トピックス

EGFR 遺伝子ステータスに応じた非小細胞肺がんに対する分子標的薬

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要　旨

遺伝子変異陽性肺がんに対する EGFR-TKI（ゲフィチニブ，エルロチニブ）の治療効果が，本邦を含めた複数の第Ⅲ相試験で明らかになった．奏効率，無増悪生存期間とも EGFR-TKI が有意に良好であるが，クロスオーバーが起こるため生存期間には差がない．現段階では，EGFR-TKI を二次治療以降より一次治療に用いるほうが良いという明確なエビデンスはない．しかし，EGFR-TKI がキードラッグであることは疑いなく，EGFR-TKI の投与の時期を逸することがないよう注意が必要である．

はじめに

2004 年，ヒト上皮成長因子受容体（EGFR）遺伝子変異が米国の2つのグループから報告された．肺がんに関連した遺伝子異常はそれまでも幾つも報告されていたが，それらと EGFR 遺伝子変異の発見との決定的な違いは，その遺伝子変異を持つ肺がんに適合する治療薬が存在することである．EGFR 遺伝子発見前から，ゲフィチニブがアジア人の非喫煙女性の腺がんに効果が高いことが知られていたが，その効果がある群に EGFR 遺伝子変異を持つ患者群が多いことが原因と分かった．EGFR 遺伝子変異を肺がん診断時に決定することが，肺がん診断・治療の流れに完全に組み込まれることになった．ここ数年，さまざまな EGFR 遺伝子変異に関する臨床研究の成果が報告された．本稿ではそれらを概説するとともに，EGFR 検査結果に基づく治療戦略について触れる．

IPASS サブグループ解析

2008 年，欧州癌治療学会で IPASS 試験が発表された[1]．試験は日本を含む東アジア中心に，非喫煙者もしくは軽喫煙者腺がんにおいて従来の化学療法とゲフィチニブ投与を比較した第Ⅲ相試験である．主要評価項目にした無増悪生存期間（PFS）では，ゲフィチニブが有意に PFS を延長したが，初期は抗がん剤が優位で，その後分子標的薬が優位になるというクロスする PFS 曲線であった．そのサブグループ解析として，高感度法の1つである scorpion ARMS 法を用いて，EGFR 遺伝子が野生型か変異型かを確定した症例のみで EGFR 変異型，野生型それぞれに分けて PFS を検討すると，それぞれの治療群でクロスすることなく PFS 曲線が分かれた（図1）．EGFR 遺伝子変異のあるサブグループでは，ゲフィチニブ投与群で有意に PFS が延長した．一方，野生型 EGFR 遺伝子変異のサブグループにおいて，ゲフィチニブ群が化学療法群を大きく下回った．野生型 EGFR 遺伝子群に対するゲフィチニブ投与で効果があったのは 91 人中1人のみで，野生型 EGFR 遺伝子の症例にゲフィチニブを投与することの問題が浮き彫りにされた．この IPASS の衝撃的な結果も，1,217 例中 EGFR

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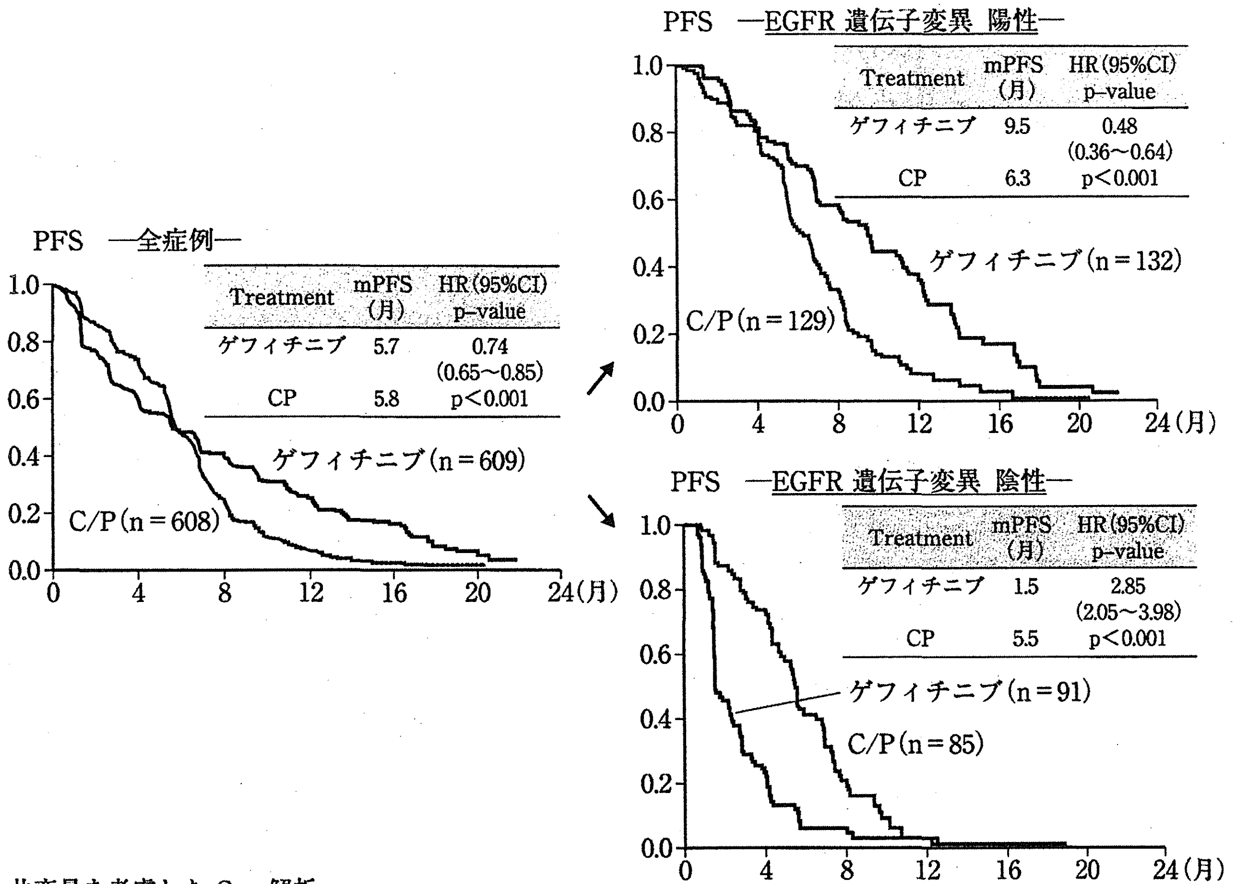
図1 IPASS試験の無増悪生存期間（PFS）（文献[1]より改変引用）



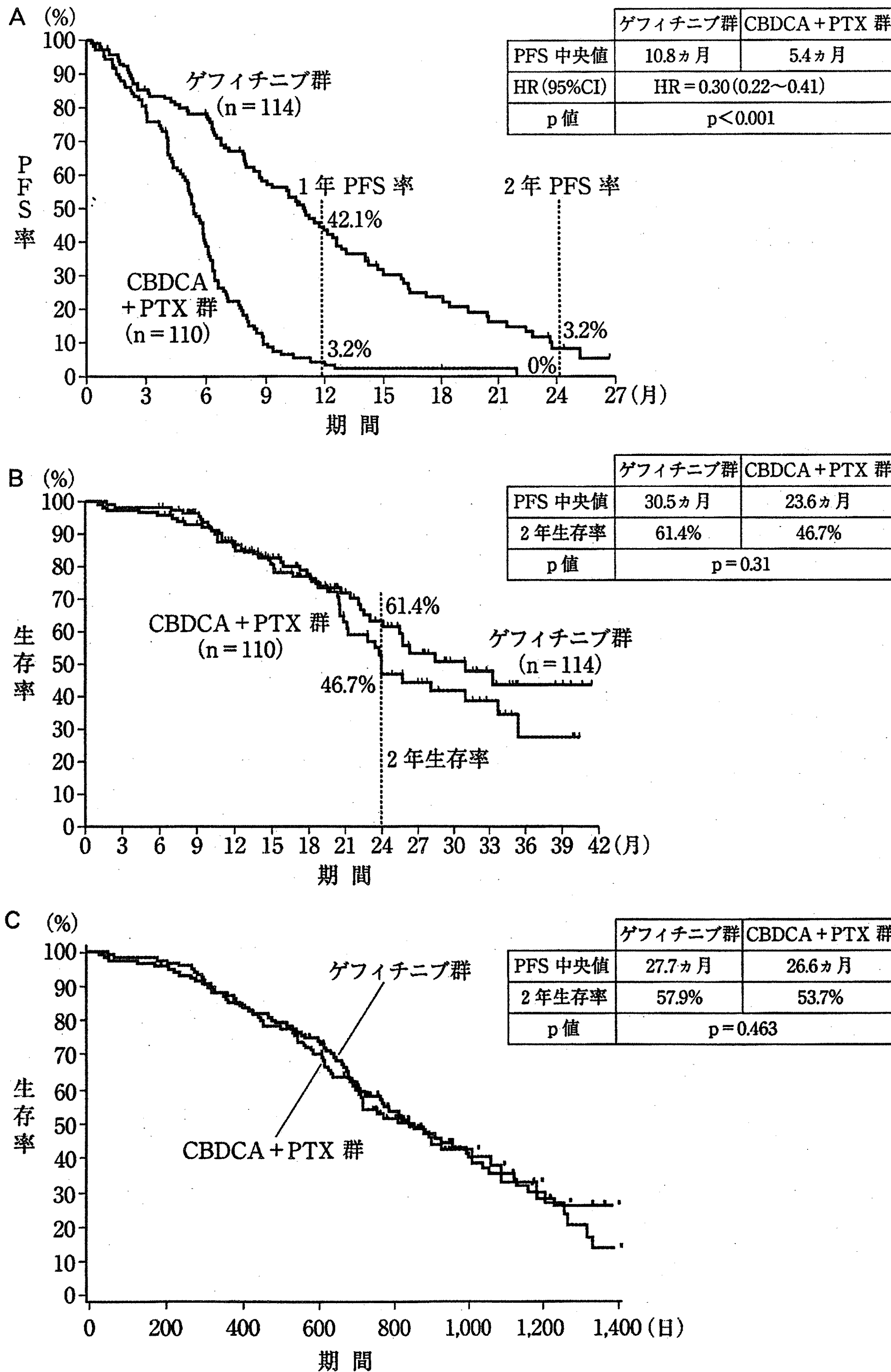
共変量を考慮したCox解析.
ハザード比＜1は，化学療法群に比べてゲフィチニブ群の病勢進行のリスクが低値であることを示す.
HR：hazard ratio，CI：confidence interval，PFS：progression-free survival

遺伝子解析結果が判明した437例（36%）のサブグループ解析の結果であり，エビデンスレベルとしては高くない．この事象を検証するには，EGFR遺伝子変異を有する症例のみを登録し，ゲフィチニブと化学療法を比較する第Ⅲ相試験が必要であった．

NEJ002試験とWJTOG3405試験

NEJ002試験は，感受性EGFR変異症例に絞り，ゲフィチニブ（G群）と本邦で汎用されているカルボプラチン（CBDCA）＋パクリタキセル（PTX）（CP群）とを1：1で割り付けて比較する第Ⅲ相試験である．主要評価項目はPFSとし，副次的評価項目に生存期間（OS），奏効率（RR），安全性を据えた．2006年4月より登録開始となり，症例集積が200例を超えた2009年5月にあらかじめ規定していた中間解析を行い，その結果PFS，RRともにG群がCP群を大きく上回ったため登録を230例で終了し，その解析した結果を『N Engl J Med』に報告した[2]．主要評価項目であるPFSはG群が10.8月で，CP群の5.4月を2倍に延長した（HR 0.3）（図2A）．RRにおいてもG群，CP群それぞれ73.7%，30.7%と有意差が認められた．OSについては副次的評価項目としたため，OSにおいて有意な差を検出するための十分な症例数を設定していなかったが，当初，生存期間中央値（MST）はG群30.5月，CP群23.6月，2年生存率がG群61.4%，CP群46.7%と報告された（図2B）．このデータはイベント数が40%前後であったため2011年にアップデートされ，MSTがG群27.7月，

図2　NEJ002 試験の PFS，OS（文献[2]より改変引用）



A

	ゲフィチニブ群	CBDCA＋PTX 群
PFS 中央値	10.8ヵ月	5.4ヵ月
HR (95%CI)	HR＝0.30 (0.22〜0.41)	
p 値	p＜0.001	

B

	ゲフィチニブ群	CBDCA＋PTX 群
PFS 中央値	30.5ヵ月	23.6ヵ月
2年生存率	61.4%	46.7%
p 値	p＝0.31	

C

	ゲフィチニブ群	CBDCA＋PTX 群
PFS 中央値	27.7ヵ月	26.6ヵ月
2年生存率	57.9%	53.7%
p 値	p＝0.463	

CP 群 26.6 月と全く差がない結果となった（図2C）．これは，G群の 98% に後治療としてゲフィチニブの投与が行われたためである．毒性は予想どおりG群では皮疹，下痢が多く，CP群では骨髄抑制，神経毒性が有意に多かった．2010 欧州癌治療学会（ESMO）で NEJ002 の QOL 調査成績が発表されたが，疼痛/呼吸困難の項と日常生活の項において有意にG群で良好であった[3]．G群で 2.1% に重篤な肺毒性が発生しており，EGFR 遺伝子変異がある肺がん症例であっても肺毒性に注意が必要である．

もう1つの本邦で行われた WJTOG3405 試験では，ファーストラインにおいてゲフィチニブ（G群）とシスプラチン（CDDP）＋ドセタキセル（DTX）（CD 群）を比較した[4]．NEJ002 試験との違いはコントロール群の化学療法の種類だけでなく，WJTOG3405 試験では術後再発の患者の割合が多いことである（WJ 試験 41%，NEJ 試験9%）．WJTOG3405 試験においても，PFS，RR ともに有意にG群が良好であった．（PFS：HR 0.49，G群 9.2 月，CD 群 6.3 月，RR：G群 56.3%，CD 群 25.3%）．この2つの日本発の第Ⅲ相臨床試験と IPASS 試験のサブグループ解析により，EGFR 遺伝子変異陽性症例に限定し，ファーストラインでの EGFR チロシンキナーゼ阻害薬（EGFR-TKI）投与が選択可能となった（2010 年日本肺癌学会肺癌診療ガイドライン）．

EGFR-TKI をいつ投与すべきか

上述した NEJ002 試験および WJTOG3405 試験の結果は，いずれもゲフィチニブをファーストラインで投与した結果であるが，EGFR-TKI 投与をファーストラインとそれ以降のラインとの間で比較した試験ではない．現在のところ，この課題に対して答えを出すような臨床試験も組まれていない．EGFR-TKI を遺伝子変異陽性患者に対しファーストラインで投与するか，セカンドライン以降で投与するか，本邦ではいまだ争点となっている．これまで，キードラッグは生存期間の短い肺がん患者に対しては優先して投与することが治療の基本であった．EGFR 遺伝子変異陽性の場合の平均生存期間は優に2年を超えるといっても，その基本は変わりないと考える．効果が最も高いと分かっている薬剤を後にとっておいて，それが結局使えないで終わる寓はだれしも犯したくない．欧米でのセカンドライン移行率は 60% 程度，本邦では 70% 前後である．これは，化学療法から投与開始した場合，30% の患者が EGFR-TKI まで進めない可能性を示唆する．また，EGFR-TKI をファーストラインに持ってくる場合，セカンドライン以降に化学療法が入りづらくなるといった批判がある．NEJ002 のデータでは，確かに初回ゲフィチニブ投与群の 35% の患者にセカンドライン以降の化学療法が投与されていない．しかしこのデータには，ゲフィチニブが長期に奏効し，カットオフ時点でも病変進行（PD）とならずにゲフィチニブの投与を続けている症例が8%含まれている．また，RECIST 上 PD 判定であっても，ゲフィチニブ投薬の利益が残っていると考えてゲフィチニブ投与が続けられている患者が4人いる．これらの症例が今後，化学療法の投与を受ける可能性を含んでおり，セカンドライン移行率は 70% を超えるものと思われる．一方，NEJ002 初回化学療法投与群の実に 98% の患者に，セカンドライン以降 EGFR-TKI が入っている．これこそが驚異的なデータであり，臨床試験を行っている医師が EGFR 遺伝子変異陽性患者に対する EGFR-TKI 治療の重要性を強く認識していたことがうかがえる．いずれにせよ，EGFR 遺伝子変異陽性患者に対してキードラッグである EGFR-TKI を投与しそこなうことがなきよう，マネージメントしなければならない．

EGFR 遺伝子変異陽性患者に対するキードラッグである EGFR-TKI をいつ止めるかについては，ファーストラインで始めた場合は，明らかな増悪が認められた時期と考える．EGFR 遺伝子変異陽性患者に EGFR-TKI だけで治療

しても，2年を超える生存期間をもたらすことは難しい．化学療法をいかに組み入れていくかが重要で，そのためには明らかな PD となったら速やかに化学療法へ移行することが必要となる．EGFR-TKI 耐性機序の半数を占めるのが，T790M 遺伝子変異である．この耐性遺伝子変異出現後は，現在使用可能な第1世代の EGFR-TKI では無効と考えられていた．しかし最近，EGFR-TKI を止めて化学療法で治療している間に耐性遺伝子変異が検出できなくなり，TKI に対する感受性が回復した症例の報告があった[5]．T790M 遺伝子変異を持つ腫瘍細胞自体の腫瘍増大スピードが遅いという報告もあり，T790M 遺伝子変異をめぐる話題は混沌としてきた．セカンド TKI の可能性や新たな TKI 耐性患者に対する治療戦略の開発が行われている中，ファーストラインで TKI を投与した場合，PD 後いったん TKI を見合わせて化学療法で腫瘍コントロールを行い，新たな戦略を待つ方針が現段階では妥当と言える．幸い EGFR 遺伝子変異が多い腺がんに対しては，血管新生阻害薬ベバシズマブを併用した化学療法やペメトレキセドを含む化学療法が使用可能である．幾つかの治療法をタンデムに組み合わせ，長期生存を目指すことが重要と思われる．一方 NEJ グループでは，化学療法と TKI の同時併用も検討している．TKI と相性が良いとされるペメトレキセドと CBDCA をゲフィチニブに加えることにより，ゲフィチニブ単独療法に生存期間で上回ることができるかどうかを EGFR 遺伝子変異陽性未治療患者で比較する (NEJ009)．化学療法と EGFR-TKI を併用したほうがより薬剤耐性ができにくいといった基礎データもあり[5]，この試験の結果が注目される．

PS 不良，高齢者に対する治療

一方，PS が悪く，次の化学療法の適応にならない患者に対しては，EGFR-TKI の投与を継続することは十分許容される．複数臓器に転移がある場合，肺内転移には効果があるが，骨転移が悪化するなどといったケースは少なからず経験する．次治療のない患者に対しては，毒性をうまくコントロールし，急激な増悪でない限り TKI 治療を持続することが勧められる．TKI を継続しながら局所放射線治療（全脳照射，骨照射）を一時的に併用することもしばしばある．ここで，高齢者に対しても PS 不良患者と同様に TKI を継続投与するかというと，高齢者の場合は PS 不良患者と異なる．高齢者の EGFR 遺伝子変異陽性患者に限定してゲフィチニブを投与した試験では，生存期間が若年者よりさらに長い傾向がある (NEJ003) (JTO in print)．高齢者であっても，EGFR 遺伝子変異陽性であれば，2年の生存期間を期待できる．TKI 耐性になった場合でも，毒性を考慮した化学療法を治療ラインに組み入れたほうが生存期間が延長する可能性がある．

おわりに

EGFR 遺伝子変異および EGFR-TKI に関する研究は，EGFR 変異肺がんの自然耐性，獲得耐性の問題や野生型 EGFR に対する TKI 治療などまだまだ研究課題は多い．また，野生型 EGFR の中に K-ras，EML4-ALK，BRAF などさまざまな遺伝子異常が存在する可能性がある．単一な遺伝子変異型ではなく，各遺伝子ステータスの全体像を表すジェノタイプという用語が使用されるようになってきている．こういったさまざまな遺伝子変異状況の研究が進み，さらに有効な治療アルゴリズムが見いだされることを期待する．

文　献

1) Mok T S, et al: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361 (10): 947-957, 2009.
2) Maemondo M, et al: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 362 (25): 2380-2388, 2010.

● 特 集 ● 抗がん剤または分子標的薬治療の効果判定と変更のタイミング
—私はこうしている—(その1)

非小細胞肺癌治療の効果判定と治療変更のタイミング

前門戸 任*

〔*Jpn J Cancer Chemother* 39(9): 1316-1319, *September*, 2012〕

Timing the Change of Chemotherapy for Non-Small Cell Lung Cancer: Makoto Maemondo (*Dept. of Respiratory Disease, Miyagi Cancer Center*)

Summary

Lung cancer patients thus far have been given a non-treated period called "treatment holiday", followed by 4-6 courses of platinum-doublet. Now, maintenance therapy has attracted much attention after the approval of pemetrexed and bevacizumab. Treatments with both drugs are effective, with mild toxicity. However, it was not established how to apply maintenance therapy to patients. Maintenance therapy with pemetrexed requires the selection of patients by the efficacy of induction therapy (PR or SD), performance status, and the tendency of disease progression. In contrast, bevacizumab monotherapy should be applied to all patients having PR or SD, because the therapy is less toxic and no predictive factor has been identified. On the other hand, the efficacy of EGFR-TKI therapy for patients with EGFR activating mutation has been established. EGFR-TKIs are key drugs for patients with EGFR-mutation, and are recommended as standard first-line therapy. I consider that chemotherapies are the second important drugs for prolonging patient survival. It has not been confirmed which is superior, the first-line therapy with EGFR-TKIs or the second line. But when EGFR-TKIs are administered in first-line, we should change TKIs to chemotherapy immediately after determination of PD on the basis of RECIST. When EGFR-TKIs are administered after chemotherapy in the second line or more line, continuation of EGFR-TKIs after PD can be accepted as long as there is no obvious exacerbation. **Key words**: Maintenance therapy, EGFR mutation, EGFR-TKI, **Corresponding author**: Makoto Maemondo, Department of Respiratory Disease, Miyagi Cancer Center, 47-1 Nodayama Medeshima-shiote Natori, Miyagi 981-1293, Japan

要旨 肺癌領域では，これまで4～6コースでプラチナ併用化学療法を終了して増悪まで無治療期間を置くことが通例であったが，アバスチン，ペメトレキセドといった抗癌剤の登場で無治療期間を置かずに単剤で治療する維持療法が脚光を浴びている。維持療法をすべてのSD以上の効果を示した症例に行うか，そのなかで維持療法のメリットがある患者を選択し得るかどうか不確定である。ペメトレキセドであれば，患者のperformance status (PS)，プラチナ併用期間での効果(PR or SD)，増大傾向の有無など治療医の選択が必要である。アバスチンについては，単剤であれば毒性は少なく効果予測因子のない現状では増悪していない症例すべてに投与してみることでいいだろう。また，EGFR遺伝子変異陽性患者におけるEGFR-TKIはキードラッグであり，どのラインで使用するかにより耐性時にすぐに化学療法に変更するか，EGFR-TKIを継続するか対応が異なる。EGFR遺伝子変異陽性症例においても化学療法が投与されたほうが予後改善につながると考えており，EGFR-TKIの使用がファーストラインであれば，PD判定後速やかに化学療法に移行することが望まれる。二次治療以降で用いるのであれば，明らかな増悪が認められるまでPD後の継続も許容されるであろう。

は じ め に

ここ5年で非扁平上皮癌に対する新規抗癌剤と分子標的薬の遺伝子ステータスに基づく治療が進歩し，非扁平上皮癌の生存期間の延長がもたらされるようになった。一方で様々な化学療法を行えるようになった反面，治療薬をどのタイミングでどのように使うか複雑になってきた。本稿では進歩する肺癌治療にどのように対応していくか，最新のエビデンスを説明するとともに当センターでのアプローチを紹介する。

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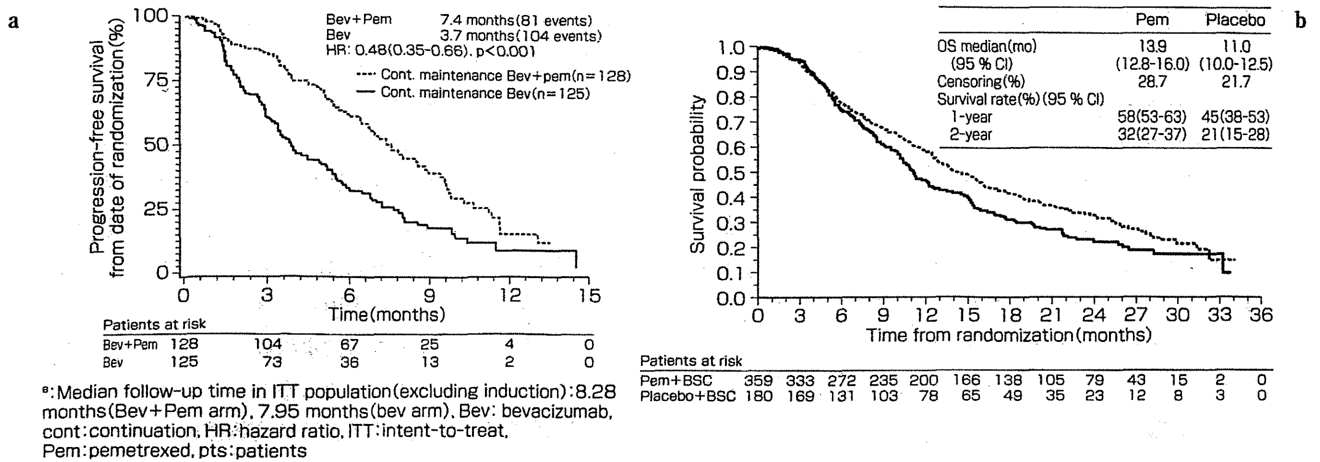

Fig. 1
a: PFS from randomization[a]
b: Paramount: final OS from randomization

I．新規抗癌剤と維持療法

抗癌剤の領域では，代謝拮抗剤の一種であるペメトレキセドが2009年に胸膜中皮腫に続いて非小細胞肺癌(非扁平上皮癌）にも適応となり，毒性が軽く効果の高い非扁平上皮癌治療の第一選択薬の一つになっている。近年，注目されているのがこのペメトレキセドを用いた維持療法である。肺癌領域では一次治療としてプラチナ併用2剤療法が行われ，4〜6コース投与後は再増大まで無治療期間を置くことが常であった。維持療法は，この無治療期間を置かずに引き続き薬剤を増悪（PD）になるまで投与する方法である。維持療法には，プラチナ併用2剤療法のうちプラチナ以外の薬剤を続けるcontinuation maintenanceと，2剤療法で使う薬剤を別の薬剤に切り替え引き続き投与するswitch maintenance（early second）がある。これまでも数々の維持療法についての臨床試験が行われてきたが，90年代から使われるようになった第三世代化学療法では十分な結果をだすことはできなかった。しかし，ペメトレキセドとエルロチニブが使われるようになり，まずswitch maintenanceで無増悪生存期間（PFS）だけでなく生存期間（OS）についても有意な成績が認められ，引き続いて昨年米国癌治療学会（ASCO）でペメトレキセドを用いたcontinuation maintenanceが有意にPFSを延長する報告がなされた(Fig. 1a)。この試験はシスプラチンとペメトレキセドの2剤併用後，維持療法としてペメトレキセドを継続してPDまで投与するデザインで，昨年に引き続きASCO2012でOSに関しても有意な延長が認められることが報告された（Fig. 1b)。本邦でも実臨床としてペメトレキセド維持療法を取り入れる施設が増えてきている。

維持療法という戦略そのものが新たに開発されたわけではなく，毒性の軽い薬剤の開発に伴って維持療法という戦略が有用なものとなってきた。無治療期間には意味があり，化学療法の毒性による疲弊した骨髄を回復させ，腫瘍再増大時に次の治療を投与しやすくする。化学療法をやみくもに長期に続けることが高齢者の多い肺癌患者に有効とは思われないが，毒性が少ないペメトレキセドやエルロチニブが出現したことによって初めて維持療法が有効となった。それでも，プラチナ併用2剤療法を終えたすべての患者に有効というわけではないだろう。患者のperformance status（PS)，プラチナ併用期間での効果［partial response（PR）or stable disease（SD)]，増大傾向の有無など治療医の選択が必要である。具体的な患者説明は，患者にプラチナ併用2剤投与前から維持療法の話をしてしまうと患者に化学療法から逃れられない印象を与え，化学療法に対し少なからず恐れを抱いている多くの患者に精神的な負担を与えてしまうことになる。当センターではプラチナ併用2剤療法の2〜3コース投与終了後に，化学療法の効果があり十分維持療法に進めそうな患者に限って維持療法の説明をする。その効果についてもPRの患者とSDの患者いずれにも投与するかどうか，PSと毒性が考慮される。効果，毒性，PSのバランスを勘案し，さらに患者の意向を確認した上で維持療法に進むかどうかを検討する。たとえPRが得られた症例であってもプラチナ併用2剤投与終了時に増大傾向を来す症例では，維持療法の効果が望めないことは自明である。

肺癌治療維持療法でのもう一つの話題は，アバスチン併用化学療法終了後維持療法として単剤のアバスチンの投与を続けるかどうかの問題がある。アバスチン維持療法のない試験がこれまで行われてこなかったために，アバスチン維持療法が標準療法として行われている。アバ

スチン単剤で肺癌に効果があるかどうかはエビデンスが不足しているといわざるを得ない。しかし，これからアバスチン単剤維持療法が有効であるかどうかを検証するのは時代遅れの感があり，それよりアバスチンにさらに殺細胞薬を加えるかどうか，またアバスチン単剤で再発した時にアバスチン投与を継続したまま次の薬剤を加えるかどうか（beyond PD）を検討するほうがより有意義であろう。アバスチン維持療法の適応について考えると，これは先に述べた殺細胞薬であるペメトレキセドとは異なる。アバスチンは骨髄にダメージを与えることなく投与を継続することができ，最も注意しなければならない喀血もプラチナ併用療法時に出現する可能性が高い。そのことからすると，アバスチン併用プラチナ2剤療法を行いPDに至っていない患者には投与しておいて患者に不利益は少ないと思われる。

II．EGFR-TKI投与，中止のタイミング

EGFR遺伝子変異陽性患者に対するEGFR-TKI（ゲフィチニブ，エルロチニブ）の効果は複数のIII相試験で確立したが，プロトコール治療後のクロスオーバーは避けられずいずれの試験も生存期間に差がない[1-4]。EGFR-TKIの投与時期，中止時期について述べる。

EGFR-TKIを遺伝子変異陽性患者に対しファーストラインで投与するか，セカンドライン以降で投与するかしばしば議論となる。EGFR遺伝子変異陽性患者の場合にEGFR-TKIがキードラッグであることは間違いない。これまで，生存期間の短い肺癌患者に対しては効果の高い薬剤から優先して投与することが肺癌患者の治療の基本であった。EGFR遺伝子変異陽性の場合の平均生存期間は優に2年を超えるとはいっても，その基本は変わりない。効果が最も高いとわかっている薬剤を後にとっておいて，それが結局使えないで終わることは避けなければならない。EGFR-TKIをファーストラインにもってくる場合の批判に，EGFR-TKIをファーストラインにもってくるとセカンドライン以降が入りづらくなるといった問題があげられる。NEJ002のデータでは，確かに初回ゲフィチニブ投与群の35％の患者にセカンドライン以降の化学療法が投与されていない。しかし，このデータはゲフィチニブが長期に奏効しカットオフ時点でもPDとならずにゲフィチニブの投与を続けている症例が8％含まれている。また，RECIST上PD判定であってもゲフィチニブ投薬の利益が残っていると考えゲフィチニブ投与が続けられている患者が4名いる。これらの症例が今後化学療法に移行する可能性を含んでおり，セカンドライン移行率は70％を超えるものと思われる。この移行率は，日本で行われた他のトライアルなどと比較しても遜色ない。一方，NEJ002初回化学療法投与群の実に98％の患者にセカンドライン以降EGFR-TKIが入っていることこそが驚異的なデータであり，臨床試験を行っている医師がEGFR遺伝子変異患者に対するEGFR-TKI治療の重要性を強く認識していたことがうかがえる。いずれにせよ，EGFR遺伝子変異陽性患者に対してキードラッグであるEGFR-TKIを投与し，損なうことがないよう注意深くマネジメントすることが重要で，それができるならば選択肢の一つとして化学療法ファーストも許容されるだろう。

EGFR遺伝子変異陽性患者に対するキードラッグであるEGFR-TKIをいつやめるかについては，ファーストラインとセカンドライン以降で異なる。ファーストラインで始めた場合は，明らかな増悪が確認されればEGFR-TKIをやめ化学療法を行う。EGFR遺伝子変異陽性患者にEGFR-TKIだけで治療しても2年を超える生存期間をもたらすことは難しい。化学療法をいかに入れていくかが重要で，そのためには明らかなPDとなったら速やかに化学療法へ移行することが必要となる。EGFR-TKI耐性機序の半数を占めるのがT790M耐性遺伝子変異である。この耐性遺伝子変異出現後のEGFR-TKIは無効と考えられていたが，T790M出現後化学療法を行っている間に耐性遺伝子変異が消失し，TKIに対する感受性が回復した症例が報告されるなど耐性遺伝子変異の扱いも混沌としている[5]。セカンドTKIの可能性やTKI耐性患者に対する新たな治療戦略の開発が行われているなか，ファーストラインでEGFR-TKIを用いた場合，耐性後いったんTKIを見合わせ化学療法で腫瘍コントロールを行い，新たな戦略を待つ方針が現段階では妥当といえる。幸いEGFR遺伝子変異が多い腺癌に対しては，前述したベバシズマブを含めいくつかの新しい有効な化学療法が使用可能である。いくつかの治療法を組み合わせ長期生存をめざすことが重要と思われる。一方，セカンドライン以降でEGFR-TKIを用いた場合，EGFR-TKIの何らかの効果が認められる間はEGFR-TKIを粘って使うことが多い。また，PSが悪く次の化学療法の適応にならない患者に対しても，EGFR-TKIの投与をPD後も継続することは十分許容される。複数臓器に転移がある場合，肺内転移には効果があるが骨転移が悪化するなどといったケースは少なからず経験する。next lineのない患者に対しては毒性をうまくコントロールし，急激な増悪でない限りTKI治療を持続している。

もう一つ，EGFR-TKIを中止する時のフレアが問題とされる。フレアはEGFR-TKI中止後一気に病変が悪化する現象のことで，最近の欧米論文で23％の頻度と報

告されている[6]。経験的にこれほど多いとは思わないが，確かにRECISTでPD判定であっても一部に腫瘍制御力を残している場合はある。次の化学療法を間隔を置かずに行うことで対応可能と考え，EGFR-TKI中止後1週間以内に次治療を行うようにしている。当センターではEGFR-TKIが耐性になってからもTKIを継続し，化学療法にTKIを重ねることはしない。いったん耐性となったTKIを化学療法と併用してまで投与する必要性を感じない。

維持療法とEGFR-TKIの投与方法を中心に述べた。肺癌領域には基礎分野でいくつかの新たな遺伝子変異が発見されている。分子標的薬をどう使っていくかには背景となる分子生物学的な知識がその一助となる。いかに基礎データ，臨床データを目前の患者に生かしていけるかがポイントになる。今後ますます進歩していく肺癌研究により，予後不良だった肺癌患者のより多くに福音がもたらされることを期待している。

文　献

1) Maemondo M, Inoue A and Kobayashi K: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* **362**(25): 2380-2388, 2010.
2) Mitsudomi T, Morita S, Yatabe Y, *et al*: Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* **11**(2): 121-128, 2010.
3) Zhou C, Wu YL, Chen G, *et al*: Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* **12**(8): 735-742, 2011.
4) Rosell R, Carcereny E, Garvais R, *et al*: Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* **13**(3): 239-246, 2012.
5) Sequist LV, Waltman BA, Dias-Santagata D, *et al*: Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* **3**(75): 75ra26, 2011.
6) Chaft JE, Oxnard GR, Sima CS, *et al*: Disease flare after tyrosine kinase inhibitor discontinuation in patients with EGFR-mutant lung cancer and acquired resistance to erlotinib or gefitinib: implications for clinical trial design. *Clin Cancer Res* **17**(19): 6298-6303, 2011.

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Epigenetic therapy with 3-deazaneplanocin A, an inhibitor of the histone methyltransferase EZH2, inhibits growth of non-small cell lung cancer cells

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ABSTRACT

EZH2 (enhancer of zeste homolog 2) is the catalytic subunit of PRC2 (polycomb repressive complex 2), which mediates histone methyltransferase activity and functions as transcriptional repressor involved in gene silencing. EZH2 is involved in malignant transformation and biological aggressiveness of several human malignancies. We previously demonstrated that non-small cell lung cancers (NSCLCs) also overexpress EZH2 and that high expression of EZH2 correlates with poor prognosis. Growing evidence indicates that EZH2 may be an appropriate therapeutic target in malignancies, including NSCLCs. Recently, an S-adenosyl-L-homocysteine hydrolase inhibitor, 3-Deazaneplanocin A (DZNep), has been shown to deplete and inhibit EZH2. The aim of this study was to determine the effect of DZNep in NSCLC cells. Knockdown of EZH2 by small-interfering RNA (siRNA) resulted in decreased growth of four NSCLC cell lines. MTT assays demonstrated that DZNep treatment resulted in dose-dependent inhibition of proliferation in the NSCLC cell lines with a half maximal inhibitory concentration (IC_{50}) ranging from 0.08 to 0.24 μM. Immortalized but non-cancerous bronchial epithelial and fibroblast cell lines were less sensitive to DZNep than the NSCLC cell lines. Soft agarose assays demonstrated that anchorage-independent growth was also reduced in all three NSCLC cell lines that were evaluated using this assay. Flow cytometry analysis demonstrated that DZNep induced apoptosis and G1 cell cycle arrest in NSCLC cells, which was partially associated with cyclin A decrease and $p27^{Kip1}$ accumulation. DZNep depleted cellular levels of EZH2 and inhibited the associated histone H3 lysine 27 trimethylation. These results indicated that an epigenetic therapy that pharmacologically targets EZH2 via DZNep may constitute a novel approach to treatment of NSCLCs.

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1. Introduction

Lung cancer is a leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases. Despite some advances in early detection and recent improvements in treatment, the prognoses of patients with lung cancer remain poor [1,2]. The current challenges are to identify new therapeutic targets and strategies and to incorporate these strategies into existing treatment regimens with the goal of improving treatment outcomes.

Epigenetic gene silencing is an important mechanism that causes loss of gene expression and that mediates, along with genetic mutation, the initiation and progression of human cancer [3]. Polycomb group (PcG) proteins regulate and mediate epigenetic transcriptional silencing. They are involved in the maintenance of embryonic and adult stem cells and in repression of key tumor-suppressor pathways, which might contribute to their oncogenic function [4]. The enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2), which also includes the suppressor of zeste 12 (SUZ12) protein and embryonic ectoderm development (EED) protein. EZH2 acts as a histone lysine methyltransferase that mediates trimethylation of lysine 27 of histone H3 (H3K27me3) to silence expression of PRC2 target genes involved in lineage differentiation [5,6].

EZH2 is overexpressed in a variety of malignancies including prostate cancer [7,8], breast cancer [7,9], melanoma [7], uterine cancer [7], gastric cancer [10], and renal cell cancer [11]. EZH2 expression levels are correlated with aggressiveness, metastasis,

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and a poor prognosis in most types of these cancers [7–11]. EZH2 is barely expressed in normal tissues of various types [12]. More recently, we found that NSCLCs also overexpress EZH2 and that high expression of EZH2 is correlated with poor prognosis [13]. Furthermore, an activating mutation in EZH2 has been identified in a subset of B-cell lymphomas [14]. Overexpression of EZH2 enhanced aggressiveness in prostatic cancer cells [15] and produced a neoplastic phenotype characterized by anchorage-independent growth and cell invasion in immortalized mammary epithelial cells and in bronchial epithelial cells [9,16]. Conversely, depletion of EZH2 results in reduced proliferation, increased apoptosis, and inhibition of tumorigenicity in cancer cells [8,15,17,18] including NSCLC cells [12,19]. These findings indicate that EZH2 may be an appropriate therapeutic target in various types of cancers, including NSCLCs.

A cyclopentenyl analog of 3-deazaadenosine, 3-deazaneplanocin A (DZNep), inhibits the activity of S-adenosyl-L-homocysteine (AdoHcy) hydrolase, the enzyme responsible for the reversible hydrolysis of AdoHcy to adenosine and homocysteine [20]. This inhibition results in the intracellular accumulation of AdoHcy, which leads to inhibition of the S-adenosyl-L-methionine-dependent lysine methyltransferase activity. Recently, DZNep was shown to reduce levels of the PRC2 complex, including EZH2, in breast cancer cells and cause concomitant loss of H3K27me3 and derepression of epigenetically silenced target genes [21]. Moreover, DZNep inhibits proliferation and promotes apoptosis in several types of cancer cells [22–28]. Currently, however, data on the activity of DZNep in NSCLC cells are scarce. The aim of the present study was to assess the effects of DZNep on NSCLC cells.

2. Materials and methods

2.1. Cell lines and reagent

The four human NSCLC cell lines—NCI-H1299 (H1299), NCI-H1975 (H1975), and A549 (American Type Culture Collection, Manassas, VA, USA) and PC-3 (Japan Cancer Research Resources Bank, Tokyo, Japan)—were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 0.03% glutamine at 37 °C in an atmosphere of 5% CO_2. The PC-3 cell line used in the study is not a prostate cancer cell line, but a NSCLC cell line with an epidermal growth factor receptor (EGFR) mutation, a deletion of exon 19 [29].

HBEC3 KT cell line was generously provided by John D Minna (University of Texas Southwestern Medical Center, Dallas, TX, USA). HBEC3 KT cell line was derived from primary human bronchial epithelial cells and immortalized by CDK4 and hTERT [30], and cultured in Keratinocyte-SFM media (Invitrogen, Carlsbad, CA, USA) containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract (Invitrogen Life Technologies) on collagen-coated dishes at 37 °C in an atmosphere of 5% CO_2. 16HBE14o-cell line was kindly provided by Dieter C. Gruenert (University of California, San Francisco, CA, USA). 16HBE14o-cell line was derived from primary human bronchial epithelial cells and immortalized by SV40 large T antigen [31], and cultured in EMEM (Invitrogen Life Technologies) containing 10% FBS on collagen-coated dishes in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. WI-38 VA-13 2RA cell line (American Type Culture Collection) was human embryo lung fibroblast WI38 cell line immortalized by SV40 virus, and cultured in EMEM containing 10% FBS in an atmosphere of 5% CO_2.

DZNep was synthesized in the National Cancer Institute by Marquez, V.E.

2.2. Transfection of siRNAs

RNA interference of EZH2 was performed using 21-bp (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Ambion (s4916, Ambion Inc., Austin, TX, USA). An unrelated siRNA comprising a 19-bp scrambled sequence was used as the negative control siRNA. Transfection was carried out using 11 nM of the siRNA oligonucleotide duplexes and Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

2.3. Cell proliferation assays

Cells were seeded at 500–3000 cells/well in 96-well plates in normal growth medium. Transfection was performed at a confluence of 30–50% with 11 nM of a siRNA and Lipofectamine 2000 reagent every 72. After 6 days, anchorage-dependent growth was measured in 96 well plates using an MTT (dimethyl thiozolyl-2′,5′-diphenyl-2-*H*-tetrazolium bromide)-based assay (CellTiter 96 non-radioactive cell proliferation assay, Promega Corp., Madison, WI, USA). At least three independent experiments were performed to determine the half maximal inhibitory concentration (IC_{50}) values for each cell line. IC_{50} values were determined using Graphpad Prism 4.0c (GraphPad Software, San Diego, CA).

Anchorage-independent growth assays were performed using 0.4% soft agarose (Seaplaque, FMC Corp., Rockland, ME, USA) in 6 well plates with or without DZNep (200 nM, 1 μM) as previously described [32]. After 2 weeks of incubation, colonies were stained with p-iodonitrotetrazolium violet (Sigma–Aldrich Co., St. Louis, MO, USA) and counted using NIH Image version 1.62 software (NIH, Bethesda, MD, USA).

2.4. Cell cycle analysis

Cells were cultured in 100-mm plates. Transfections were performed at a confluence of 30–50% with 11 nM of a siRNA. After 72 h, cells were treated with trypsin, washed twice with PBS, and fixed in 70% ethanol at −20 °C. Fixed cells were subjected to centrifugation and then resuspended in 250 μg/ml RNase and 50 μg/ml propidium iodide (PI) (Sigma) to label the DNA. DNA content was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and two software packages: CellQuest 3.1 (BD Pharmingen, San Diego, CA, USA) and ModFit LT 2.0 (Verity Software House, Topsham, ME, USA).

2.5. Analysis of apoptosis

Cells were stained with FITC-conjugated annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (Calbiochem, Darmstadt, Germany). Briefly, cells were treated with trypsin, subjected to centrifugation at 1000 × *g* for 5 min, washed one time with ice-cold PBS, and then resuspended in 500 μl of binding buffer. Thereafter, 1.1 μl of Annexin V-FITC and 10 μl of PI were added to the cell suspensions, and the components were mixed for 15 min in the dark. The percentage of apoptotic cells was measured using a FACScan flow cytometer (Becton Dickinson). Data analysis was performed using CellQuest 3.1 (BD Pharmingen).

2.6. Western blotting

Cell lysates derived from each NSCLC cell line were prepared by disrupting the cells in radioimmune precipitation assay buffer [150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM Tris (pH 7.4)], which was supplemented with 100 μg/ml leupeptin, 100 μg/ml aprotinin, and 10 mM phenylmethylsulfonyl fluoride. The cell lysates were subjected first to sonication and then centrifugation to remove debris; the protein concentration in each sample

Table 1
IC50 values of DZNep for the inhibition of proliferation in NSCLC and non-cancerous cell lines.

Cell type	Cell line	IC50 of DZNep (μM)[a]
NSCLC cells	H1299	0.21 ± 0.01
	H1975	0.08 ± 0.02
	A549	0.24 ± 0.01
	PC-3	0.19 ± 0.01
Non-cancerous cells	HBEC3 KT	0.58 ± 0.09*
	16HBE14o-	1.03 ± 0.11*
	WI-38 VA-13 2RA	0.63 ± 0.07*

[a] Data are presented as mean ± SD of three independent experiments. IC50 values of DZNep for the inhibition of proliferation in NSCLC and non-cancerous cell lines.
* $p < 0.001$ compared with each NSCLC cell line by one-way ANOVA with Tukey's multiple comparison test.

was determined using the Bio-Rad Protein Assay kit (BioRad Laboratories, Hercules, CA, USA). Samples containing equal amounts of protein were loaded onto gels, and the proteins in each sample were separated in 12% or 15% SDS gels; separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, St. Albans, UK), and the membranes were incubated with the following antibodies: anti-EZH2 (11/EZH2; BD Transduction Laboratories, San Jose, CA, USA), anti-SUZ12 (clone 3C1.2, Millipore, Billerica, MA, USA), trimethyl-Histone H3 Lys 27 (07-449, Millipore), anti-EED (09-774, Millipore), cyclin A (H-432, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-p27^{Kip1} (clone 57, BD Transduction Laboratories), and anti-actin (A-2066, Sigma–Aldrich Co.) antibodies. The primary antibodies were detected using anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (NA934V, NA931V, Amersham Biosciences); secondary antibodies were visualized using the Amersham ECL system after the membranes were washing with TBST six times (5 min each) after first and second antibodies incubation.

2.7. Statistical analysis

Statistical significance between two groups was determined using unpaired, two-sided Student's t-test. For multiple group comparison, statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. The level of significance was set at $p < 0.05$. All tests were performed using SPSS software (version 18.0; IBM SPSS, Chicago, IL, USA).

3. Results

3.1. siRNA-mediated knockdown of EZH2 expression inhibited growth of NSCLC cells

We investigated whether siRNA-mediated knockdown of EZH2 inhibited growth in four NSCLC cell lines. Reductions in EZH2 protein expression in all four NSCLC cell lines—H1299, H1975, A549, and PC-3—were confirmed by Western blot analysis (Fig. 1). For each NSCLC cell line, cells transfected with EZH2 siRNA exhibited less cell proliferation than did cells transfected with negative control siRNA.

3.2. DZNep inhibited growth of NSCLC cells

MTT assays demonstrated that DZNep caused dose-dependent inhibition of cell proliferation of NSCLC cell lines (Fig. S1), and the IC_{50} values ranged from 0.08 to 0.24 μM (Table 1). We also examined three immortalized but non-cancerous cell lines (HBEC3 KT, and 16HBE14o-bronchial epithelial cell lines and WI-38 VA-13 2RA fibroblast cell line). These cell lines also showed dose-dependent

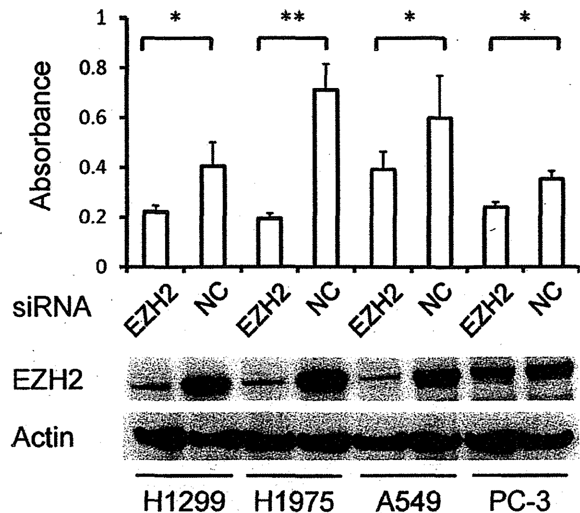

Fig. 1. Effects of EZH2 knockdown on cell proliferation in NSCLC cell lines. NCI-H1299 (H1299), NCI-H1975 (H1975), A549, and PC-3 cells were transfected with EZH2 siRNA or a negative control siRNA every 72 h. After 6 days, cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are means ± SD of quadruplet samples in one of three independent experiments. Similar results were obtained from all three independent experiments. *$p < 0.05$ and **$p < 0.01$ versus cells treated with negative control siRNA by unpaired, two-sided Student's t-test. Cell lysates were collected 72 h after transfection with EZH2 or a negative control siRNA; lysates were subjected to western blot analysis. EZH2; transfected with EZH2 siRNA, NC; transfected with negative control siRNA.

inhibition of cell proliferation by DZNep and had the IC_{50} values ranging from 0.54 to 1.03 μM, which were significantly higher than that of each NSCLC cell line ($p < 0.001$) (Table 1). Soft agarose assays showed that DZNep also reduced anchorage-independent growth in a dose-dependent fashion in all three NSCLC cell lines evaluated (Fig. 2). We excluded PC-3 cells from these assays because these cells did not form colonies within 4 weeks even in the absence of DZNep.

3.3. DZNep induced cell-cycle arrest and apoptosis

We used flow cytometry to determine whether the reduction in proliferation was due to cell cycle arrest or apoptosis in the four NSCLC cell lines. Treatment with DZNep at a concentration

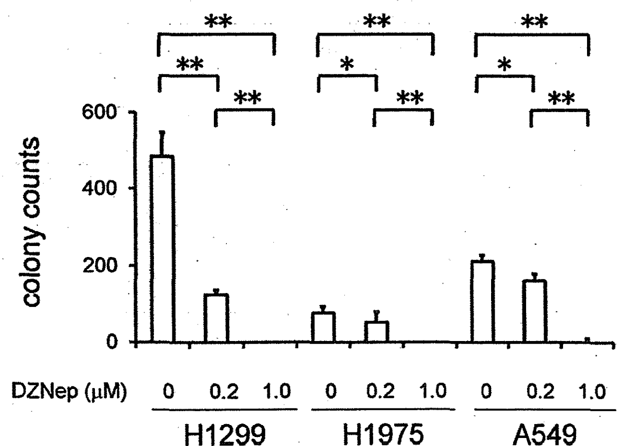

Fig. 2. Inhibition of anchorage-independent growth by DZNep. Representative data from one of three independent experiments is shown. Data are means ± SD of triplicate samples. Similar results were obtained from all three independent experiments. *$p < 0.05$ and **$p < 0.01$ between indicated groups by one-way ANOVA with Tukey's multiple comparison test.

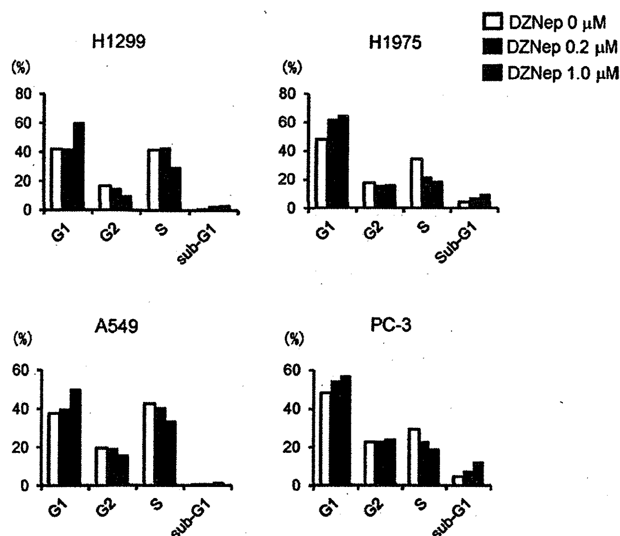

Fig. 3. Effect of DZNep on cell cycle in NSCLC cells. Cells were transfected with indicated doses of DZNep. After 72 h, the percentage of cells in each cell cycle phase was measured using a FACS flow cytometer and ModFitLT software. Representative data from one of three independent experiments is shown. Similar results were obtained in all three independent experiments.

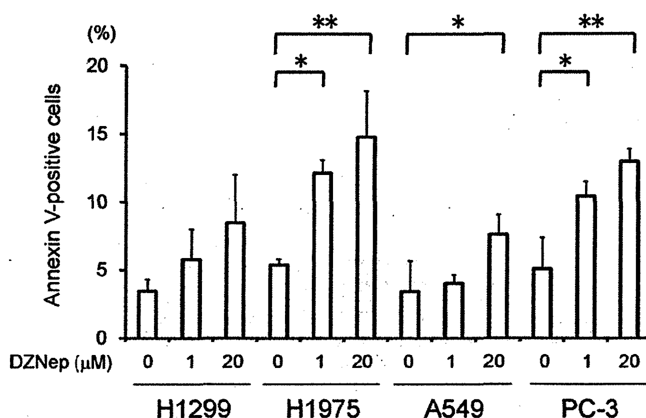

Fig. 4. Cell apoptosis analysis of NSCLC cells using flow cytometry with Annexin V-FITC and PI staining. Cells were treated with indicated doses of DZNep. After 72 h, the percentage of cells in the apoptotic fraction was measured using a FACS flow cytometer. Data are means ± SD of triplicate samples from one of three independent experiments. Similar results were obtained in all three independent experiments. *$p < 0.05$ and **$p < 0.01$ between indicated groups by one-way ANOVA with Tukey's multiple comparison test.

of 0.2–1.0 μM resulted in a slight increase in accumulation of cells in G1 phase of the cell cycle with a concomitant decrease in cells in S phase. The sub-G1 fraction also increased slightly following treatment with DZNep (Fig. 3). Flow cytometry analysis using Annexin V and PI demonstrated that the apoptotic fraction in each cell line increased in a dose-dependent manner following the treatment with DZNep; these effects were more evident in H1975 and PC-3 cells than in A549 cells and in H1299 cells, in which the difference did not reach statistical significance (Fig. 4).

3.4. Treatment with DZNep depleted EZH2, SUZ12, EED, histone H3 lysine 27 trimethylation and cyclin A, and increased p27^kip1 in NSCLC cells

Treatment with DZNep results in reduced expression of three PRC2 proteins (EZH2, SUZ12 and EED) in breast and colon cancer cells [21]. Consistent with these previous findings, treatment with DZNep resulted in reduced expression of the EZH2, SUZ12, and EED proteins in all four NSCLC cell lines, while the degree of the reduction varied between proteins and cell lines (Fig. 5). We next determined the effects of DZNep on H3K27me3 (trimethylation of lysine 27 of histone H3). A slight reduction in the repressive H3K27me3 followed treatment with DZNep. DZNep induced dose-dependent accumulation of $p27^{Kip1}$ and decreases in cyclin A in H1975 and PC3 cells, while such effect was only slightly seen in H1299 and A549 cells.

4. Discussion

EZH2 overexpression correlates with tumor aggressiveness and poor prognosis in a variety of malignancies including prostate, breast, uterine and gastric cancers [7–10]. Recently, we and others have shown that EZH2 is frequently overexpressed in NSCLCs and that high expression of EZH2 is correlated with tumor aggressiveness and poor prognosis [13,33]. Finding from the present study demonstrated that knockdown of EZH2 expression by siRNA reduced cell proliferation in four types of NSCLC cells, including A549 and H1299 cells, which were used in a previous study. In addition, our findings indicated that pharmacologic disruption of EZH2 via DZNep, which inhibits the histone methyltransferase EZH2, inhibited growth in four NSCLC cell lines in a dose-dependent manner with higher sensitivity than in non-cancerous cells; these findings were consistent with findings from previous studies on different types of tumors [22–28].

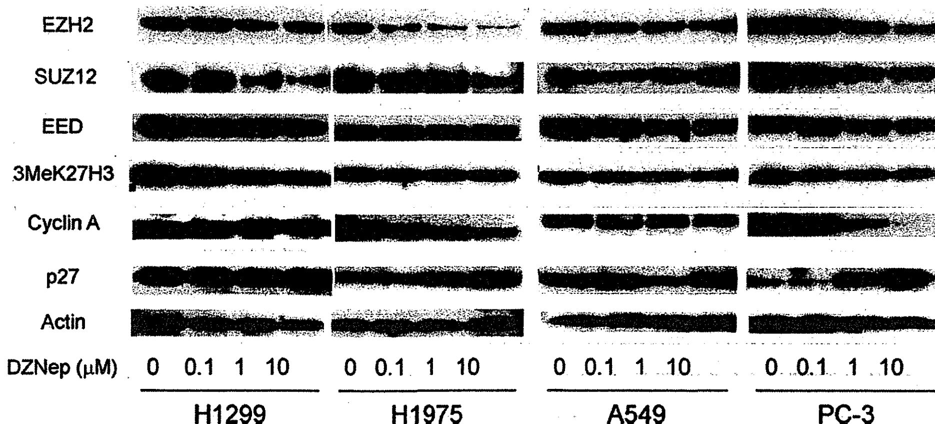

Fig. 5. Western blot analysis of NSCLC cells. Cell lysates were collected 72 h after the indicated doses of DZNep were administered; lysates were then subjected to western blot analysis. Representative western blots of EZH2, SUZ12, EED, trimethylation of lysine 27 on histon H3 (H3K27me3), cyclin A, $p27^{Kip1}$, and actin are shown.

The reduction of PRC2 components (EZH2, SUZ12, and EED) and the associated H3K27me3 by DZNep were consistent with finding from previous studies [21,27]. Meanwhile, DZNep was originally identified as an AdoHcy hydrolase inhibitor [20], which leads to the indirect inhibition of various S-adenosyl-methionine-dependent methylation reactions [34]. A recent study showed that DZNep globally decreases histone methylation including H3K9me3, H3K4me3 and H4K20me3, except for H3K9me3 and H3K36me3, suggesting histone methyltransferases other than EZH2 could be also susceptible to inhibition by DZNep [26]. Nevertheless, EZH2 knockdown alone caused significant inhibition of cell proliferation in all NSCLC cells in the present study, suggesting that the effect of DZNep are mediated by EZH2 depletion and the associated H3K27me3 reduction at least in part.

To our knowledge, the suppressive effects of DZNep on the proliferation of lung cancer cells have not been demonstrated previously, although DZNep has been shown to enhance deoxyazacytidine-mediated upregulation of some cancer-testis antigens in cells of lung cancers and to augment recognition and lysis of these cancer cells by T cells specific for these antigens [35]. Results of the cell cycle analysis in the present study indicated that the growth suppression by DZNep was associated with G1 cell cycle arrest in NSCLC cells; this conclusion is consistent with the findings from acute myeloid leukemia (AML) cells treated with DZNep [27]. Knockdown of EZH2 by siRNA has also been shown to induce G1 cell cycle arrest in Ras-transformed bronchial epithelial cells [16], A549 cells [12], and colon cancer cells [36]. Interestingly, DZNep induced decreases in cyclin A and accumulation of $p27^{Kip1}$ in H1975 and PC3 cells, but had minimal effects on these markers in the other two NSCLC cell lines. EZH2 is shown to reverse pRB2/p130-HDAC1 mediated transcriptional repression of cyclin A [37]. Genetic depletion of EZH2 results in reactivation of the $p27^{Kip1}$ gene in pancreatic cancer [17]. Association between either cyclin A repression or $p27^{Kip1}$ accumulation and G1 cell cycle arrest has been shown in various types of cells [38,39]. Meanwhile, Fiskus et al. reported that DZNep treatment induces p16, p21, $p27^{Kip1}$, and FBXO32 while reducing cyclin E and HOXA9 levels in the human AML cells [27]. Taken together, these results indicate that growth suppression by DZNep was associated with G1 arrest in NSCLC cells, partly via cyclin A repression and $p27^{Kip1}$ accumulation, while different cell lines may employ distinct cellular programs in responding to DZNep.

Our finding that DZNep induced apoptosis was consistent with findings from previous studies of other types of cancer cells including breast, hepatoma, and AML cells [21,24,27]. Recently, Wu et al. reported that EZH2 directly inhibits E2F1-dependent apoptosis through epigenetically modulating Bim expression in A549 and H1299 NSCLC cells [19]; this finding is also consistent with our findings. Notably, the apoptotic response was distinct in gefitinib-sensitive PC-3 cells, which have an EGFR mutation (a deletion of exon 19), and in gefitinib-resistant H1975 cells, which have two EGFR mutations (L858R and T790M); this observation indicated that DZNep may be useful for treating NSCLCs that have an EGFR gene mutation, including gefitinib-resistant mutations. Recent evidence indicates that oncogenic RAS can activate EZH2 expression through MEK/ERK signaling [40]. Taking together, our results suggest that activating EGFR mutations, which often occur in NSCLCs, may activate EZH2 through RAS/MEK/ERK signaling and modulate apoptotic response by DZNep. Besides, PC-3 and H1975, in which DZNep-induced apoptosis was more evident, displayed strong EZH2 silencing upon DZNep treatment, suggesting that their strong apoptotic responses may be due to higher intracellular accumulation or less efficient degradation of DZNep. The precise mechanisms by which DZNep induced apoptosis in NSCLCs have yet to be determined.

In conclusion, we showed that, via G1 arrest and apoptosis, the histone methyltransferase EZH2 inhibitor, DZNep, inhibited growth in four different cell lines that represented different types of NSCLC cells. An epigenetic therapy that pharmacologically targets EZH2 may be a potent cancer therapeutic for treatment of NSCLCs.

Conflict of interest statement

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2012.08.003.

References

[1] Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010;60:277–300.
[2] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
[3] Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet 2004;38:413–43.
[4] Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 2006;6:846–56.
[5] Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 2004;14:155–64.
[6] Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res 2008;647:21–9.
[7] Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 2006;24:268–73.
[8] Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419:624–9.
[9] Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA 2003;100:11606–11.
[10] Matsukawa Y, Semba S, Kato H, Ito A, Yanagihara K, Yokozaki H. Expression of the enhancer of zeste homolog 2 is correlated with poor prognosis in human gastric cancer. Cancer Sci 2006;97:484–91.
[11] Wagener N, Macher-Goeppinger S, Pritsch M, Husing J, Hoppe-Seyler K, Schirmacher P, et al. Enhancer of zeste homolog 2 (EZH2) expression is an independent prognostic factor in renal cell carcinoma. BMC Cancer 2010;10:524.
[12] Takawa M, Masuda K, Kunizaki M, Daigo Y, Takagi K, Iwai Y, et al. Validation of the histone methyltransferase EZH2 as a therapeutic target for various types of human cancer and as a prognostic marker. Cancer Sci 2011;102:1298–305.
[13] Kikuchi J, Kinoshita I, Shimizu Y, Kikuchi E, Konishi J, Oizumi S, et al. Distinctive expression of the polycomb group proteins Bmi1 polycomb ring finger oncogene and enhancer of zeste homolog 2 in nonsmall cell lung cancers and their clinical and clinicopathologic significance. Cancer 2010;116:3015–24.
[14] McCabe MT, Graves AP, Ganji G, Diaz E, Halsey WS, Jiang Y, et al. Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hypertrimethylation of histone H3 on lysine 27 (H3K27). Proc Natl Acad Sci USA 2012;109:2989–94.
[15] Karanikolas BD, Figueiredo ML, Wu L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. Prostate 2010;70:675–88.
[16] Watanabe H, Soejima K, Yasuda H, Kawada I, Nakachi I, Yoda S, et al. Deregulation of histone lysine methyltransferases contributes to oncogenic transformation of human bronchoepithelial cells. Cancer Cell Int 2008;8:15.
[17] Ougolkov AV, Bilim VN, Billadeau DD. Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2. Clin Cancer Res 2008;14:6790–6.

[18] Wagener N, Holland D, Bulkescher J, Crnkovic-Mertens I, Hoppe-Seyler K, Zentgraf H, et al. The enhancer of zeste homolog 2 gene contributes to cell proliferation and apoptosis resistance in renal cell carcinoma cells. Int J Cancer 2008;123:1545–50.
[19] Wu ZL, Zheng SS, Li ZM, Qiao YY, Aau MY, Yu Q. Polycomb protein EZH2 regulates E2F1-dependent apoptosis through epigenetically modulating Bim expression. Cell Death Differ 2010;17:801–10.
[20] Glazer RI, Hartman KD, Knode MC, Richard MM, Chiang PK, Tseng CK, et al. 3-Deazaneplanocin: a new and potent inhibitor of S-adenosylhomocysteine hydrolase and its effects on human promyelocytic leukemia cell line HL-60. Biochem Biophys Res Commun 1986;135:688–94.
[21] Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 2007;21:1050–63.
[22] Kemp CD, Rao M, Xi S, Inchauste S, Mani H, Fetsch P, et al. Polycomb repressor complex-2 is a novel target for mesothelioma therapy. Clin Cancer Res 2012;18:77–90.
[23] Crea F, Hurt EM, Mathews LA, Cabarcas SM, Sun L, Marquez VE, et al. Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer. Mol Cancer 2011;10:40.
[24] Hayden A, Johnson PW, Packham G, Crabb SJ. S-adenosylhomocysteine hydrolase inhibition by 3-deazaneplanocin A analogues induces anti-cancer effects in breast cancer cell lines and synergy with both histone deacetylase and HER2 inhibition. Breast Cancer Res Treat 2011;127:109–19.
[25] Suva ML, Riggi N, Janiszewska M, Radovanovic I, Provero P, Stehle JC, et al. EZH2 is essential for glioblastoma cancer stem cell maintenance. Cancer Res 2009;69:9211–8.
[26] Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, et al. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. Mol Cancer Ther 2009;8:1579–88.
[27] Fiskus W, Wang Y, Sreekumar A, Buckley KM, Shi H, Jillella A, et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. Blood 2009;114:2733–43.
[28] Jiang X, Tan J, Li J, Kivimae S, Yang X, Zhuang L, et al. DACT3 is an epigenetic regulator of Wnt/beta-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. Cancer Cell 2008;13:529–41.
[29] Nagai Y, Miyazawa H, Huqun, Tanaka T, Udagawa K, Kato M, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res 2005;65:7276–82.
[30] Ramirez RD, Sheridan S, Girard L, Sato M, Kim Y, Pollack J, et al. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Cancer Res 2004;64:9027–34.
[31] Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, et al. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol 1994;10:38–47.
[32] Sabichi AL, Hendricks DT, Bober MA, Birrer MJ. Retinoic acid receptor beta expression and growth inhibition of gynecologic cancer cells by the synthetic retinoid N-(4-hydroxyphenyl) retinamide. J Natl Cancer Inst 1998;90: 597–605.
[33] Huqun, Ishikawa R, Zhang J, Miyazawa H, Goto Y, Shimizu Y, et al. Enhancer of zeste homolog 2 is a novel prognostic biomarker in nonsmall cell lung cancer. Cancer 2011;118:1599–606.
[34] Chiang PK. Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. Pharmacol Ther 1998;77:115–34.
[35] Rao M, Chinnasamy N, Hong JA, Zhang Y, Zhang M, Xi S, et al. Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. Cancer Res 2011;71:4192–204.
[36] Fussbroich B, Wagener N, Macher-Goeppinger S, Benner A, Falth M, Sultmann H, et al. EZH2 depletion blocks the proliferation of colon cancer cells. PLoS ONE 2011;6:e21651.
[37] Tonini T, Bagella L, D'Andrilli G, Claudio PP, Giordano A. Ezh2 reduces the ability of HDAC1-dependent pRb2/p130 transcriptional repression of cyclin A. Oncogene 2004;23:4930–7.
[38] Girard F, Strausfeld U, Fernandez A, Lamb NJ. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 1991;67:1169–79.
[39] Resnitzky D, Hengst L, Reed SI. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27Kip1. Mol Cell Biol 1995;15:4347–52.
[40] Fujii S, Fukamachi K, Tsuda H, Ito K, Ito Y, Ochiai A. RAS oncogenic signal upregulates EZH2 in pancreatic cancer. Biochem Biophys Res Commun 2012;417:1074–9.

A Phase I Study of Amrubicin and Fixed Dose of Irinotecan (CPT-11) in Relapsed Small Cell Lung Cancer

Japan Multinational Trial Organization LC0303

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Purpose: To determine the maximum tolerated dose of amrubicin (AMR) with a fixed dose of irinotecan (CPT-11).

Methods: Patients having pathologically proven small cell lung cancer (SCLC) relapsed after one or two chemotherapies, and Eastern Cooperative Oncology Group performance status of 0 to 2 were eligible for the study. CPT-11 was delivered as 50 mg/m^2 on days 1 and 8, every 21 days. AMR was delivered on day 1. Doses of AMR were level 1: 80 mg/m^2, level 2: 90 mg/m^2, and level 3: 100 mg/m^2. Dose elevation was determined using the modified continuous reassessment method. Tolerability was assessed after the first cycle. Another two cycles were conducted when disease progression or unacceptable toxicities were not observed.

Results: Eighteen patients (mean age: 66.3 years) were enrolled. A total of 40 courses were conducted. Grade 3/4 toxicities of the first cycle were leukocytopenia: 11 (61%, grade 3/4: 8/3); neutropenia: 15 (83%, grade 3/4: 6/9); and thrombocytopenia: three (17%, grade 3/4: 2/1). Other grade 3 toxicities observed were febrile neutropenia, one; infection, three; diarrhea, one; and dyspnea, one. Dose-limiting toxicity was observed in two of six patients at level 2 (neutropenia and febrile neutropenia) and in one of six at level 3 (thrombocytopenia and infection). The maximum tolerated dose was level 3, and so, the recommended dose for phase II trials was judged to be 90 mg/m^2. Objective response was obtained in four of eight patients who were able to evaluate responses. Median survival time was 13 months, with 68% at 1-year survival rate.

Conclusions: This combination was well tolerated and showed encouraging activities in SCLC. Randomized phase II trials are being planned in chemonaive SCLC.

Key Words: Small cell lung cancer, Amrubicin, Irinotecan, Modified continuous reassessment method, Relapse.

(*J Thorac Oncol.* 2012;7: 1845–1849)

Lung cancer is the most common and deadliest cause of cancer death worldwide.[1] Small cell lung cancer (SCLC) represents 13% of all lung cancer and is the most aggressive form of lung cancer, with an overall 5-year survival rate less than 5%.[2] SCLC is one of the most chemo-sensitive solid tumors, and the outcome for patients with SCLC is slowly improving.[3,4] Nevertheless, despite a good initial response to therapy, most patients with SCLC develop chemotherapy resistance and relapse. Second-line chemotherapy should then be applied. Although the introduction of new agents has improved the outcome of patients with SCLC,[5] the development of more active chemotherapy, and especially the introduction of more effective new drugs, is warranted to continue to improve the survival of patients with SCLC.

Amrubicin (AMR), a totally synthetic 9-aminoanthracycline, inhibits DNA topoisomerase II activity. AMR is converted to an active metabolite, amrubicinol, by reduction of its C-13 ketone group to a hydroxy group. AMR has either an equivalent or a stronger antitumor effect in comparison with doxorubicin in nude mice transplanted with human tumor cells.[6–8] In a phase I trial of AMR for 3 consecutive days at 3-week intervals in patients with advanced non-SCLC (NSCLC) without prior chemotherapy, the maximum tolerated dose (MTD) and the recommended dose were estimated to be 50 mg/m^2 and 45 mg/m^2, respectively. The major dose-limiting toxicity (DLT) was myelosuppression.[9] In a phase II study of AMR using a schedule of 45 mg/m^2 on days 1 to 3 every 3 weeks, in 33 previously untreated patients with extensive disease (ED) SCLC, an overall response rate of 76% and a complete response rate of 9% were reported.[10]

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Disclosure: The authors declare no conflicts of interest.

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Median survival time was 11.7 months. When 3-day AMR is combined with another chemotherapeutic agent such as cisplatin, carboplatin, or irinotecan (CPT-11), the incidence of myelosuppression increases, which makes it impossible to use clinically.[11]

On the other hand, in a phase I study of the administration of AMR on day 1, 29 evaluable courses of treatment were conducted in groups at doses increasing from 10 to 130 mg/m^2 of AMR on day 1.[12] Myelosuppression was the DLT, and a MTD was 130 mg/m^2. Nonhematologic side effects were mild grastrointestinal symptoms and hair loss. The recommended dose was 100 mg/m^2.

Although there is no study to compare the 3-day AMR with single-day AMR, the decision of 3-day AMR regimen is mainly derived from the results of NSCLC.

Irinotecan is a water-soluble semisynthetic camptothecin derivative that inhibits topoisomerase I. Irinotecan is one the most active drugs used in the treatment of SCLC and NSCLC.[13,14] The Japan Clinical Oncology Group reported that the combination of cisplatin and irinotecan allows for significantly better survival than the combination of cisplatin and etoposide for previously untreated ED SCLC.[15]

Combinations of topoisomerase I and topoisomerase II inhibitors have been reported to be active against SCLC based on in vitro and in vivo animal model.[16]

Based on this background, a phase I study was designed to determine the MTD of 1-day AMR and irinotecan by applying the modified continual reassessment method (CRM) and to obtain preliminary evidence of the therapeutic activity of this combination in patients with relapsed SCLC.

PATIENTS AND METHODS

Patient Selection

Patients were required to fulfill the following eligibility criteria: pathologically or cytologically diagnosed SCLC; relapsed after one or two regimens of chemotherapy; adequate reserves of hematological function (neutrophil count ≥ 1500/μl and ≤7000/μl, platelet count ≥ 100,000/μl, and hemoglobin > 8.5 mg/dl); adequate hepatic function (bilirubin ≤ 1.5 mg/dl, aspartate aminotransferase [≤2x the upper limit of normal], and alanine aminotransferase [≤2x the upper limit of normal]); adequate renal function (creatinine [≤2x the upper limit of normal] and pulmonary function [Pa_{O_2} ≥ 70 torr]); Eastern Cooperative Oncology Group performance status (PS) of 0, 1, or 2; expected survival more than 3 months; and acquisition of written informed consent. Baseline pretreatment evaluations included a complete history, physical examination, laboratory tests, chest radiograph, electrocardiogram, computed tomography scans of the chest and abdomen, magnetic resonance imaging of the brain, and a radionuclide bone scan. The protocol was approved by the institutional review board of each participating institute.

Exclusion criteria included the following: massive pleural effusion, pericardial effusion, or ascites; symptomatic brain metastasis; uncontrollable hypertension, unstable angina, heart failure, and myocardial infarction within 1 year; uncontrollable diabetes mellitus; watery diarrhea and ileus; pulmonary fibrosis in chest x-ray; history of anthracyclin use; and severe infection.

Drug Administration

The protocol treatment consisted of three courses, each requiring 21 days to complete. In each course, irinotecan was diluted in 500 ml of normal saline for administration and then administered to the patient at a fixed dose of 50 mg/m^2 as an intravenous infusion on days 1 and 8. After the completion of irinotecan infusion, AMR dissolved in 20-ml saline was delivered intravenously as a 5-minute infusion on day 1 alone, every 21 days. The dose levels of AMR consisted of level 1: 80 mg/m^2, level 2: 90 mg/m^2, and level 3: 100 mg/m^2. Which dose level of AMR should be allocated to each of the patients except the first three patients was determined by applying the CRM.[17] The CRM, which is comparable with the traditional phase I design in terms of study duration and proportion of patients treated at a dose greater than the MTD, can take account of the cumulative DLT data of all the treated patients and determine the dose allocation to the next cohort.

Granulocyte colony stimulating factor (G-CSF) was allowed to use at more than grade 3 leukocytopenia or neutropenia, and prophylactic use were not allowed.

Trial Design

Toxicity was graded according to the National Cancer Institute-Common Toxicity Criteria version 2.0. The scheme of this trial is shown in Figure 1. Tolerability was assessed after the first course. Another two courses were conducted when disease progression or unacceptable toxicities were not observed. DLT was defined using the National Cancer Institute Common Toxicity Criteria (version 2.0) as development of at least one of the following adverse events occurring during the first course (21 days) of protocol treatment: grade 4 neutropenia lasting more than 7 days; febrile neutropenia (>38.5°C, grade 3 or 4 neutrophils) for more than 1 day; grade 4 thrombocytopenia (<10,000/μl); more than 2 weeks delay of next course due to neutropenia (<1500/μl) or thrombycytopenia (<75,000/μl); >grade 2 liver or renal dysfunction; and other >grade 2 toxicities except for alopecia, nausea, vomiting, and appetite loss. The MTD was defined a priori as the highest dose at which a maximum of 33% of patients were expected to experience a DLT during the first course. The first three patients were treated at level 1 for reasons of safety. Nevertheless, if one of three or more patients had the DLT at level 1, then AMR would be decreased

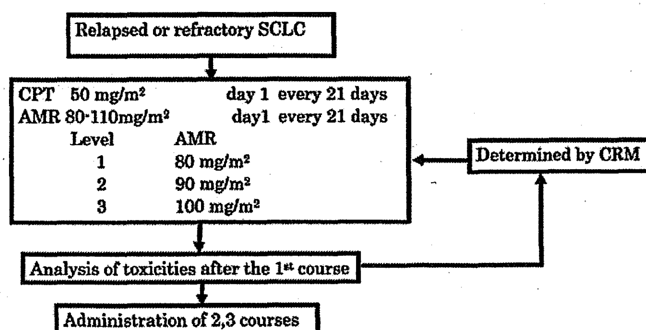

FIGURE 1. Schema of phase I study using CRM. SCLC, small cell lung cancer; CPT, irinotecan; AMR, amrubicin; CRM, continuous reassessment method.

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to the dose level of 70 mg/m^2. The dose level that was the closest to the current estimate of the MTD was identified by the CRM and allocated to each of the succeeding patients. Both dose escalation and deescalation were permitted.

Statistical Analysis

The MTD was estimated by the CRM at the end of the trial. For accrued patients into whom a part or all the protocol treatment are administered, the incident frequency and severity of adverse events, including DLTs, were reported. Objective tumor assessments were conducted using Response Evaluation Criteria Solid Tumors.[18] Overall survival was measured from the date of study treatment to the date of death from any cause. One-year survival rate were estimated by Kaplan-Meier method.

RESULTS

Between June 2004 and October 2006, 18 patients participated in this trial at five institutions. Patient characteristics are listed in Table 1. Fifteen patients were male, and three were female, and the median age was 66 years. Four patients had PS 0, and 14 patients had PS 1. Two patients had limited disease, and 16 patients had ED at relapse. Numbers of prior chemotherapy regimens were 1 in 11 patients and 2 in seven patients. Previous chemotherapy consisted of cisplatin and etoposide in 10 patients; cisplatin and irinotecan in six patients; carboplatin and etoposide in four patients; carboplatin and irinotecan in three patients; and etoposide in one patient. The responses to the prior chemotherapy were complete response in two patients and partial response in 15 patients. All the responders were sensitive relapse, which is defined as disease that responded to first-line chemotherapy and relapsed later than 90 days after the last dose of first-line chemotherapy. Only one had progression of disease. Other treatments included thoracic radiotherapy in 12 patients and surgery in one patient.

Toxicities during the first course are listed in Table 2. Six patients each were enrolled at dose levels 1 (AMR, 80 mg/m^2), 2 (AMR, 90 mg/m^2), and 3 (AMR, 100 mg/m^2). No DLT were observed during the first course of level 1. At level 2, DLT was observed in two patients. One patient had neutropenia lasting > 7 days, and the other patient had febrile neutropenia. At level 3, DLT was observed in two patients: one had neutropenia and the other had thrombocytopenia and infection.

On the basis of these cumulative DLT data on 18 patients, the DLT probabilities at levels 1, 2, and 3 were calculated by the CRM as 0.000, 0.205, and 0.348, respectively. Because the target DLT probability was specified as 33% as mentioned (see Trial Design section), the MTD was estimated to be level 3.

A total of 40 courses were conducted. Grade 3/4 hematological toxicities of first cycle were leukocytopenia: 61% (grade 3/4: 8/3); neutropenia: 83% (grade 3/4: 6/9); and thrombocytopenia: 17% (grade 3/4: 2/1). Grade 3 febrile neutropenia occurred in one patient (6%). Grade 3 infection occurred in three patients (17%). Grade 3 diarrhea and dyspnea occurred in one patient (6%) each. In the first course, DLT was observed in two of six patients at dose level 2 (prolonged grade 4 neutropenia and febrile neutropenia) and in one of six at dose level 3 (thrombocytopenia and infection).

For supportive care during the entire courses, G-CSF was administered in 16 patients (88%). Antibiotics were used for three patients (17%). Blood transfusion was required in one patient (6%).

TABLE 1. Patient Characteristics

Characteristics	
Total no. of patients	18
Sex	
Male	15
Female	3
Age (yr)	
Mean	66.3
Range	57–79
Performance status (ECOG)	
0	4
1	14
Disease extent at relapse	
Limited disease	2
Extensive disease	16
Prior therapy	
No. of prior chemotherapy regimens	
1	11
2	7
Chemotherapy	18
Cisplatin + etoposide	10
Cisplatin + irinotecan	6
Carboplatin + etoposide	4
Carboplatin + irinotecan	3
Etoposide	1
Response to prior chemotherapy	
CR	2
PR	15
SD	
PD	1
Chemotherapy-free interval (d)	
<60	1
≥60	17
Thoracic radiotherapy	12
Surgery	1

ECOG, Eastern Cooperative Oncology Group, CR, complete response, PR, partial response; SD, stable disease; PD, progression disease.

Response and Survival

Tumor responses were observed in eight patients. Four patients of eight patients had partial response, three had stable disease, and one had disease progression. The overall response rate was 50.0% (4/8). The median survival time was 13 months, with 68% at 1-year survival rate.

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TABLE 2. Toxicities During the First Course

Amrubicin	Grade (National Cancer Institute-Common Toxicity Criteria)															
	Level 1 ($n = 6$) 80 mg/m², Day 1 50 mg/m², Days 1 and 8					Level 2 ($n = 6$) 90 mg/m², Day 1 50 mg/m², Days 1 and 8					Level 3 ($n = 6$) 100 mg/m², Day 1 50 mg/m², Days 1 and 8					
Irinotecan	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**Grades 3 and 4 in All Levels**
Leukopenia		1	2	3			1	1	3	1			2	2	2	11/18 (61%)
Neutropenia		1	2	2	1				1	5				3	3	15/18 (83%)
Thrombocytopenia	4	2				4	1		1		2	2		1	1	3/18 (17%)
Hemoglobin febrile		5	1			1	4		1		1	2	3			1/18 (6%)
Neutropenia	1															1/18 (6%)
Infection	5		1			6					3			3		3/18 (17%)
Nausea	4	2				3	3				3	1	2			0/18 (0%)
Diarrhea	2	4				5			1		3	2	1			1/18 (6%)
Dyspnea	6					5			1		6					1/18 (6%)

DISCUSSION

This demonstrated that the MTD of AMR determined by the CRM was level 3, 100 mg/m², and thus, the recommended dose for phase II trials was judged to be 90 mg/m², one dose level below the MTD, with the fixed dose of irinotecan delivered as 50 mg/m² on days 1 and 8, every 21 days.

This study was for previously treated SCLC. Objective response was obtained in four patients (29%). Median survival time was 13 months with 68% at 1-year survival rate. It should be noted that AMR was administered on day 1 alone without the use of G-CSF. So far, there has been no study using AMR only on day 1 with irinotecan.

Combinations of irinotecan and 3-day AMR in lung cancer have been reported recently. Yanaihara et al.[19] conducted a phase I trial, in which 11 patients with NSCLC were treated at 3-week intervals with AMR on days 1 to 3 plus 60 mg/m² of irinotecan on days 1 and 8. The 30 mg/m² of AMR dose was one dose level above the MTD. Diarrhea and leukopenia were the DLT. The recommended dose for phase II studies is 60 mg/m² of irinotecan on days 1 and 8 and 25 mg/m² of AMR on days 1 to 3 every 3 weeks. They also reported that AMR did not affect the pharmacokinetics of irinotecan, SN-38 or SN-38 glucuronide.

Kaneda et al.[11] started the dose of AMR from 35 mg/m² on days 1 to 3 and irinotecan 50 to 60 mg/m² after the completion of AMR on days 1 and 8, every 3 weeks in phase I for patients with advanced lung cancer. The most frequent toxicities were bone marrow suppression followed by infection, diarrhea, and pneumonitis. As a consequence of these toxicities, the MTD and the recommended dose could not be determined. They concluded that this combination is not tolerated and is inactive against both NSCLC and SCLC. This indicates that 35 mg/m² of AMR on days 1 to 3 may be too toxic.

Oshita et al.[20] conducted dose escalation study of AMR with fixed-dose irinotecan in patients with ED SCLC. Thirteen previously untreated patients were treated with irinotecan at 60 mg/m² day 1 and dose-escalated AMR on days 1 to 3 with prophylactic G-CSF on days 5 to 9, every 2 to 3 weeks. A total of 31 courses were administered at dose level 2 (35 mg/m²/d) in six patients, and grade 4 neutropenia was observed during five courses (16.1%). Irinotecan at 60 mg/m² on day 1 and AMR at 35 mg/m²/d on days 1 to 3 with G-CSF support every 3 weeks are recommended. The above data indicate that 35 mg/m²/d on 1 to 3 could not be administered without G-CSF.[20] There are three phase II trials published on AMR monotherapy for patients with relapsed SCLC. The Thoracic Oncology Research Group conducted a single-arm phase II study AMR on 16 chemotherapy refractory and 44 sensitive patients.[21] When given at a dose of 40 mg/m²/d (days 1–3) every 3 weeks, their results demonstrated a 50% response rate in the refractory group and 52% in the sensitive group. The progression-free survival, overall survival, and 1-year survival in the refractory group and the sensitive group were 2.6 and 4.2 months, 10.3 and 11.6 months, and 40% and 46%, respectively. In previously treated patients with SCLC, AMR 40 mg/m²/d (days 1–3) every 3 weeks was administered in a phase II trial.[22] Twenty-six patients (nine sensitive and 17 refractory patients) received a median number of three cycles of therapy. The response rate was 46.2% (55.6% in sensitive patients and 41.2% in refractory patients). The median survival time was 9.4 months (11.0 months for sensitive patients and 5.7 months for refractory patients). Grade 4 neutropenia occurred in 73.1% of patients. Grade 3 or 4 thrombocytopenia occurred in 50% of patients. A comparison of AMR (40 mg/m²/d, d1–3) with topotecan (1.0 mg/m²/d, days 1–5) for previously treated SCLC ($n = 59$, 36 sensitive relapsed and 23 refractory relapsed) in a randomized phase II trial was reported by Inoue et al.[23] Overall response rates were 38% (53% in sensitive relapse and 17% in refractory) for the AMR arm and 13% (21% and 0% in sensitive and refractory) for the topotecan arm. AMR may be superior to topotecan. Nevertheless, neutropenia was severe, and one treatment-related death due to infection was observed in the AMR arm.

Although this study is a phase I study, the response rate of 50% and median survival time of 13 months for the previously treated patients with SCLC indicate that the combination of AMR and irinotecan is active and encouraging.

As a result of our study, AMR 90 mg/m² on day 1 with irinotecan 50 mg/m² on days 1 and 8 were recommended for further

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study because of the increased incidence of grade 4 myelosuppression at level 3 (AMR, 100 mg/m^2). Our data indicate that AMR on day 1 alone with fixed-dose irinotecan without G-CSF was feasible and demonstrated activity in relapsed SCLC.

A randomized phase II trial comparing AMR (90 mg/m^2, day 1) and irinotecan (50 mg/m^2) with cisplatin (60 mg/m^2 on day 1) and irinotecan (60 mg/m^2 on days 1 and 8) is ongoing for patients with chemonaive ED SCLC.

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REFERENCES

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71–96.
2. Fischer B, Arcaro A. Current status of clinical trials for small cell lung cancer. *Rev Recent Clin Trials* 2008;3:40–61.
3. Janne PA, Freidlin B, Saxman S, et al. Twenty-five years of clinical research for patients with limited-stage small cell lung carcinoma in North America. *Cancer* 2002;95:1528–1538.
4. Chute JP, Chen T, Feigal E, et al. Twenty years of phase III trials for patients with extensive-stage small-cell lung cancer: perceptible progress. *J Clin Oncol* 1999;17:1794–1801.
5. Ettinger DS. New drugs for chemotherapy-naive patients with extensive-disease small cell lung cancer. *Semin Oncol* 2001;28:27–29.
6. Yamaoka T, Hanada M, Ichii S, et al. Cytotoxicity of amrubicin, a novel 9-aminoanthracycline, and its active metabolite amrubicinol on human tumor cells. *Jpn J Cancer Res* 1998;89:1067–1073.
7. Morisada S, Yanagi Y, Noguchi T, et al. Antitumor activities of a novel 9-aminoanthracycline (SM-5887) against mouse experimental tumors and human tumor xenografts. *Jpn J Cancer Res* 1989;80:69–76.
8. Noguchi T, Ichii S, Morisada S, et al. In vivo efficacy and tumor-selective metabolism of amrubicin to its active metabolite. *Jpn J Cancer Res* 1998;89:1055–1060.
9. Sugiura T, Ariyoshi Y, Negoro S, et al. Phase I/II study of amrubicin, a novel 9-aminoanthracycline, in patients with advanced non-small-cell lung cancer. *Invest New Drugs* 2005;23:331–337.
10. Yana T, Negoro S, Takada M, et al. Phase II study of amrubicin in previously untreated patients with extensive-disease small cell lung cancer: West Japan Thoracic Oncology Group (WJTOG) study. *Invest New Drugs* 2007;25:253–258.
11. Kaneda H, Kurata T, Tamura K, et al. A phase I study of irinotecan in combination with amrubicin for advanced lung cancer patients. *Anticancer Res* 2006;26:2479–2485.
12. Inoue K, Ogawa M, Horikoshi N, et al. Phase I and pharmacokinetic study of SM-5887, a new anthracycline derivative. *Invest New Drugs* 1989;7:213–218.
13. Masuda N, Fukuoka M, Kusunoki Y, et al. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1992;10:1225–1229.
14. Masuda N, Fukuoka M, Takada M, et al. CPT-11 in combination with cisplatin for advanced non-small-cell lung cancer. *J Clin Oncol* 1992;10:1775–1780.
15. Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 2002;346:85–91.
16. Takigawa N, Ohnoshi T, Ueoka H, et al. Comparison of antitumor activity of new anthracycline analogues, ME2303, KRN8602, and SM5887 using human lung cancer cell lines. *Acta Med Okayama* 1992;46:249–256.
17. Goodman SN, Zahurak ML, Piantadosi S. Some practical improvements in the continual reassessment method for phase I studies. *Stat Med* 1995;14:1149–1161.
18. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors: European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
19. Yanaihara T, Yokoba M, Onoda S, et al. Phase I and pharmacologic study of irinotecan and amrubicin in advanced non-small cell lung cancer. *Cancer Chemother Pharmacol* 2007;59:419–427.
20. Oshita F, Saito H, Yamada K. Dose escalation study of amrubicin in combination with fixed-dose irinotecan in patients with extensive small-cell lung cancer. *Oncology* 2008;74:7–11.
21. Omoda S, Masuda N, Seto T, et al. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. *J Clin Oncol* 2006;24: 5448–5453.
22. Hasegawa Y, Takeda K, Kashii T, et al. Clinical experiences of amrubicin hydrochloride (Calsed) monotherapy in previously treated patients with small-cell lung cancer. *Jpn J Cancer Chemother* 2005;45:811–815.
23. Inoue A, Sugawara S, Yamazaki K, et al. Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. *J Clin Oncol* 2008;20:5401–5406.

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