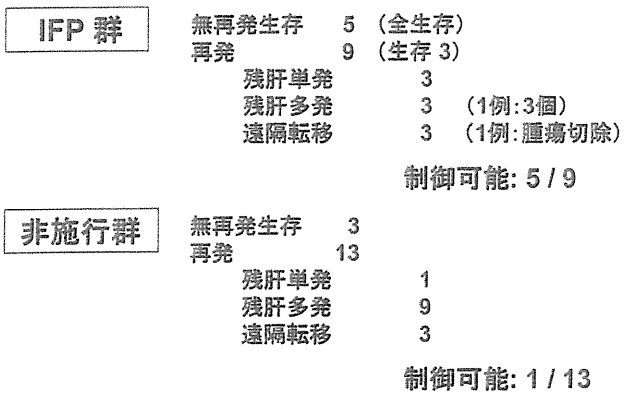

図6．IFP群，非施行群における再発パターンの比較
非施行群では制御不能な再発がほとんどであったが，IFP施行群では再発時に局所治療により病勢コントロールが可能な症例が多かった。

生存率を検討すると，累積生存率はIFP施行群（n＝8）で1年100%，3年86%，非施行群（n＝8）で1年0%，無再発生存率はIFP施行群で1年50%，3年50%，非施行群で1年0%とVp3-4の高度進行例ではより大きな差を認めた（図7）。

IFP療法により長期無再発生存している高度門脈侵襲陽性肝癌症例

50代の男性，HCV陽性。肝右葉を占拠する径16cmの巨大腫瘍および右門脈から本幹さらには左門脈二次分枝まで伸びる腫瘍栓を認めた（図8）。肝機能は良好であり，肝外病変もなかったことから拡大肝右葉切除術，門脈内腫瘍栓摘出術を施行した。術後6週間後よりIFP療法を4週間施行した。以後，外来通院で経過観察しているが術後11年経過した現在も無再発生存中である。

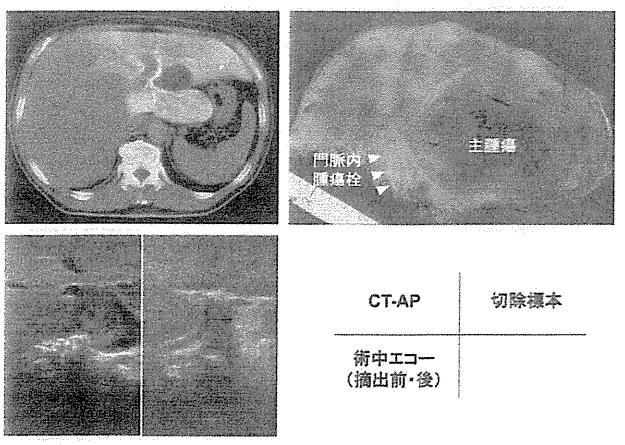

図8．長期無再発生存している高度門脈侵襲陽性肝癌（Vp4）症例
肝右葉を占拠する巨大肝癌で，門脈左枝（対側）まで腫瘍栓が進展していた。術後IFP療法（4週間）を施行し，術後11年経過し無再発生存中である。

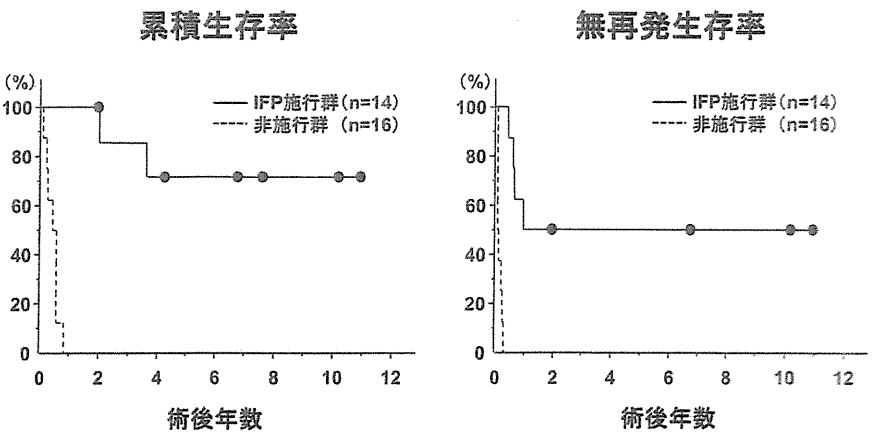

図7．Vp≧3症例における累積・無再発生存率
Vp3-4の高度進行例における累積・無再発生存率ともIFP施行群（n＝8）で有意に良好であった（累積生存率：IFP施行群1年100%，3年86%，非施行群1年0%，無再発生存率：IFP施行群1年50%，3年50%，非施行群1年0%）。

考　察

近年の画像診断技術の進歩に伴い，肝癌は比較的早い段階で発見されることが多くなった[6]。治療の面からも，肝切除・経皮的焼灼療法・肝動脈塞栓術等各種治療法の進歩，さらには肝移植治療の普及により，肝細胞癌の予後は全体として改善した[7]。しかしながらその一方で，肉眼的門脈侵襲特にVp 2以上の進行肝癌となると，標準的かつ効果的な治療法はなく，その予後は未だ著しく不良である[8]。「科学的根拠に基づく肝癌診療ガイドライン」においても，肉眼的脈管侵襲を伴わない肝細胞癌に関しては，積極的治療が勧められている[4]。すなわち，肝障害度A/Bかつ単発あるいは3 cm，3個以内の肝細胞癌に対しては，切除もしくは局所療法，肝障害度Cで3 cm，3個以内の肝細胞癌に対しては肝移植，良好な成績とともに推奨されている。しかしながら，脈管侵襲あるいは肝外転移を伴うものに関しては標準的かつ効果的な治療法は推奨されておらず，肝癌治療の中でそれら高度進行肝癌の治療のみが，いわば手つかずの状態である[4,8]。2009年度版の肝癌診療ガイドラインでは「インターフェロン併用化学療法は有用か？」というclinical questionに対して，「インターフェロン併用化学療法は有用と考えられ，行うことを考慮してもよいが，十分な科学的根拠がない」（グレードC1）と記載されている。しかし，高度の門脈侵襲を伴う進行肝癌に対するIFNα全身投与の併用療法は，門脈本幹に腫瘍栓を有する肝癌に対し，シスプラチン単独肝動注との比較試験（RCT）でその有用性が証明されている[9]。また，IFNα併用シスプラチン全身投与では奏功率は13.3%であり，生存期間の延長もみられたという報告もある[10]。

IFN/5FU療法は高度進行肝癌に対する治療として，わが国を中心として行われてきた[11,12]。歴史的にはまずIFNのみの単独全身投与が行われたが，それらの成績は，奏功率10%以下と芳しくなかった[13,14]。また，全身化学療法とIFNの併用では，奏功率が10～25%とIFN単独投与よりは高い傾向にあったが，まだ不十分な結果であった[15,16]。しかしながら，これら全身投与に使用された薬剤は，ほとんどがdoxorubicinであった。その後，肝動注療法とIFNとの併用では，奏効率は30～50%と非常に良好な結果が報告された[11,12]。肝動注に使用された薬剤は殆どがcisplatinあるいは5FU系薬剤であった。Otaら[11]の報告では，Vp 4あるいは多発肝内転移のため切除不能な症例に対して，IFN併用5FU肝動注療法による1年生存率は49%，CR症例（15%）に限ると1年生存率は83%と報告されている。Obiら[12]の報告でも，全症例の1年生存率は34%，CR症例（16%）の1年生存率は81%と報告されている。さらに興味深いことに，既に肝外転移をきたしている症例に関するIFN併用5FU肝動注療法の有効例はほとんど報告されておらず，PD症例において1年以上の予後が得られた症例はほとんどみられない。すなわち，IFN/5FU療法は，主腫瘍の進展の抑制のみならず，血液中癌細胞の着床・転移を抑制している可能性がある。そのような概念から，われわれは肉眼的門脈侵襲陽性肝癌に対する治癒切除後の補助療法として，IFN併用のLow-dose FP（5FU/CDDP）経静脈的全身投与を行うことにした。

今回の検討では，IFP施行群において累積および無再発生存率は，非施行群と比較して有意に良好であった。特に，Vp 3 - 4症例16例では，8例に肝切除後IFP療法を，8例には肝切除のみを行ったが，IFP施行群の1年生存率が100%であったのに対して，非施行群では全例1年以内に死亡していた。しかしながら，無再発生存率をみると，IFP施行群は非施行群より良好ではあるが，多くは術後早期に再発をきたしていた。IFP施行群における良好な累積生存率には，再発形式の違いが影響しているのでないかと考えている。実際，IFP群の再発の多くは，残肝内への単発再発や制御可能（局所治療が可能）な肺転移であったのに対し，非施行群は全て制御不能な再発であった。

IFNの抗腫瘍効果に関しては，マウス肝癌細胞を用いた実験で増殖抑制，浸潤抑制効果および脾注肝転移モデルでの肝転移抑制効果を証明した[17]。臨床的には単独での抗主応効果は認めないため，5FUあるいはCDDPとの組合せで，ある種の相乗効果をしめしているものと考えられる。Takaokaら[18]は，IFNが5FUによる腫瘍細胞アポトーシスを増強させるp53を誘導することを報告

した。また，Eguchi ら[19]は，細胞周期関連蛋白である p27の発現増強を介して IFN の効果が発揮されることを報告している。5FU の中間代謝物質（FdMP）の細胞内農道上昇効果，あるいは thymidylate syntase 阻害率の上昇などの報告もある[20,21]。また，IFN/5FU あるいは IFN/FP 療法の臨床的効果からは，今回のわれわれの結果が示すように肝癌細胞の着床・転移抑制効果も推測され，今後のさらなる研究が期待される。

結　語

われわれが行っている IFP 療法は，肉眼的門脈侵襲陽性肝癌術後の制御不能な再発を予防し，予後を改善する可能性がある有用な治療戦略と考えられる。

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Strong anti-tumor effect of NVP-AUY922, a novel Hsp90 inhibitor, on non-small cell lung cancer

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ABSTRACT

The anti-tumor activity of a newly developed Hsp90 inhibitor, NVP-AUY922 (AUY922), against non-small cell lung cancer (NSCLC) was examined. Twenty-one NSCLC cell lines were used, the somatic alterations of which were characterized. Cell proliferation was analyzed using a modified MTS assay. Expression of the client proteins was assessed using Western blotting. The cell cycle was analyzed using flow cytometry. The IC_{50} value of AUY922 for the NSCLC cell lines ranged from 5.2 to 860 nM (median, 20.4 nM). Based on previous data, cells with an IC_{50} of less than 50 nM were classified as sensitive cells and 19 of the 21 NSCLC cell lines were judged to be sensitive. The IC_{50} of five malignant pleural mesothelioma (MPM) cell lines revealed that the MPM cells had a significantly higher IC_{50} value (median, 89.2 nM; range, 22.2–24,100 nM) than the NSCLC cells ($p = 0.015$). There was significant depletion of both the total and phosphorylated client proteins – EGFR, MET, HER2 and AKT – at low drug concentrations (50–100 nM) in drug-sensitive cell lines. Cell-cycle analysis was performed for two sensitive cell lines, H1975 and H838. Following AUY922 treatment, an increase in the sub-G_0-G_1 cell population, as well as appearance of cleaved PARP expression, indicated the induction of apoptosis. In conclusion, AUY922 was effective against most NSCLC cell lines, independent of the type of known molecular alteration, and appears to be a promising new drug for the treatment of NSCLC.

1. Introduction

Lung cancer is associated with various types of molecular alteration, including epidermal growth factor receptor (EGFR) mutation, *K-ras* mutation, *HER2* amplification and, as recently found, *EMK4-ALK* gene fusion [1–3]. Improvements in our understanding of the molecular alterations involved in lung cancer have brought significant advancements in molecular-targeted therapy [4]. Among these alterations, *EGFR* mutations, which are frequent alterations in lung adenocarcinoma, are a predictive factor for the efficacy of EGFR-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib [1,2]. These EGFR-TKIs have a marked anti-tumor effect on NSCLCs with common *EGFR* mutations. However, acquired resistance from, for example, a secondary *EGFR* T790M mutation or *MET* amplification is a major problem that is responsible for treatment failure [5–7].

The heat-shock protein 90 (Hsp90) complex is a chaperone protein that facilitates the refolding of unfolded or misfolded proteins. It plays a pivotal role in cancer cell survival, as it stabilizes a large set of proteins, so-called client proteins, many of which are essential for apoptosis, cell-cycle regulation, proliferation, and other characteristic properties of cancer cells [8,9]. In NSCLC, Hsp90 stabilizes oncogenic proteins such as EGFR, MET, HER2 and AKT [9,10]. We and some other studies have shown that geldanamycin (GM) and its analogues, the benzoquinone ansamycin class (17 allylamino-17demethoxygeldanamycin [17-AAG] and 17 dimethylaminoethylamino-17-demethoxygeldanamycin [17-DMAG]), are effective against *EGFR*-mutated cell lines, even those that contain the *EGFR* T790M mutation that causes resistance to EGFR-TKI [11–14]. However, the results of clinical trials for 17-AAG and 17-DMAG were somewhat disappointing [15–19] and new potent Hsp90 inhibitors have therefore been pharmacologically designed and synthesized to offer improved efficacy and acceptable toxicity. NVP-AUY922 (AUY922) is one of these newly designed small-molecule Hsp90 inhibitors based on the 4,5-diarylisoxazole scaffold; it has a much higher affinity for Hsp90 than previous GM

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analogues [20]. AUY922 is bound to the ATP binding site of Hsp90α at the N-terminal domain, and its X-ray crystal structure confirms a crucial network of hydrogen bonding interactions. It exhibits the tightest binding of any small-molecule Hsp90 ligand because the entropy of binding to Hsp90 is almost negligible. Indeed, preclinical data from various types of human cancer have shown an anti-proliferative effect of AUY922, with low nanomolar potency both *in vivo* and *in vitro*, with no major adverse effects being observed in mice [20–24]. In these studies, AUY922 suppressed the client proteins (EGFR, MET, HER2 and AKT) that participate in the progression of various cancer cells, and AUY922 is considered to be a promising agent for NSCLC. However, to our knowledge, the efficacy of AUY992 has been reported in only one NSCLC cell line (A549) to date [25], although Phase II clinical trials for patients with advanced NSCLC have recently started.

In this study, we examined the anti-tumor effect of AUY922 against NSCLC cell lines containing several known genetic alterations, including *EGFR* mutations.

2. Materials and methods

2.1. Drugs and cell lines

AUY922 was obtained from Novartis (Nuremberg, Germany) and dissolved in dimethyl sulfide (DMSO) at stocked concentrations of 10 mM and stored at −20 °C. Working dilutions were always freshly prepared. Most of NSCLC and MPM cell lines used in this study were established at two institutions. The prefix NCI-H- (abbreviated as H-) indicates cell lines established at the National Cancer Institute-Navy Medical Oncology Branch, National Naval Medical Center, Bethesda, MD, and the prefix HCC- indicates lines established at the Hamon Center for Therapeutic Oncology Research, the University of Texas Southwestern Medical Center at Dallas, Dallas, TX. These cell lines were kindly provided by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). A549 was purchased from American Type Culture Collection (Manassas, VA). NCI-H3255 was provided from Dr. Bruce Johnson (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA). PC-9 was provided from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Gefitinib-resistant PC-9 cell line (RPC-9) was provided from the Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Japan [26]. All the cancer cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Determination of cell proliferation

Cell proliferation was determined by a modified MTS assay with CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI). Cells were seeded on 96-well flat-bottomed tissue culture plates (Becton Dickinson, San Jose, CA) at a concentration of 3×10^3 cells/well with complete culture medium and allowed to adhere to the plate for 24 h. Then the cells were incubated in the presence of the drug of each concentration ranging from 0 (control) to 10 μM of for another 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the treatment, 20 μL of CellTiter 96® AQueous One Solution Reagent was dropped into each well of plates. After the incubation of another 60 min, the optical densities (ODs) of these samples were directly measured using an Immuno Mini NJ-2300 (Nalge Nunc International, Rochester, NY). A reference wavelength at 490 nm was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance. The OD of control samples was regarded as 100 and others were compared to the control. Each drug concentration was distributed in 4-replicate wells and each experiment was repeated thrice. The anti-proliferative activity of AUY922 was shown as IC_{50}, which is the concentration of the drug required to inhibit cell proliferation by 50%.

2.3. Western blot analysis and immunoprecipitation

Protein expression analysis was assessed by Western blotting. The lysate was extracted and 20 μg of total protein was then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with anti-EGFR, anti-phospho-EGFR (Ty1068), anti-Met (25H2), anti-phospho-Met (3D7, Tyr1234/1235), anti-HER2, anti-phospho-HER2 (Tyr877), anti-Akt, anti-phosphor-Akt (Ser473), anti-p44/42 mitogen-activated protein kinases (MAPK), anti-phosphor-MAPK (Thr202/Tyr204), anti-Cyclin D1, anti-cdc2 and anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) (19F4) antibodies (Cell Signaling Technology, Beverly, MA), anti-Hsp90 (Novocastra, Newcastle, UK), anti-Hsp70 (Stressgen Bioreagents, Ann Arbor, MI), anti-CDK4 (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Actin (used as loading control, Millipore, Billerica, MA) and then with goat anti-rabbit and goat anti-mouse IgG-HRP coupled to horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After the incubation with antibodies, the membranes were developed by ECL Plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

2.4. Flow cytometric analysis

Cells were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase for 5 min at room temperature and then stained with propidium iodide at 50 μg/mL to determine subdiploid DNA content using a FACScan. Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was done using CellQuest version 3.3 software.

3. Results

3.1. Anti-proliferative effect of AUY922 in NSCLC cell lines

The concentrations of AUY922 at IC_{50} in each cell line are shown in Table 1 and Fig. 1. The molecular characteristics of NSCLC cell lines are also described (Table 1). The IC_{50} values in the NSCLC cell lines ranged from 5.2 to 860 nM, whereas those in the MPM cell lines ranged from 22.2 to 24,100 nM ($p = 0.015$), indicating a significant difference in AUY922 sensitivity between NSCLC and MPM cell lines. For NSCLC, AUY922 exhibited a strong anti-proliferative effect in cell lines with *EGFR* mutations that were either sensitive to EGFR-TKI or that had acquired resistance to EGFR-TKI, similar to the effects of GM analogues. Furthermore, AUY922 also exhibited anti-proliferative effects on cell lines with wild-type *EGFR*, a *K-ras* mutation, *EML4-ALK* fusion gene, or other genetic alterations.

We also determined the IC_{50} value of the SKBR3 breast cancer cell line to validate the IC_{50} value determined with our MTS assay by comparing it with published data [22]. Our and previously published IC_{50} values were 9.7 ± 3.5 nM and 3.3 ± 0.9 nM, respectively, suggesting that the IC_{50} value measured using our system was not remarkably different from the published data [22]. Thus, in accordance with the published criteria, an IC_{50} value of less than 50 nM was regarded as being a sensitive cell line [22]. On the basis of this criteria, 19 of the 21 NSCLC cell lines and two of the five MPM cell lines were classified as being sensitive ($p = 0.034$).

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Table 1
IC_{50} inhibition values for NVP-AUY922 in NSCLC and MPM cell lines.

Cancer type	Cell lines	Histological subtypes	AUY922		Genetic alterations	
			Sensitivity[a]	IC_{50} (nM)		
NSCLC	PC-9	AD	Sensitive	8.6 ± 0.5	*EGFR* mutation	Exon19 del.
	HCC2935	AD	Sensitive	9.2 ± 0.1		Exon19 del.
	HCC827	AD	Sensitive	16.9 ± 0.4		Exon19 del.
	HCC2279	AD	Sensitive	26.3 ± 3.6		Exon19 del.
	HCC4011	AD	Sensitive	17.9 ± 0.1		L858R
	H3255	AD	Sensitive	29.5 ± 5.8		L858R
	RPC-9	AD	Sensitive	20.4 ± 1.4		Exon19 del. + T790M
	H1975	AD	Sensitive	5.2 ± 0.3		L858R + T790M
	H1650	AD	Sensitive	23.5 ± 2.9		Exon19 del. + PTEN del.
	H1299	LC	Sensitive	32.4 ± 0.1	*N-ras* mutation	
	A549	AD	Sensitive	16.3 ± 0.6	*K-ras* mutation	
	H2009	AD	Sensitive	21.4 ± 0.8	*K-ras* mutation	
	H358	AD	Sensitive	28.1 ± 4.1	*K-ras* mutation	
	H2170	SQ	Sensitive	9.1 ± 0.3	*HER2* amplification	
	H1648	AD	Sensitive	9.6 ± 0.1	*HER2* amplification	
	H1819	AD	Sensitive	23.9 ± 1.0	*HER2* amplification	
	Calu3	AD	Resistant	248 ± 8.5	*HER2* amplification	
	H1993	AD	Sensitive	7.7 ± 0.2	*MET* amplification	
	H1395	AD	Resistant	860 ± 7.1	*B-raf* mutation	
	H2228	AD	Sensitive	20.4 ± 6.5	*EML4-ALK* fusion gene variant E6a/b;A20	
	H838	AD	Sensitive	17.1 ± 0.6	None	
MPM	H211	Biphasic	Sensitive	22.2 ± 3.8		
	H290	Epithelial	Sensitive	27.3 ± 3.8		
	H28	Sarcomatoid	Resistant	89.2 ± 8.2		
	HP1	Biphasic	Resistant	1070 ± 10		
	H2052	Epithelial	Resistant	24,100 ± 4900		
BC	SKBR3		Sensitive	9.7 ± 3.5		

NSCLC, non-small cell lung cancer; MPM, malignant pleural mesothelioma; BC, breast cancer; AD, adenocarcinoma; LC, large cell carcinoma; SQ, squamous cell carcinoma.
[a] Sensitivity: sensitive cell lines, IC_{50} value ≤ 50 nM; resistant cell lines, IC_{50} value > 50 nM; del, deletion; NVP-AUY922 exhibited strong effects to most NSCLC cell lines with *EGFR* and *K-ras* mutation or *HER2* and *MET* amplification.

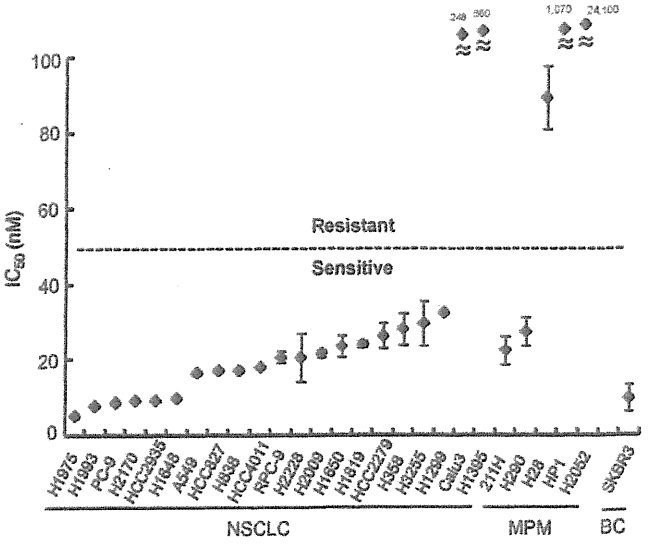

Fig. 1. IC_{50} values of non-small cell lung cancer and malignant pleural mesothelioma cell lines.

Two cell lines, H1395 and Calu3, were considered to be resistant. H1395 contains a *B-raf* mutation as a known molecular alteration, while Calu3 has a strong amplification of *HER2* and increased copy numbers of *EGFR* and *PIK3CA*. However, the H2170 cell line, which also exhibited strong *HER2* amplification and an increased copy number of *EGFR*, was classified as a sensitive cell line (IC_{50} = 9.1 ± 0.3), suggesting that amplification of *HER2* or *EGFR* is not the factor that causes resistance to AUY922.

3.2. Effects of AUY922 on molecular signature in NSCLC cell lines

Subsequent experiments focused on NSCLC. The effect of AUY922 on protein expression was examined according to concentration and exposure time in three sensitive cell lines (H1975, A549, and H838) and two resistant cell lines (H1395 and Calu3). Cells were harvested 24 h after drug treatment in a concentration gradient experiment (Fig. 2 and Supplementary Fig. 1). In sensitive cell lines, the depletion of both the total and the phosphorylated client proteins, such as EGFR, MET, HER2, AKT, and Cyclin D1 (CCND1), was observed after treatment with 50 nM of AUY922. Suppression of phospho-MAPK (p-MAPK) but not total-MAPK (t-MAPK) may be caused by down-regulation of its upstream molecules, which are the client proteins of Hsp90. Although inhibition of Hsp90 activity with drugs is generally correlated with Hsp70 protein levels after treatment [22,27], Hsp70 expression increased in both sensitive and resistant cell lines. In terms of the resistant cell lines, although expression of the client proteins was not depleted after treatment with a high concentration of AUY922 in Calu3 (IC_{50} = 248 nM), H1395 – another resistant cell line (IC_{50} = 850 nM) – showed depletion of client proteins after treatment with AUY922 at a low concentration (Fig. 2 and Supplementary Fig. 1).

For exposure time analysis, each cell line except H1395 was treated with the AUY922 concentration, which was five times as high as each IC_{50}. H1395, the IC_{50} of which was 850 nM, was exposed to 100 nM of AUY922. Although variation of protein depletion and recovery was observed according to proteins or cell lines, decreased expression of the majority of proteins was observed from 12 to 72 h (Fig. 3 and Supplementary Fig. 2). Of note, there was no major difference in the pattern of the protein expression profile time course between sensitive cell lines and H1395-resistant cell lines.

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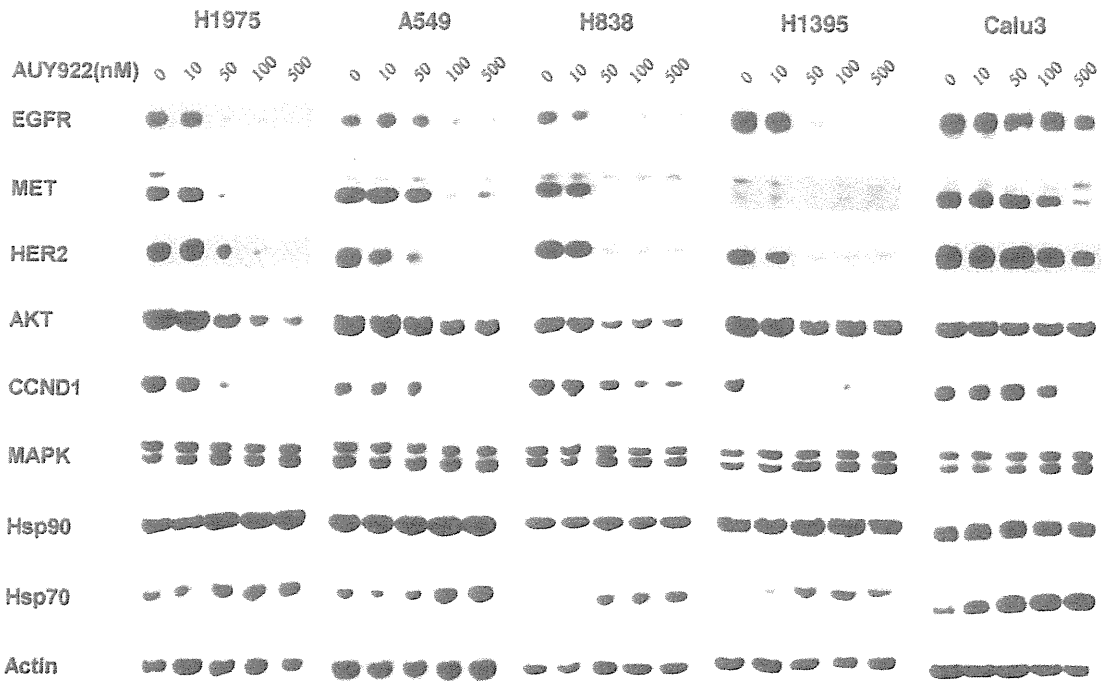

Fig. 2. The profiles of protein expression under the treatment of different AUY922 concentrations for 24 h.

3.3. Effects of AUY922 on cell cycle and apoptosis

We analyzed the cell cycle in two sensitive cell lines (H1975 and H838) to examine the impact of AUY922 on cell-cycle distribution, especially induction of apoptosis. Whereas the pattern of cell-cycle distribution after treatment of AUY922 was different between two cell lines, sub-G_0-G_1 DNA content increased in a time-dependent manner for both cell lines. Cleaved PARP also increased with AUY922 treatment, indicating that AUY922 induced apoptosis in these two cell lines (Fig. 4).

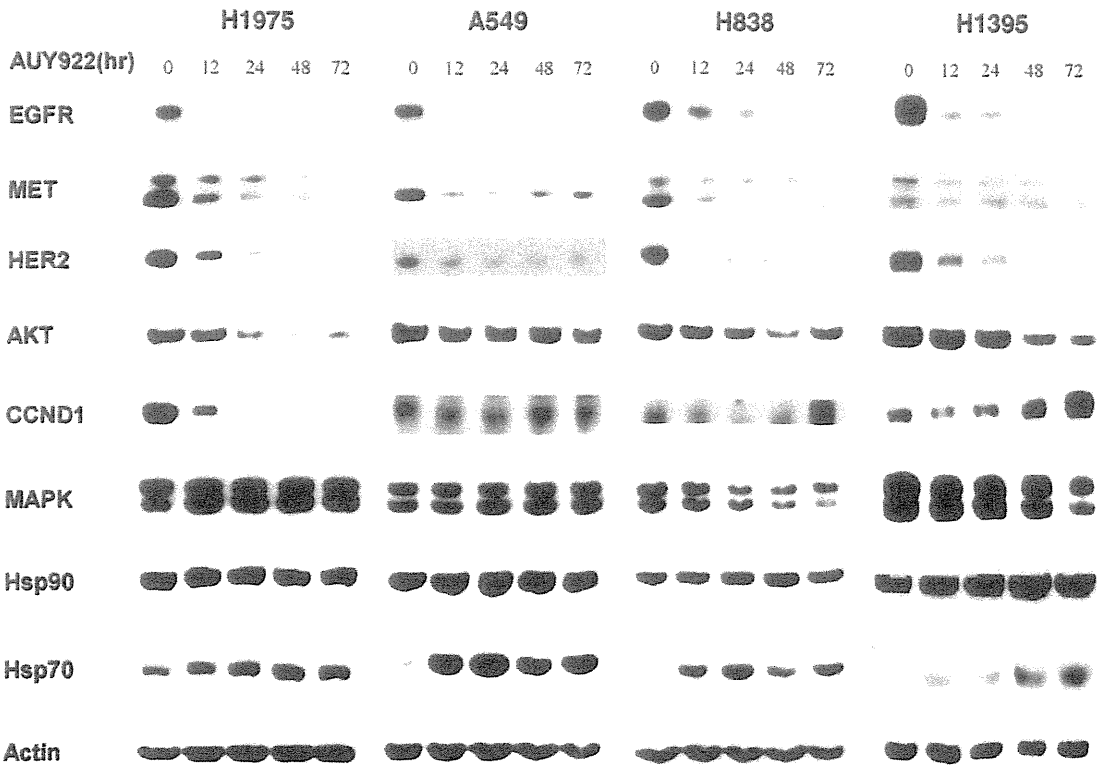

Fig. 3. The profiles of protein expression according to exposure time with AUY922. Each NSCLC cell line (H1975, A549, and H838) was treated with AUY922 of which concentration was five times as high as each IC_{50}. H1395 was exposed to 100 nM of AUY922.

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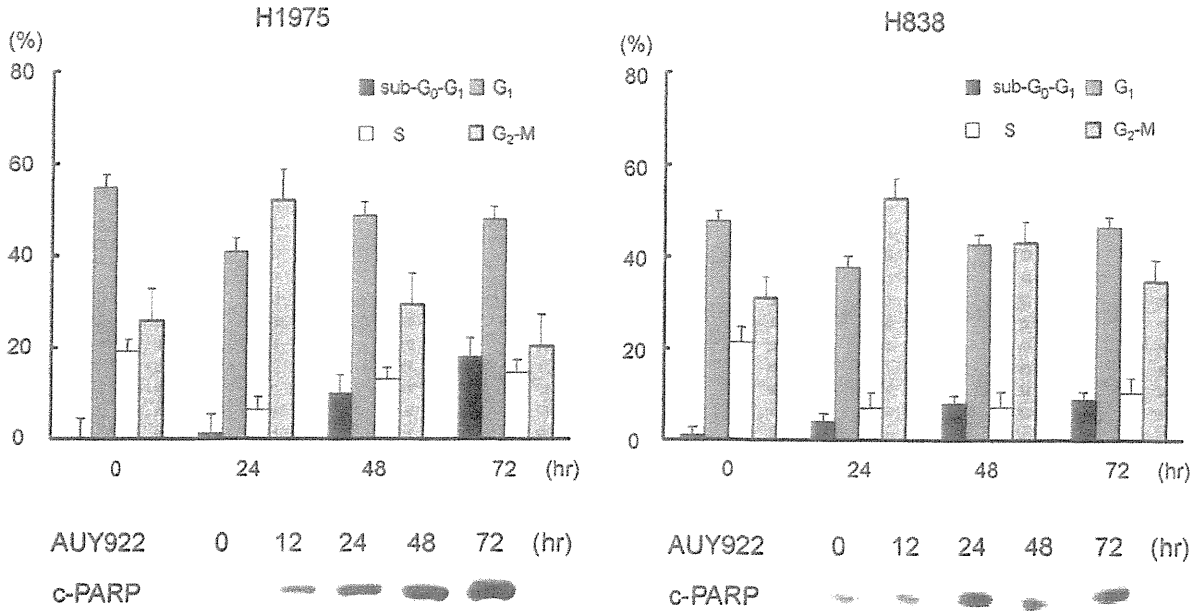

Fig. 4. The impact of AUY922 on cell cycle distribution and induction of apoptosis. Using two sensitive cell lines, cell cycle distribution was analyzed using flow cytometry and cleaved PARP expression was examined using Western blotting. After treatment of AUY922, sub-G_0-G_1 DNA content increased in a time-dependent manner and cleaved PARP also increased with AUY922 treatment.

4. Discussion

In this study, we found that AUY922 had a strong anti-proliferative effect on most NSCLC cell lines. Previous studies have indicated that GM analogue Hsp90 inhibitors have an anti-tumor effect on *EGFR* mutant NSCLC cell lines, including acquired TKI-resistant NSCLC. This suggests that Hsp90 inhibitors are promising agents for resistance to EGFR-TKI in the treatment of NSCLC [12]. However, a recent clinical trial for IPI-504, an analogue of 17-AAG, failed to show its significant effectiveness for *EGFR* mutant NSCLC patients [17]. On the other hand, IPI-504 showed response to 2 of 3 NSCLC patients with *EML4-ALK* fusion gene. One of the reasons is that enrolled patients with *EGFR* mutation had been treated at least two prior EGFR-TKI agents, suggesting that the biological features of these *EGFR* mutant tumors might be different from those of untreated tumors with single oncogene addicted status. In addition, cancer cell lines with *EML4-ALK* might be more sensitive for 17-AAG than those with *EGFR* mutation [17]. Unlike GM analogues including 17-AAG, AUY992 exhibited similar anti-tumor effect not only in *EGFR* mutant tumors, but also in wild-type *EGFR* tumors with various molecular alterations including *K-ras* mutation, *EML4-ALK* fusion gene, or *MET* or *HER2* amplification. One reason is that AUY922 has a much higher affinity for the N-terminal nucleotide-binding site of human Hsp90 than other Hsp90 inhibitors and can strongly suppress the expression of many client proteins at low concentrations [20].

Cell-cycle distribution was examined in two cell lines to assess the induction of apoptosis, but the pattern of distributions was not identical. Many client proteins of Hsp90 are thought to be involved in the pathogenesis of cancers. The degree and manner of involvement of each client protein should vary according to the cancer type, resulting in variation of the cellular response, such as cell-cycle distribution and degree of apoptosis. This would account for the difference in pattern of cell distribution and degree of apoptosis even in the sensitive cell lines.

In our series, the two cell lines Calu3 and H1395 were regarded as being resistant to AUY922. The client proteins in Calu3 were not depleted with AUY922 treatment as much. The fact that expression of Hsp70 was induced in Calu3 confirmed the inhibition of Hsp90 with AUY922, which suggested that drug transporters or metabolic activity might not be responsible for the resistance of Calu3. The cause of preserved expression of client proteins is unclear. In contrast, H1395 showed decreased expression of the client proteins at a low concentration of AUY922, which was similar to the response in sensitive cell lines. As early recovery of client proteins under AUY922 treatment was related to drug resistance in glioblastoma [28], we examined whether there was a difference in the recovery time of depleted proteins between sensitive and resistant cell lines. However, there was no difference between them in NSCLC and the mechanism of resistance was unclear. One possible explanation for the observed resistance is that although Hsp90 has many client proteins that are generally essential for tumor proliferation and survival in the majority of cancers, when cancer cells do not depend on these client proteins for survival, the inhibition of Hsp90 may not be effective. Of clinical relevance, this point may suggest that the selection of patients suited to AUY922 treatment based on molecular properties is difficult. Further investigation to identify the factors that can predict sensitivity or resistance to AUY922 is necessary.

Our results suggest that AUY922 is not effective in MPM compared to NSCLC. Although the precise mechanism of resistance is not clear, the molecular characteristics of MPM are different from those of NSCLC [29,30]. Regarding the clinical use of AUY922, Phase I/II trials of intravenously administered AUY922 are currently ongoing (http://clinicaltrials.gov/) for patients with various types of cancer. From February 2011 to present, two interesting clinical trials have begun for advanced NSCLC. The NCT01124864 trial is for patients who have received at least two lines of prior chemotherapy, and the patients are stratified according to *K-ras* and *EGFR* mutation status. The NCT01259089 trial is for patients with lung adenocarcinoma with "acquired resistance" to EGFR-TKI. It is noteworthy that our data strongly support the use of AUY922 for the treatment of NSCLC patients with various somatic alterations or with acquired resistance to EGFR-TKI.

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In conclusion, our study suggests that AUY922 is a potent candidate for the treatment of the majority of NSCLCs, independent of the major known genetic alterations.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2011.09.011.

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Inhibition of mTOR by temsirolimus contributes to prolonged survival of mice with pleural dissemination of non-small-cell lung cancer cells

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Temsirolimus (CCI-779), a recently synthesized analogue of rapamycin, specifically inhibits mTOR and has been approved for clinical use in renal cell carcinoma. Recent reports have indicated the growth inhibitory effect of temsirolimus in some cancers including non-small-cell lung carcinoma (NSCLC). In this study, we aimed to explore the potential therapeutic use of temsirolimus as a treatment for NSCLC. Using cultured NSCLC cells (A549, H1299, and H358), we determined the effect of temsirolimus on cell proliferation and its antitumor effects on subcutaneous tumors, as well as its contribution to the survival of mice having pleural dissemination of cancer cells, mimicking advanced NSCLC. Temsirolimus suppressed proliferation of NSCLC cells in a dose-dependent manner, with an IC_{50} of <1 nM. Western blot analysis revealed that temsirolimus treatment specifically inhibited the phosphorylation of mTOR and its downstream effectors in 1 h, accompanied by an increased cell population in the G_0/G_1 phase, but according to flow cytometry, the cell population did not increase in the sub-G_0 phase. When NSCLC subcutaneous tumor-bearing mice were treated with temsirolimus, tumor volume was significantly reduced (tumor volume on day 35: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$). Furthermore, prolonged survival was observed in pleural disseminated tumor-bearing mice with temsirolimus treatment (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$). These results suggest that temsirolimus could be useful for NSCLC treatment, due to its antiproliferative effect, and could be a potential treatment for advanced NSCLC, giving prolonged survival. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01967.x, 2011)

Lung cancer is one of the most aggressive malignancies with poor prognosis. It is estimated that more than 160 000 and 65 000 lung cancer patients in the USA and Japan, respectively, die each year.[1,2] A wide variety of new chemotherapy medicines have been developed and introduced in clinical practice, but the mortality rate has not been improved.[1] Recently, the strategy of drug development has focused on targeting particular molecules that are supposed to be critical for cancer progression. Several molecules in the growth factor receptor pathway are specifically targeted because those molecules are well recognized as being aberrantly regulated in cancers. For example, epidermal growth factor receptor (EGFR) and its downstream molecules are often upregulated due to gene amplification or mutation;[3,4] therefore, targeting EGFR is a major therapeutic strategy for non-small-cell lung carcinoma (NSCLC).[5] Gefitinib is a well-known small molecule inhibitor that selectively suppresses EGFR tyrosine kinase activity[6] and has been applied in the treatment of NSCLC.[7] Several studies have shown that gefitinib treatment has a drastic antitumor effect in a subset of NSCLC which had acquired certain types of EGFR mutation.[8,9] Since the appearance of gefitinib, several selective EGFR inhibitors have been developed. However, these drugs only revealed a minimal effectiveness due to the aberrant regulation of molecules located downstream from the receptor tyrosine kinase pathways including Ras-Raf-MAPK and phosphatidylinositol 3′-kinase (PI3K)-Akt.[10,11] Among them, mammalian target of rapamycin (mTOR) is one of the major effectors regulated by the PI3K-Akt signaling pathway and plays a central role in this stimulated growth.[12,13] Moreover, there is an upregulation of mTOR activity in many types of cancers including NSCLC.[14,15] Therefore, several compounds that selectively inhibit mTOR activity have been developed for clinical use.[16,17] Temsirolimus (CCI-779), an analogue of rapamycin, was recently synthesized to specifically inhibit mTOR and has provided prolonged survival of patients with renal cell carcinoma. It was also reported that temsirolimus showed a certain antitumor effect on other types of cancers including breast cancer,[18] glioblastoma,[19] neuroendocrine carcinomas,[20] and mantle cell lymphoma.[21] Moreover, temsirolimus has antitumor effects in other diseases such as lymphangioleiomyomatosis.[22] Based on these observations, we questioned whether temsirolimus treatment could be a potential therapeutic option for NSCLC. In this study, we evaluated the antiproliferative and antitumor effects of temsirolimus in NSCLC *in vitro* and *in vivo*, with an assessment of its survival advantage in an animal model of advanced NSCLC.

Materials and Methods

Cell lines and cultures. Three cancer cell lines that were established from human NSCLC (A549, H1299, and H358) were used in this study. A549 was cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) and H1299 and H358 were cultured in RPMI-1640 medium (Sigma-Aldrich) at 37°C in humidified air with 5% CO_2 These media were supplemented with 10% FCS (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich).

Reagents. Temsirolimus, commercialized as Tricel by Wyeth K.K. (Madison, NJ, USA), was purchased from OZ International (Tokyo, Japan). The temsirolimus was diluted to the final concentration with culture media before an *in vitro* experiment. When temsirolimus was used *in vivo*, it was dissolved and diluted to a final concentration of 10 mg/kg with 0.9% sodium chloride.

Trypan blue exclusion assay. Cancer cells (5.0×10^3 per well) were plated directly in 24-well dishes with culture medium.

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After the cells entered into an exponential growth phase, they were treated with different concentrations of temsirolimus (0, 0.1, 1, 10, 100, or 1000 nM) for 48 h, stained with Trypan blue, and the number of viable cells was counted using a hemacytometer.

Apoptosis assay. Cells in apoptosis were determined by TUNEL assay using a MEBSTAIN Apoptosis kit II (MBL International, Woburn, MA, USA) according to the manufacturer's protocol. Briefly, cells (1.0×10^4 per well) were seeded on Lab-Tek 8-well permanox chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) and were treated with 10 nM/L of temsirolimus or with an equivalent volume of diluted DMSO (final concentration, 0.005%) as a control for 48 h. The TUNEL-positive cells were counted with a fluorescence microscope.

Cell cycle analysis by flow cytometry. For cell cycle analysis, cancer cells were plated in six well tissue culture plates and treated with different concentrations of temsirolimus (0, 1, 10, or 100 nM/L). After a 24-h treatment, the cells were harvested and stained with 20 mg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson) using CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Whole cell lysates and nuclear protein were extracted using M-PER buffer (Thermo Fisher Scientific, Rockford, IL, USA) and NE-PER buffer (Thermo Fisher Scientific) supplemented with protease inhibitors and phosphatase inhibitors. The protein concentration of the collected supernatants was determined and equal amounts of protein were electrophoresed under a reducing condition in gradient polyacrylamide gels (ATTO, Tokyo, Japan) and were then transferred onto PVDF filter membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. An Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA) was used for signal detection. The antibodies used for Western blotting were phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-S6 ribosomal protein (Ser235/236), and hydroxy-HIF-1α (Pro564) (D43B5). All of them were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Dako Cytomation (Glostrup, Denmark). Goat anti-rabbit IgG was obtained from American Qualex Antibodies (La Mirada, CA, USA).

Animal experiments. The protocol for the animal experiments was approved by the Ethics Review Committee for Animal Experiments of Okayama University (Okayama, Japan). Mice used in this study were purchased from Clea (Tokyo, Japan). A549 s.c. xenografts were produced on the backs of 6-week-old male BALB/c nu/nu mice by injecting 3×10^6 cells mixed with Matrigel (BD Biosciences) at a 1:1 ratio. After 7 days, the tumor-bearing animals were randomized into two groups that consisted of seven mice each: (i) temsirolimus (10 mg/kg given i.v. once/week for 5 weeks); and (ii) saline alone as a vehicle (given i.v. once/week for 5 weeks). Tumor volume was measured weekly (length × width × height). To create the A549 pleural dissemination model, 4×10^6 cancer cells were intrathoracically injected into the pleural cavity of 6-week-old male BALB/c nu/nu mice. After 7 days, the animals were randomized into two groups that consisted of eight mice each; (i) temsirolimus (10 mg/kg given i.p. once/week for 5 weeks); and (ii) saline alone as a vehicle. The drug was given once a week and lasted until the mice expired. Each animal experiment was repeated three times and the representative data is shown. The dose and schedule of temsirolimus treatment (10 mg/kg/week) in these animal experiments was decided based upon previous reports where the researchers used a range of 8–20 mg/kg/week.[23–25]

Immunohistochemistry. Surgically resected pleural membrane tissues from mice with disseminated pleural tumors from A549 cells were used for immunohistochemical study following procedures described previously.[26] Deparaffinized tissue sections were immersed in methanol containing 3% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in citrate buffer was done for antigen retrieval. After incubation with a blocking buffer the sections were treated with an anti-phospho-mTOR rabbit mAb (Cell Signaling Technology) for 6 h at room temperature followed by immunobridging with Avidin DH-biotinylated HRP complex (Nichirei, Tokyo, Japan). Signal detection was done for 2–5 min using 3,3′-diaminobenzidine tetrahydrochloride dissolved to 50 mM/L Tris–HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer-hematoxylin. Monoclonal anti-human mouse Ki-67 antibody (MIB-1; Dako Cytomation) was used to calculate the Ki-67 labeling index by counting the number of positively stained cells per 1000 cancer cell nuclei for each section.

Statistical analysis. Student's *t*-test was used to compare data between two groups. Data represent the mean ± SD. Overall survival was calculated using the Kaplan–Meier method and compared by the log–rank test. $P < 0.05$ was considered statistically significant.

Results

Inhibition of mTOR by temsirolimus suppresses cell growth of NSCLC cells. First, we examined how effective temsirolimus was at inhibiting the proliferation of cultured NSCLC cells using a Trypan blue exclusion assay. As shown in Figure 1, temsirolimus suppressed the cell proliferation of A549, H1299, and H358 cells in a dose-dependent manner. The IC_{50} values were measured to examine the suppression of cell proliferation by temsirolimus in NSCLC cell lines. The IC_{50} for A549 cells was 0.76 nM and those for H1299 and H358 were 0.75 nM and 0.64 nM, respectively (Fig. 1). These data indicated that temsirolimus effectively inhibited the viability of NSCLC cells at a low concentration of <1 nM.

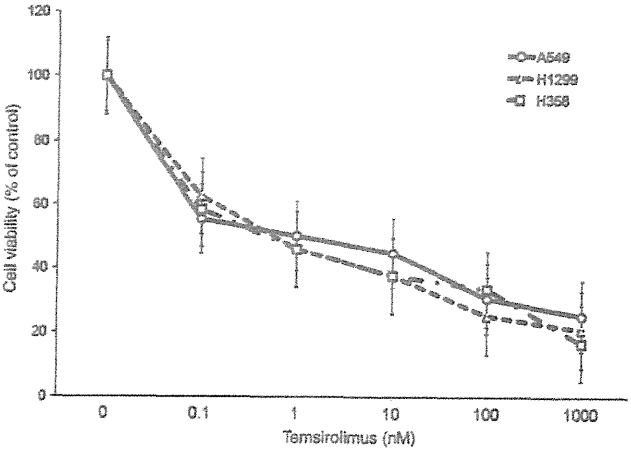

Fig. 1. Temsirolimus suppresses cell proliferation of non-small-cell lung carcinoma cells in a dose-dependent manner. Cultured cells were treated with the indicated concentrations of temsirolimus for 48 h and the number of viable cells was counted by the Trypan blue exclusion method. The IC_{50} values of A549, H1299, and H358 cells were 0.76, 0.75, and 0.64 nM, respectively.

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Temsirolimus inhibited mTOR pathway in a dose- and time-dependent manner. Next, in order to evaluate the effect of temsirolimus on regulating activation in the mTOR pathway, we examined the phosphorylation of mTOR and its downstream effectors by Western blot analysis. As expected, the treatment with temsirolimus suppressed the activations of mTOR, p70 ribosomal S6 kinase, and S6 in a dose-dependent fashion in A549 (Fig. 2A). This inhibitory effect occurred after 1 h, and lasted at least 4 h (Fig. 2B). Similar results were obtained in another NSCLC cell line, H1299 (Fig. 2C,D). Furthermore, we assessed the expression status of cell cycle markers including $p21^{cip1}$, $p27^{kip1}$, and cyclinD1, whose expression is often modified by the inactivation of p70 S6 kinase and S6.[27] Interestingly, $p21^{cip1}$ was apparently induced by temsirolimus treatment, but we did not observe any change in cyclinD1 or $p27^{kip1}$ (Fig. S1). Because the upregulation of $p21^{cip1}$ is known to contribute to cell cycle arrest, these data suggest that the inhibition of the mTOR pathway using temsirolimus is a promising strategy to diminish the proliferation of NSCLC cells.

Temsirolimus treatment leads to G_1 cell cycle arrest but not cell death. Our next question was whether temsirolimus treatment is lethal to NSCLC cells. In order to answer this question, we carried out flow cytometry to analyze the cell cycle distribution in A549 and H1299 cell lines under temsirolimus treatment. Interestingly, temsirolimus treatment increased the cell population in the G_0/G_1 phase, but not in the sub-G_0 phase, which accounted for dead cells (Fig. 3). Furthermore, when we examined the amount of apoptosis by TUNEL assay, we did not observe a significant number of apoptotic cells (data not shown). Taken together, these results suggested that temsirolimus suppressed NSCLC cell proliferation by its cytostatic effect, not by cytotoxicity.

Temsirolimus reduces s.c. tumor growth of NSCLC cells. Next, we investigated the effect of temsirolimus on *in vivo* tumor growth. A549 s.c. xenografts were made. When 10 mg/kg temsirolimus was given weekly i.v. to the mice bearing the s.c. tumor, a significant delay of s.c. tumor growth was observed on day 35 (tumor volume: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$) (Fig. 4). None of the mice died of drug-induced toxicity and no other significant adverse events were observed. Moreover, during the observation period (up to 35 days after cell inoculation), there was no significant change in body weight in either group (data not shown).

Temsirolimus treatment prolonged survival of mice with disseminated pleural tumors of NSCLC cells. As shown above, we found that temsirolimus had a cytostatic effect on NSCLC cells and showed a delay of s.c. tumor growth. Based on these results, we predicted that a major advantage of temsirolimus treatment would be an improvement in the survival of patients bearing NSCLC tumors, similar to renal cell carcinoma.[28] To investigate the effect of temsirolimus on survival, we made a pleural dissemination animal model by injecting A549 cells into the intrapleural cavity, to mimic an advanced clinical stage of NSCLC. A weekly i.p. injection of 10 mg/kg temsirolimus significantly prolonged the survival period of these pleural disseminated tumor-bearing mice (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$) (Fig. 5A,B).

Macroscopic observation by opening the thoracic cavity of the mice showed that temsirolimus treatment obviously reduced the number and the volume of pleural disseminated tumors on day 21 after the inoculation of A549 cells in the thoracic cavity (Fig. 5C,D), although tumors were recognized in a bilateral thoracic cavity regardless of temsirolimus treatment. This result led us to speculate that temsirolimus reduced the growth of pleural disseminated tumors, leading to the prolonged survival of the tumor-bearing mice. Furthermore, immunohistochemical analysis revealed that phosphorylation of mTOR was strongly suppressed in the tumor tissues of the temsirolimus-treated mice (Fig. 5E,F).

The immunohistochemical analysis for Ki-67 using disseminated pleural tumor tissues revealed a significant decrease in the number of proliferating cells (determined by calculating the

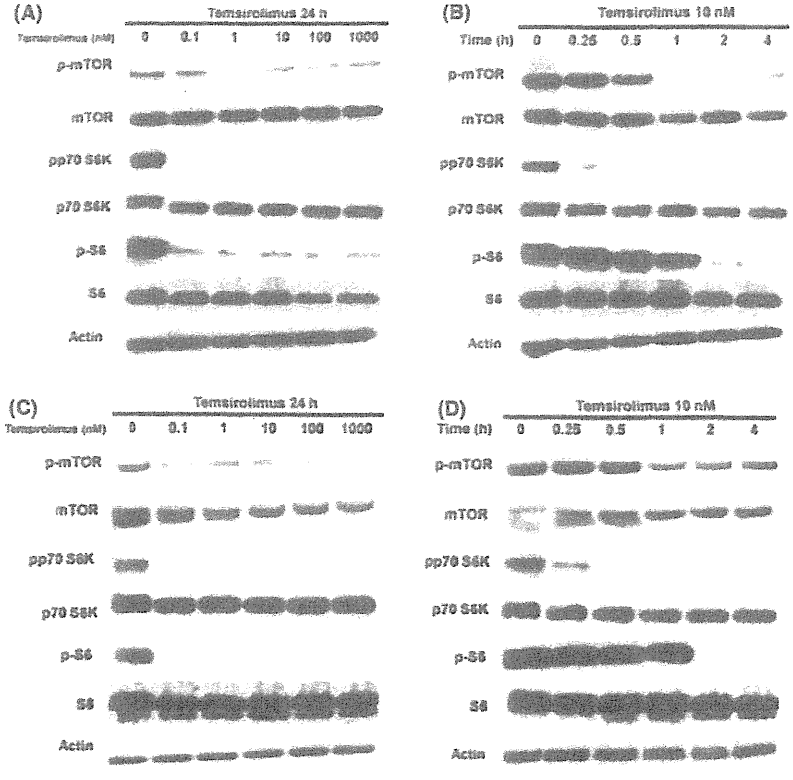

Fig. 2. Temsirolimus suppresses the activation of mTOR and its downstream effectors. Whole cell lysates of A549 (A,B) and H1299 (C,D) non-small-cell lung carcinoma cells that were treated with the indicated concentrations of temsirolimus were used for Western blot to determine the inhibitory effects on mTOR and its downstream effectors in dose-dependent (A,C) and time-course (B,D) studies. p-mTOR, phospho-mTOR; pp70 S6K, phospho-p70 S6 kinase; p-S6, phospho-S6 ribosomal protein.