表2　男女別がん検診受診における市町村国保を1とした場合のオッズ比―多変量調整ロジスティック回帰分析の結果*

	大腸がん検診		胃がん検診		肺がん検診		乳がん検診	子宮がん検診
	男性	女性	男性	女性	男性	女性	女性	女性
共済組合本人	3.16 (2.97～3.36)	2.80 (2.59～3.02)	5.18 (4.87～5.52)	4.58 (4.24～4.95)	3.00 (2.83～3.20)	2.78 (2.58～3.00)	3.18 (2.94～3.45)	2.59 (2.43～2.75)
健保組合本人	2.29 (2.20～2.39)	1.85 (1.75～1.95)	3.32 (3.19～3.46)	2.59 (2.47～2.73)	2.31 (2.21～2.41)	1.84 (1.75～1.94)	1.80 (1.71～1.88)	1.66 (1.60～1.73)
協会けんぽ本人	1.44 (1.37～1.51)	1.42 (1.35～1.50)	1.91 (1.83～2.00)	1.91 (1.82～2.00)	1.52 (1.45～1.59)	1.48 (1.40～1.55)	1.51 (1.44～1.58)	1.52 (1.46～1.57)
いずれかの被用者保険本人	1.65 (1.57～1.73)	1.22 (1.13～1.33)	2.17 (2.08～2.27)	1.85 (1.72～2.00)	1.64 (1.56～1.72)	1.29 (1.19～1.40)	1.54 (1.43～1.65)	1.56 (1.46～1.65)
被用者保険被扶養者	1.04 (0.93～1.15)	1.05 (1.01～1.09)	1.12 (1.01～1.24)	1.09 (1.05～1.14)	1.06 (0.95～1.19)	1.09 (1.04～1.13)	1.23 (1.18～1.27)	1.24 (1.20～1.28)
組合国保	1.18 (1.06～1.31)	0.89 (0.79～1.00)	1.17 (1.06～1.29)	0.90 (0.81～1.01)	1.16 (1.04～1.29)	0.87 (0.77～0.98)	0.92 (0.83～1.02)	1.07 (0.98～1.17)
その他（生活保護）	0.80 (0.69～0.93)	0.63 (0.53～0.76)	1.09 (0.95～1.24)	0.80 (0.68～0.94)	0.88 (0.76～1.02)	0.65 (0.54～0.78)	0.81 (0.71～0.93)	0.90 (0.81～1.01)

*年齢，学歴，婚姻，失業，持ち家，喫煙，政令指定都市について調整した．

（　）内は95％信頼区間

り，特定健診の導入も影響してがん検診業務に手が回らない市町村職員の状況が伝えられている．

おわりに

がん検診受診率50％以上という課題を達成することは容易ではなく，国民全体を対象としたキャンペーンだけでは解決できない．受診率向上策に関して，若年者，壮年者，高齢者では異なったアプローチが必要である[3]のと同様に，本研究が明らかにした医療保険に基づく国保加入者等のハイリスクポピュレーションに照準を定めた重点的な対策を実施し，がん検診受診率の向上を目指すべきである．

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実地医家がいかに肺癌を疑い，いかに早期発見するか

—肺癌早期発見のありかたと進めかた—

中山富雄

東京 文光堂 本郷

実地医家がいかに肺癌を疑い，いかに早期発見するか

—肺癌早期発見のありかたと進めかた—

中山富雄
大阪府立成人病センター・がん予防情報センター／なかやま・とみお

はじめに●

本稿におけるテーマである「いかに肺癌を疑い，いかに早期発見するか」とは，「特定の症状から肺癌であることを疑い，早期発見する」ということではない．大腸癌を除けば，ほぼすべての癌では症状がないものを指すため，症状から肺癌を疑い早期発見することは不可能である．**図1**に早期発見の概念図を示す．すなわち早期発見とは症状を自覚し外来を受診する前(無症状の間：前臨床期)に適切な検査を行い，診断することであり，例えば咳が続くとか血痰が出るといった肺癌を強く疑うべき症状を有して患者が来たときに肺癌が診断されても，早期発見である可能性はかなり低い．いかに疑うか？ ではなく，例えば高血圧などの慢性疾患で定期的受診を続ける患者に対して，定期的検査をどう行うかということである．**表1**に検診と診療の違いをまとめた．診療は有症状で受診する初診患者への診療をここでは指す．有病率が高いことから迅速かつ正確な対応を要するが，検診は無症状で有病率が低いことから，大多数を占める健常者に誤って癌の疑いをかけたり，検査による偶発症などの不利益を与えないことが重要視されている．したがって，実地臨床においては，目的が診療なのか検診なのかで対応を変えることが望まれる．

対象者は？●

健康増進法に基づいて市町村が実施する肺癌検診の対象者は，40歳以上の男女と定義されている．高齢者に対する上限は設けられていないものの，気管支鏡などの精密検査や治療が耐えられる体力がないと，たとえ陰影が見つけられても何もできないことになる．肺癌のハイリスクは喫煙者である．喫煙者に対しては，感冒などを訴えて受診した際も，直ちに投薬して終わりではなく，健康診断の受診歴がなければ，下記に記すような検査を提案するべきである．禁煙者についても過去5年以内の禁煙では肺癌罹患リスクはほとんど下がらない[1]ので，現在喫煙者と同様に扱うべきである．一方非喫煙者の肺癌リスクは諸外国と比べれば無視できないほど高いので，基本的には年1回の定期検査が望まれる．

定期検査として何が推奨されるか？●

肺癌を見つけるために行う定期検査とは，胸部単純X線撮影であり，現時点で「低線量CT」はま

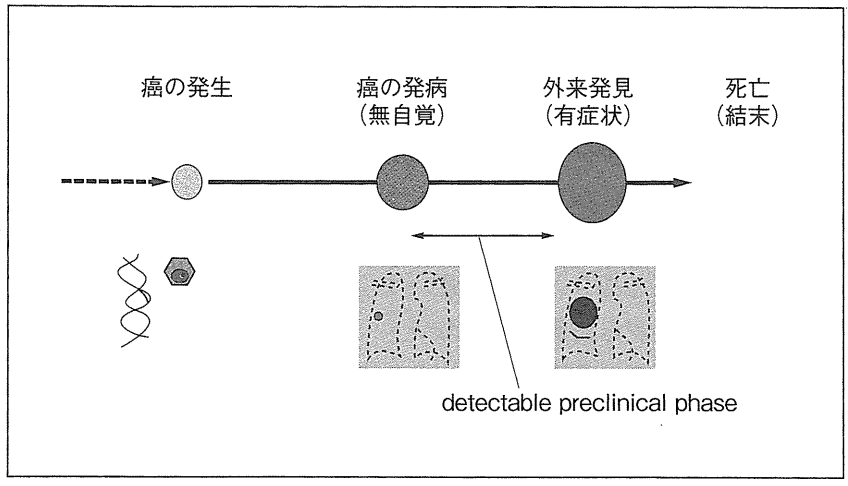

図1　癌の発生から早期発見・外来発見までの概念図
癌が検査法の診断限界(下限)を超え，診断可能になった時期(癌の発病)から，放置して症状が出て外来受診するころまでの期間をdetectable preclinical phase(前臨床期)と呼ぶ．早期発見とは前臨床期の間に適切な検査法を行うことで可能となる．

0910-1551/12/ ¥100/頁 /JCOPY

●癌の早期発見を試みる場合，症状が出てからでは遅い．無症状者へのスクリーニング検査が重要．
●診療の場合は，感度が優先され，侵襲的な検査や治療法も許される場合がある．
●検診の場合は，特異度が優先され，健常者に害や不利益を与えないことが重要視される．
●肺癌のハイリスクは喫煙者であるが，非喫煙者にも早期診断を試みるべきである．

表1　診療と検診の違い

	診療	検診
対象者	一般的には有症状で生活に支障がある	無症状で生活に支障がない
有病率	高い．数分の1～数十分の1	低い．数百分の1～数千分の1
感度・特異度の重要度	標的疾患をもれなく診断すること(高い感度)が要求される	病気をもっていないのに病気の疑いをかけないこと(高い特異度)が要求される
検査方法・診断方法	精度の高い方法であり，ときには侵襲的検査も可能	費用が安価であり安全性が高い
実施手順および指針	診療ガイドラインに沿うことが望ましい．ただし裁量権はかなり許される	国の指針や学会などの検診ガイドラインに沿うことが要求される．裁量権はあまり許されない

表2　肺癌検診用の胸部X線撮影条件

アナログ撮影	被験者―管球間距離を1.5 m以上とし，定格出力150 kV以上の撮影装置を用い，120 kV以上の管電圧および希土類システム(希土類増感紙＋オルソタイプフィルム)による撮影
デジタル撮影*	管球検出器間距離(撮影距離)180～200 cm，X線管電圧120～140 kV，撮影mAs値4 mAs程度以下，入射表面線量0.3 mGy以下，グリッド比12：1以上

*デジタル撮影の画像処理に関しては，文献3)を参考にされたい．

(日本肺癌学会：肺癌集団検診の手引きより改変引用)

だ推奨されていない．ここでいう「低線量CT」とは検診に特化した低線量撮影を行ったCTであり，通常診断用のCT撮影とは異なる．通常診断用のCTを検診目的で定期的に健常者に行うことは，被曝線量のリスクが高く推奨されていない．胸部単純X線撮影では正面撮影のみが用いられて側面撮影は行われない．日本肺癌学会肺癌取扱い規約[2]によれば，肺癌集団検診として用いる胸部単純X線撮影は，定格出力150 kV以上の撮影装置が求められている(**表2**)．現行で販売されている装置でこの基準を満たさない機器はないと考えられるが，条件や希土類増感紙，オルソタイプフィルムなどの使用については，メーカーの定期的な管理が必要であり，画質の悪いフィルムはもちろんのことながら読影に値しない．

現在発売されている撮影機器の主力であるデジタル撮影に関しては，アナログ撮影よりもやや低線量でもよい画質のものが撮影できるとされている(表2)．電子カルテの普及に伴い，画像がモニタで読影できるようになってきたが，通常のPC用モニタのような輝度の低いカラー液晶モニタは，胸部X線の読影用には適していない．単純X線の読影はCTやMRIとは異なり，画質の設定がシビアであることから，少なくとも2メガピクセル以上の医療用の高精細モニタを用いるべきである．

早期発見するために，指摘すべき陰影は？●

肺癌の場合，早期癌は明確には定義されていないが，厚生省(当時)がん研究助成金池田班の定義によると腫瘍径2 cm以下でリンパ節転移のないものとされている．胸部X線上で2 cm以下とは，おおむね肋骨1本分の幅であり，この程度の小さい陰影を標的とすべきである．また陰影の性状であるが，予後のよい高分化腺癌は細胞密度が低いことから淡い陰影を呈するものである．読影経験

●肺癌の早期発見には，胸部単純X線検査が現在推奨されている．
●診断線量の胸部CTは被曝線量のリスクが高く，推奨されていない．低線量CTが注目されている．
●胸部単純X線のデジタル撮影の読影には，専用の高輝度モニタが必要である．
●市町村が実施する住民検診の場合，二重読影・比較読影が義務づけられている．

の少ない医師は，周辺との濃度差がはっきりした濃い陰影を拾い上げる傾向があるが，肺癌の早期発見を目的とするのであれば，ごくわずかな濃淡の変化に着目し過去のフィルムと比較する，あるいは精密検査を勧めることが重要である．

一般的に単純X線検査での要精検率は2～4%となっている．10%以上となった場合は，判定の基準に問題があるといわざるを得ない．

精密検査はどうしたらよいのか？●

胸部単純X線で指摘された陰影が，どんな原因疾患によるものなのか？ を突き詰める方法としては，胸部CTが必須である．確かに肺炎が疑われるような陰影の経過観察の場合は，単純X線検査の再検査で済まされる場合が多いが，単純X線検査での質的診断は専門医にとっても困難な場合が多い．X線検査で指摘した陰影とは別の部位に腫瘍が見つかる場合もあるので，CTの撮影はできる限り行った方がよい．CTの所見をもって悪性が疑われる場合は，自施設に呼吸器専門医がいない場合は，専門医療機関に直ちに紹介すべきである．肺癌の場合経過観察をしてよいかどうかの判断は困難で，リスクを回避する意味でも専門医への紹介が望ましい．

市町村検診を受託した場合は●

市町村が実施主体である健康増進法に基づく癌検診の委託を受けた場合は，医療としての実施とは異なり，国が定めた指針[3]に添うものでないといけない．これは日本肺癌学会肺癌集団検診の手引き[2]と基本的に同じものであり，二重読影と比較読影が必須となっている．二重読影は2人の読影医が別個に読影することにより見落としを防ぐという意味合いと，見落とした場合の個人の法的リスクを分散するという意味合いがある．市町村からの委託事業であっても読影医が1人の場合は，その読影医師に直接法的責任が問われるものであり，是非とも二重読影・比較読影システムを構築した上で受託していただきたい．また市町村検診の場合は，判定DとEが要精検となるが，肺癌が少しでも疑われる場合は，必ずE判定とし，専門医療機関にご紹介いただきたい．肺癌以外の疾患(結核，肺炎，気胸，心大血管異常)を疑いDと判定され，精査の結果，もし肺癌が見つかっても，発見肺癌には含めない取り決めとなっているのでご注意をいただきたい．

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First-Line Gefitinib in Patients Aged 75 or Older With Advanced Non–Small Cell Lung Cancer Harboring Epidermal Growth Factor Receptor Mutations

NEJ 003 Study

Makoto Maemondo, PhD,† Yuji Minegishi, PhD,‡ Akira Inoue, PhD,‡ Kunihiko Kobayashi, PhD,‖ Masao Harada, PhD,¶ Shoji Okinaga, PhD,# Naoto Morikawa, MD,** Satoshi Oizumi, PhD,†† Tomoaki Tanaka, PhD,‡‡ Hiroshi Isobe, PhD,§§ Shoji Kudoh, PhD,‖‖ Koichi Hagiwara, PhD,‡‡ Toshihiro Nukiwa, PhD,†‡ and Akihiko Gemma, PhD ‡*

Introduction: Recent studies have demonstrated that first-line treatment with gefitinib, an epidermal growth factor receptor (EFGR)–targeted tyrosine kinase inhibitor, is significantly superior to standard chemotherapy for advanced non–small-cell lung cancer (NSCLC) harboring EGFR sensitive mutations. Meanwhile, the efficacy of gefitinib therapy among elderly populations diagnosed with EGFR-mutated NSCLC has not yet been elucidated. The purpose of this study was to investigate the efficacy and feasibility of gefitinib for chemotherapy-naive patients aged 75 or older with NSCLC harboring EGFR mutations; generally, these patients have no indication for treatment with platinum doublets.

Methods: Chemotherapy-naive patients aged 75 years or older with performance status 0 to 1 and advanced NSCLC harboring EGFR mutations, as determined by the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method, were enrolled. The enrolled patients received 250 mg/day of gefitinib orally.

Results: Between January 2008 and May 2009, 31 patients were enrolled, all of whom were eligible. The median age was 80 (range, 75–87) years. Twenty-five patients (81%) were women, and 30 patients (97%) had adenocarcinoma. The overall response rate was 74% (95% confidence interval, 58%–91%), and the disease control rate was 90%. The median progression-free survival was 12.3 months. The common adverse events were rash, diarrhea, and liver dysfunction. One treatment-related death because of interstitial lung disease occurred.

Conclusions: This is the first study that verified safety and efficacy of first-line treatment with gefitinib in elderly patients having advanced NSCLC with EGFR mutation. Considering its strong antitumor activity and mild toxicity, first-line gefitinib may be preferable to standard chemotherapy for this population.

Key Words: Non–small cell lung cancer, Epidermal growth factor receptor mutation, Gefitinib

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For the North-East Japan Study Group. *Department of Respiratory Medicine, Miyagi Cancer Center, Miyagi, Japan; †Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; ‡Department of Internal Medicine, Division of Pulmonary Medicine, Infections Disease and Oncology, Nippon Medical School, Tokyo, Japan; §Department of Respiratory Medicine, Tohoku University Hospital, Sendai, Japan; ‖Department of Respiratory Medicine, Saitama Medical University International Medical Center, Saitama, Japan; ¶Department of Respiratory Medicine, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan; #Department of Geriatrics and Gerontology, Tohoku University Hospital, Sendai, Japan; **Department of Respiratory Medicine, Tohoku Employees' Pension Welfare Hospital, Miyagi, Japan; ††First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan; ‡‡Department of Respiratory Medicine, Saitama Medical University, Saitama, Japan; §§Department of Medical Oncology, KKR Sapporo Medical Center, Sapporo, Japan; and ‖‖Department of Respiratory Medicine, Fukujuji Hospital, Tokyo, Japan.

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Non–small-cell lung cancer (NSCLC), which accounts for 80% of lung cancer, remains the major cause of cancer-related death in both Western and Asian countries. With prolongation of life expectancy, both the incidence and mortality of lung cancer in the elderly are rising. In Japan, 48 500 individuals aged 70 years or older were estimated to die of lung cancer in 2009[1]; moreover, the ratio of elderly patients dying from lung cancer increased from 57% in 1989 to 72% in 2009. Treatment strategy in elderly patients with lung cancer has, thus, become an important issue.

About half of the newly diagnosed NSCLC patients have advanced disease, with no indication for local therapy such as surgery and radiotherapy. Chemotherapy for the elderly shows similar efficacy to that observed in younger

patients. However, it is generally more toxic, in terms of both incidence and severity, because of age-related weakening of organ function.[2] Consequently, standard chemotherapy for elderly NSCLC patients, especially those aged 75 years or older, is performed as monotherapy with vinorelbine, gemcitabine, or docetaxel instead of platinum doublets, which are the standard for younger patients.[3–7] Although a recent phase III study suggested that the platinum doublet of monthly carboplatin and weekly paclitaxel may be superior to the gemcitabine or vinorelbine monotherapy in the elderly population, the treatment-related death rate of the doublet group was determined to be 7%.[8] Thus, investigation into safer and more effective treatments for elderly NSCLC patients is required.

Gefitinib, an orally administered tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR), is a key molecularly targeted drug used for the treatment of advanced NSCLC. In May 2004, seminal studies showed that the presence of somatic mutations in the kinase domain of EGFR strongly correlated with increased responsiveness to EGFR TKIs in patients with NSCLC.[9,10] Before this observation, it had been known that subgroups of NSCLC patients, including those of Asian race, female sex, nonsmoking status, and having adenocarcinoma, displayed significant responses to gefitinib.[11,12] These subgroups turned out to have a high incidence of EGFR mutations.[13] Recently, two phase III studies comparing gefitinib treatment with chemotherapy in chemo-naive patients selected on the basis of EGFR mutations were reported from Japan.[14,15] These studies revealed the superiority of gefitinib treatment over standard chemotherapy by demonstrating that first-line gefitinib administration doubled progression-free survival (PFS) as compared with standard chemotherapy. One of two studies we conducted, namely the NEJ002 study, demonstrated that treatment with gefitinib provided patients with a better quality of life as compared with chemotherapy.[16] The eligibility criteria in these studies was limited to patients aged 75 years or younger, as the treatments with platinum doublets were considered to be inappropriate for more elderly populations because of increased toxicity. Moreover, it has been reported in Japan that this more elderly group of patients develop interstitial lung disease (ILD) frequently when treated with gefitinib.[17] In previous studies, we demonstrated that patient selection by EGFR mutation can dramatically improve the risk–benefit balance of gefitinib treatment; however, no study thus far has investigated the efficacy and feasibility of first-line gefitinib treatment in elderly NSCLC patients with EGFR mutation. Thus, the current phase II study was conducted.

METHODS

Patient Selection

This multicentric phase II study was approved by the institutional review board of each participating institute. The main eligibility criterion was to select chemotherapy-naive patients with NSCLC harboring sensitive EGFR mutations. Namely, patients with exon 19 deletions, L858R, L861Q, G719A, or G719S were included, but those with a resistant T790M mutation were excluded. Patients who were 75 years of age or older with Eastern Cooperative Oncology Group performance status (PS) 0 to 2 were also deemed eligible. Other eligibility requirements were stage IIIB to IV or postoperative recurrent NSCLC, presence of a measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST), adequate organ function including liver function (aspartate transaminase and alanine aminotransferase ≤100 U/liter, total bilirubin <2.0 mg/dL), and written informed consent.

EGFR Mutation

Cytological or histological specimens were examined for EGFR mutation by the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method.[18] Briefly, genomic DNA fragments containing mutation hot spots of the EGFR gene were amplified via PCR in the presence of a peptide nucleic acid clamp primer synthesized from a peptide nucleic acid with a wild-type sequence. This method leads to preferential amplification of the mutant sequence, which is then detected by a fluorescent primer that incorporates locked nucleic acids to increase the specificity. As a result, the mutant EGFR sequence is detected in specimens that contain 100 to 1000 excess copies of wild-type EGFR sequence. The sensitivity and specificity of the peptide nucleic acid-locked nucleic acid PCR clamp method are 97% and 100%, respectively.

Drug Administration

Gefitinib was administered orally once a day at a dose of 250 mg. Patients continued to receive gefitinib until progression of disease, occurrence of intolerable severe toxicity, or withdrawal of consent. When severe toxicity was observed, patients were allowed to receive a reduced dose of gefitinib in accordance with the protocol.

Treatment Assessment

Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were determined based on RECIST version 1.0. The primary end point of this study was overall objective response rate (ORR), which was the rate of patients with CR + PR; secondary end points were PFS, overall survival (OS), and toxicities. Computer

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Address for correspondence: Yuji Minegishi, MD, PhD, Department of Internal Medicine, Division of Pulmonary Medicine, Infectious Disease and Oncology, Nippon Medical School, 1-1-5 Sendagi Bunkyo-Ku, Tokyo, 113–8602 Japan. E-mail: yminegishi@nms.ac.jp

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tomography (CT) scans were taken every month until CR or PR was observed. CR and PR required confirmation via reassessment no earlier than 4 weeks after the first assessment meeting the criteria for response. After the confirmation, CT scans were taken every other month until PD was observed. The CT films of all patients were extramurally reviewed for confirmation of response. PFS was defined as the time from the date of randomization to the first observation of disease progression or death. OS was defined as the time from the date of randomization to the date of death or the most recent follow-up. Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0.

Statistical Consideration

Sample size was determined using the data as follows. Response rates greater than 70% had been previously observed in nonage-restricted patients with EGFR-mutated NSCLC.[15] Meanwhile, clinical studies with elderly patients that investigated the efficacy of first-line chemotherapies in Japan showed ORR of 28% to 55%.[7,19] Thus, we assumed that an ORR of more than 55% was clinically useful, whereas an ORR of less than 30% was not clinically useful. With $\alpha = 0.05$ and $\beta = 0.1$, the number of patients required was 27. Allowing 10% loss in follow-up, a total of 30 patients were planned for enrollment.

All enrolled patients were evaluated for efficacy of received regimen. All patients treated with gefitinib, even for a short period of time, were entered into safety analysis.

RESULTS

Patient Characteristics

Between January 2008 and May 2009, a total of 31 patients were enrolled. Baseline characteristics are described in Table 1. The median age at the time of enrollment was 80.3 years (range, 75–89 years); 52% of the patients were over the age of 80. Of the 31 patients enrolled, 25 (81%) were women and 2 (6%) had a PS of 2. Histological types were all adenocarcinoma except for one adenosquamous carcinoma. There were 7 patients (23%) with stage IIIB, 22 (71%) with stage IV, and 2 (6%) with postoperative recurrence.

Efficacy

The ORR was 74.2% (95% confidence interval [CI], 57.9%–90.5%); one patient had CR, and 22 patients had PR. Five of the remaining 8 patients (16.1%) had SD, with the resulting disease control rate (CR + PR + SD) reaching 90.3% (Table 2). This result attained the primary end point by a wide margin. The median follow-up period at the time of analysis was 27.5 months. Of all 31 patients enrolled, 15 (48.3%) were alive and free from progression for at least 6 months. The median PFS was 12.1 months (Fig. 1*A*), the 1-year OS was 83.9% (95% CI, 70.2%–97.6%), and 2-year OS was 58.1% (95% CI, 45.2%–70.9%). At the data cutoff point (December 2010), 13 patients (41.9%) had died, and the median OS was 33.8 months (Fig. 1*B*).

TABLE 1. Character

	N = 31	(%)
Sex		
Women	6	19
Men	25	81
Age		
Mean (SD)	80.3	(4.1)
Range	75–89	
Smoking status		
Nonsmoker	23	74
Smoker	8	26
Performance status		
0	16	55
1	13	39
2	2	6
Stage		
IIIB	7	23
IV	22	71
Postop	2	6
Histology		
Adenocarcinoma	30	97
Adenosquamous	1	3

Safety and Toxicity

Toxicity data for all 31 patients are presented in Table 3. Nine patients (29%) had a grade 3 adverse event (AE); 1 had a grade 5 AE ILD, and died of respiratory failure. The most common hematologic AE was elevation of transaminases; grade 3 to 4 elevation occurred in three patients (19%). The most common nonhematologic AEs were rash in 21 patients (71%), diarrhea in 10 patients (32%), and appetite loss in 9 patients (29%). Dose reduction was seen in 14 patients (45%). Incidence and severity of AEs were acceptable and comparable with previous reports.[13–15]

Treatment After Progression of Disease

Patient management after the protocol treatment was retrospectively investigated. Any treatment was allowed after confirmation of PD. Gefitinib was continued in 10 of 20 patients confirmed to have PD. Three patients were treated with monotherapies of cytotoxic agents, including vinorelbine, gemcitabine, or docetaxel, and one patient was given

TABLE 2. Response Rate of Treatment With Gefitinib

Response	N = 31	(%)
CR	**1**	**3**
PR	**22**	**71**
Stable disease	**5**	**16**
Progressive disease	**3**	**10**
Overall response rate (CR + PR)	**23**	**74**
95% confidence interval		(57.9–90.5)

CR, complete response; PR, partial response.

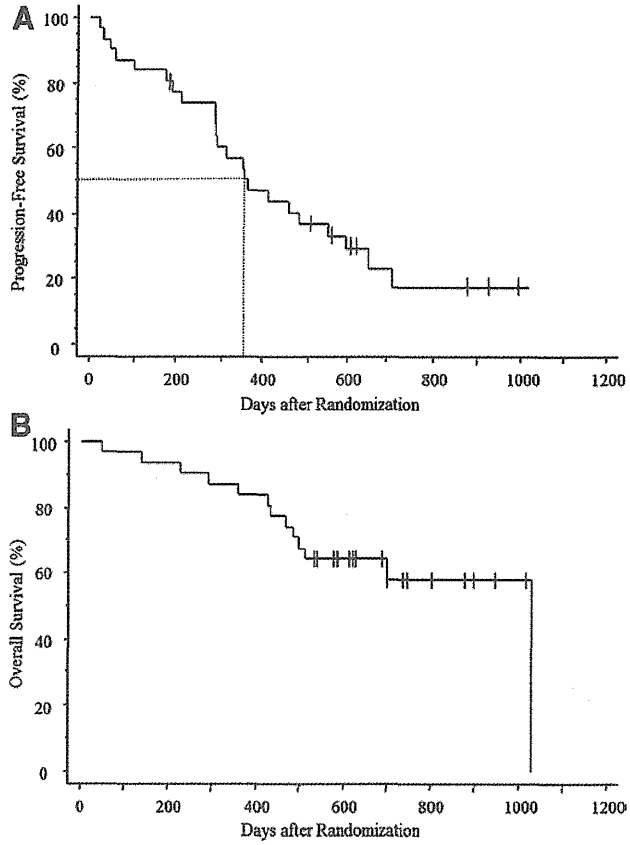

FIGURE 1. Progression-free survival and overall survival. Kaplan–Meier curves for progression-free survival are shown for the progression-free survival population (*A*), and Kaplan–Meier curves for overall survival are shown in (*B*). In (*A*) and (*B*), tick marks indicate patients for whom data were censored.

TABLE 3. Safety—Hematologic and Nonhematologic Toxicity

	NCI-CTC Grade					Grade
	1	2	3	4	5	3–4 (%)
Hematologic adverse events						
Leukocytopenia	2	1	0	0	0	0
Neutropenia	0	1	0	0	0	0
Anemia	6	4	0	0	0	0
Thrombocytopenia	2	1	0	0	0	0
AST/ALT	7	2	6	0	0	19
T-Bil	3	1	0	0	0	0
Creatinine	5	1	0	0	0	0
Hyperkalemia	7	0	0	0	0	0
Nonhematologic adverse events						
Pneumonitis	0	0	0	0	1[a]	3
Rash	12	10	1	0		3
Nail change	4	2	0	0		0
Stomatitis	3	0	0	0		0
Alopecia	3	0	0	0		0
Appetite loss	7	2	1	0		3
Nausea/vomiting	1	0	0	0		0
Diarrhea	9	2	1	0		3
Constipation	2	0	0	0		0
Fatigue	4	1	0	0		0

NCI-CTC, National Cancer Institute Common. Terminology Criteria; AST, androgen suppression therapy; ALT, alanine aminotransferase; T-Bil, total bilirubin.
[a]Treatmant-related death.

erlotinib. No patient was treated with platinum doublets. Six patients did not receive any second-line treatment.

DISCUSSION

This is the first study targeting elderly patients with EGFR-mutated NSCLC. In this study, gefitinib displayed remarkable efficacy without increased toxicity.

We have previously reported a single-arm phase II study in which gefitinib was administered to frail patients with poor PS or elderly patients who were unfit to undergo treatment with cytotoxic agents.[20] In that study, the patients enrolled were 20 to 74 years old with a PS of 3 to 4, 75 to 79 years old with a PS of 2 to 4, and aged 80 years or older (super-elderly) with a PS of 1 to 4. Patients older than 74 years of age accounted for 39% of the total enrolled patients but, nevertheless, OS was 17.8 months (Table 4). The current study strengthened the conclusion of the previous one and provided more information with respect to the efficacy of gefitinib in elderly NSCLC patients with EGFR mutation.

We defined elderly patients as those who were 75 years old and older. Many studies and subgroup analyses were performed by considering elderly cases as 70 years of age or older, especially in Western countries. We have regarded patients aged 70 to 75 years as being treatable with platinum-based chemotherapy. In fact, patients in this age group were enrolled in the NEJ002 study and were able to withstand treatment with platinum doublet. Accordingly, we excluded this group of patients from enrollment in the present study. Considering the aging of population structures and the increased longevity in Japan, we thought that the candidate selection for this study was reasonable.

Currently, in elderly patients, single-agent chemotherapy with a third generation agent (vinorelbine, gemcitabine, or taxanes) is the recommended approach according to the American Society of Clinical Oncology guidelines.[2–7] Gefitinib, which is considered minimally toxic, is often selected for the treatment of advanced NSCLC in elderly patients. Crino et al. performed a randomized phase II study (Gefitinib Versus Vinorelbine in Chemotherapy-Naïve Elderly Patients With Advanced Non-Small-Cell Lung Cancer [INVITE]) of gefitinib versus vinorelbine treatment in 196 chemotherapy-naive unselected elderly patients.[21] There were no statistical differences between gefitinib and vinorelbine in terms of PFS, OS, and ORR. Their study showed obviously lower efficacy of gefitinib in nonselected patients, as compared with the results shown from our study of EGFR-mutated patients.[22–24] These differences in effectiveness among studies highlight the importance of selection of patients by EGFR mutation analysis when administrating gefitinib. Furthermore, in another study of gefitinib treatment in Japanese patients aged

TABLE 4. Pivotal Clinical Trials of Cytotoxic Agents or EGFR-TKIs in Elder Patients With NSCLC and Recent Trials of Gefitinib in Patients selected by EGFR Mutation

Trial	Treatment	*n*	ORR (%)	PFS (mo)	MST (mo)	*p* Value
Cytotoxic agent in unselected elder patients						
ELVIS[3]	VNR	76	19.7		6.4	0.04
	BSC	78	—		4.8	
MILES[5]	VNR + GEM	232	21	4.1	6.9	NS
	GEM	233	16	4.4	6.5	
	VNR	233	18	4.4	8.3	
WJTOG9904[7]	DTX	89	22.7	5.5	14.3	$p = 0.138$
	VNR	91	9.9	3.1	9.9	
EGFR-TKI in unselected elder patients						
Ebi N.[25]	Gefitinib	49	25	4	10	—
Crino L.[21]	Gefitinib	97	3.1	2.7	5.9	NS
	VNR	99	5.1	2.9	8.0	
Jackman D. M.[27]	Erlotinib	80	10	3.5	10.9	—
Chen Y. M.[28]	Erlotinib	57	22.8	4.6	11.7	$p = 0.70$
	VNR	56	8.9	2.5	9.3	
EGFR-TKI in selected younger patients						
WJTOG3405[14]	Gefitinib	86	62.1	9.2	Immature	$p < 0.001$ (PFS)
	CDDP + DTX	86	32.2	6.3		
NEJ002[15]	Gefitinib	114	73.7	10.8	30.5	$p < 0.001$ (PFS)
	CBDCA + PTX	110	30.7	5.4	23.6	
EGFR-TKI in selected elder patients (current study)						
NEJ003	Gefitinib	31	74.2	12.1	33.8	—

ELVIS, Elderly Lung Cancer Vinorelbine Italian Study; MILES, Multicenter Italian Lung Cancer in the Elderly Study; ORR, overall response rate; PFS, progression-free survival; MST, median survival time; VNR, vinorelbine; BSC, best supportive care; NS, not significant; GEM, gemcitabine; DTX, docetaxel; EGFR-TKI, epidermal growth factor receptor–tyrosine kinase inhibitors; CDDP, cisplatin; CBDCA, carboplatin; PTX, paclitaxel.

75 or older, which included about 40% of the patients who were examined for EGFR mutations and 14% of the patients with EGFR-mutated tumors, the response rate was only 25%.[25] Meanwhile, there have been a few studies of treatment for elderly unselected patients with erlotinib, which is supposed to be more toxic than gefitinib as the administered dose was set near the maximum tolerance dose.[26,27] The response rates in these studies were 10% or less, which were similar to those from the gefitinib studies conducted in Western populations (Table 4). In the other Asian study, erlotinib was compared with vinorelbine treatment in patients aged 70 or older.[28] That study demonstrated that erlotinib yielded a higher response rate and PFS than vinorelbine. The percentage of mutation-positive patients was 30% of those who were examined for EGFR mutations in the erlotinib group. This high proportion might have contributed to the better results of the erlotinib group. The treatment of unselected NSCLC patients with erlotinib was also as ineffective as with gefitinib. Efficacy results in patients selected by EGFR mutation in the current study were substantially superior to those observed in the studies of gefitinib or erlotinib with unselected cases. Surprisingly, the median PFS and 2 year-survival rate here were comparable with results obtained in NEJ002 (12.3 versus 10.8 months, 58% versus 61%, respectively) despite the limited enrollment of an elderly population in this study. These two studies, namely NEJ002 and NEJ003, have very similar backgrounds as they were performed during almost the same time period at identical institutions. It was suggested that gefitinib displayed similar efficacy in elderly patients when compared with their younger counterparts (Table 4). Although the current phase II study could not verify whether gefitinib prolonged PFS in elderly patients in comparison with younger patients, gefitinib might still prove to be the most suitable agent for elderly patients with EGFR-mutated NSCLC.

Elderly patients generally have more comorbidities and lower organ function than younger patients. Treatment-related toxicity in the elderly is a more significant issue than for younger patients. A subgroup analysis of BR.21 showed that elderly patients treated with erlotinib displayed similar efficacy with respect to survival and quality of life as their younger counterparts but experienced greater toxicity.[27] In the current study, toxicity was generally mild and predictable. Rash, diarrhea, and elevation of transaminase were observed frequently, similar to other studies with EGFR-TKIs. The single case of treatment-related death that occurred in our study was because of ILD, although this condition was not found in other patients. The frequency of ILD in the current study was comparable with that previously reported in Japan. Unfortunately, this patient did not respond to treatment with a large dose of corticosteroid, which is generally used for such conditions.[17,29] Advanced age and smoking, preexisting ILD, and poor performance status have been reported as risk factors for ILD during treatment with gefitinib.[17] Elderly patients treated with EGFR-TKIs should be monitored with further caution for ILD. On the whole, gefitinib was found to be a well-tolerated therapy for elderly patients with mutated NSCLC.

In conclusion, first-line gefitinib treatment is highly effective with acceptable toxicity for elderly patients with advanced NSCLC harboring EGFR mutations. Together with our previous studies (NEJ001, NEJ002), gefitinib is shown to be an ideal therapy for all types of NSCLC patients with EGFR mutation.

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MiR-23a regulates TGF-β-induced epithelial-mesenchymal transition by targeting E-cadherin in lung cancer cells

MENGRU CAO[1,2], MASAHIRO SEIKE[1], CHIE SOENO[1], HIDEAKI MIZUTANI[1], KAZUHIRO KITAMURA[1], YUJI MINEGISHI[1], RINTARO NORO[1], AKINOBU YOSHIMURA[1], LI CAI[2] and AKIHIKO GEMMA[1]

[1]Department of Internal Medicine, Division of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, Tokyo 113-8603, Japan; [2]Department of Medical Oncology, The Third Affiliated Hospital of Harbin Medical University, Harbin, P.R. China

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Abstract. Transforming growth factor-β (TGF-β)-induced epithelial-mesenchymal transition (EMT) has been shown to be related to the pathogenesis of various diseases including lung cancer. Recently, microRNAs (miRNA) have been recognized as a new class of genes involved in human tumorigenesis. MiR-23a/24/27a is a miRNA cluster located in chromosome 19p13.12, which can function as an oncogene in several human cancers. In this study, we analyzed miR-23a/24/27a expression in 10 non-small cell cancer (NSCLC) cell lines by real-time PCR analysis. Correlation between expression of these miRNAs and TGF-β/Smad signaling was evaluated. We found that miR-23a could be regulated by TGF-β1 in a Smad-dependent manner in A549 lung adenocarcinoma cells showing the EMT phenomenon. Knockdown of miR-23a partially restored E-cadherin expression under conditions of TGF-β1 stimulation. In contrast, overexpression of miR-23a could suppress E-cadherin expression and stimulate EMT. Furthermore, A549 cells with overexpressed miR-23a were more resistant to gefitinib compared to the parental cells. These findings suggest that miR-23a regulates TGF-β-induced EMT by targeting E-cadherin in lung cancer cells and may be useful as a new therapeutic target in NSCLC.

Introduction

Lung cancer continues to be a leading cause of cancer death both in Japan and worldwide (1) and, despite recent improvements in chemotherapies and molecular-targeted therapies, the prognosis remains poor (2-5). Patient selection based on a specific biomarker is one strategy that could lead to improved lung cancer treatments. Although some biomarkers predictive of metastasis, prognosis and drug sensitivity have already been reported in lung cancer, more sensitive and specific biomarkers could facilitate the development of novel therapeutic applications (6-8).

Epithelial-mesenchymal transition (EMT) comprises a complex series of reversible events that can lead to the loss of epithelial cell adhesion and the induction of a mesenchymal phenotype (9). Thus, EMT is characterized by the loss of epithelial differentiation markers including E-cadherin and the induction of mesenchymal markers such as vimentin and fibronectin. EMT can be induced by transforming growth factor-β1 (TGF-β1) (10). The Smad pathway is a major transducer of TGF-β signaling (11). Smad2 and Smad3 are phosphorylated by the TGF-β type I receptor and form complexes with Smad4 (11). These complexes accumulate in the nucleus of the cell, regulating the transcription of target genes and playing critical roles in the control of cell proliferation, differentiation, apoptosis and cell migration. In response to TGF-β, the TGF-β receptors also activate alternative signaling effectors, such as mitogen-activated protein kinase, phosphatidylinositol-3 kinase, and Rho-like GTPases (11). It has been recognized that EMT plays a pivotal role in several diverse processes during embryonic development, chronic inflammation and fibrosis (12). Recently, several studies demonstrated that EMT was correlated with carcinogenesis, metastasis and poor prognosis in various human cancers, including those of the lung (13-16). Furthermore, EMT has been reported to be related to reduced sensitivity and acquired resistance to epidermal growth factor tyrosine kinase inhibitors (EGFR-TKI) in lung cancer cells (17-19). Taken together, these findings demonstrate that the suppression of EMT could be used as a potential target for treatment of lung cancer.

MicroRNA (miRNAs) are a class of short single-stranded noncoding endogenous RNAs, approximately 18-24 nucleotides in length, which post-transcriptionally modulate gene expression by either inhibiting translation or inducing mRNA degradation (20). MiRNAs have been recognized as a new class of genes involved in human tumorigenesis (21,22) and recently they have been shown to be diagnostic, prognostic and therapeutic biomarkers in lung cancer (22-25). For example, high miR-155 expression and low let-7a expression, as independent risk

Correspondence to: Dr Masahiro Seike, Department of Internal Medicine, Division of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan
E-mail: mseike@nms.ac.jp

Key words: microRNA, epithelial-mesenchymal transition, transforming growth factor-β, Smad, lung cancer

factors, have a negative prognostic impact on outcome in lung adenocarcinoma patients (23). The miR-17-92 cluster functions as an oncogene, and has been shown to promote lung cancer carcinogenesis (24). We previously reported that the inhibition of miR-21, whose upregulation is associated with EGFR mutations, can be a therapeutic strategy, either as a monotherapy or in combination with EGFR-TKI treatment (25). These findings suggest that miRNA can serve as a novel therapeutic target as well as diagnostic and prognostic marker in lung cancer.

A recent study reported that a specific cluster of miRNA, miR-23a/24/27a, was induced by TGF-β in a Smad-dependent manner in hepatocellular carcinoma (HCC) cells (26). Upregulation of these miRNAs were able to suppress TGF-β-induced growth suppressive activities in HCC cells. In this present study, we analyzed miR-23a/24/27a expression in non-small cell cancer (NSCLC) cells and evaluated the correlation between its expression and TGF-β/Smad signaling. We found that miR-23a could be induced by TGF-β in a Smad-dependent manner in A549 cells. In addition, overexpression of mature miR-23a reduced E-cadherin expression and stimulate the EMT phenomenon which is involved in tumorigenesis. Furthermore, in these A549 cells, inhibition of miR-23a could also partially suppress TGF-β-induced EMT, while overexpression was associated with the tendency for EGFR-TKI resistance. We have demonstrated that, in lung cancer cells, miR-23a is regulated by the TGF-β/Smad pathway and plays a critical role in EMT through the targeting of E-cadherin.

Materials and methods

Cell culture. We used 10 NSCLC cell lines: A549, PC9, PC14, LC2/ad, RERF-LC-KJ, RERF-LC-OK adenocarcinoma (AC) cell lines and PC1, PC10, LK2 and SQ5 squamous cell carcinoma cell lines (SCC) for this study. An immortalized tracheal cell line (BET2A) was used as a normal control cells. A549 and BET2A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); RERF-LC-KJ, RERF-LC-OK, LC2-ad, LC2/Ad and SQ5 were obtained from the RIKEN Cell Bank (Ibaraki, Japan); and PC1, PC9, PC10 and PC14 were obtained from Immuno-Biological laboratories (Gunma, Japan). NSCLC cell lines were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). BET2A was maintained in RPMI-1640 medium with 5% FBS.

RNA extraction and real-time quantitative reverse transcription-PCR. Total RNA was extracted from the BET2A and NSCLC cell lines with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The miR-23a, miR-24 and miR-27a expression levels were quantified by quantitative reverse transcription-PCR (qRT-PCR) using TaqMan® MicroRNA Assay System (Applied Biosystems, Foster City, CA). RNU66 (PN 4373382) was used as an internal control (Applied Biosystems). MiRNA expression was quantified and reported as $2^{-\Delta\Delta Ct}$ value (27).

Antibodies and western blot analysis. Cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 0.5% sodium-deoxycholate. The lysates were kept on ice for 30 min, and then centrifuged at 13000 x g for 30 min. The supernatant was collected and then 10 μg of each of the proteins was separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membrane. After being blocked in 5% skimmed milk, the membrane was incubated with Smad2/3, β-actin (Cell Signaling Technology, Beverley, MA, USA), E-cadherin, N-cadherin and vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Proteins were detected by immunoblotting using ECL-Plus reagents (GE Healthcare Bio-Science Corp, Piscataway, NJ, USA).

Oligonucleotide transfection. TGF-β1 was purchased from R&D system (Minneapolis, MN, USA). Cells were treated with 5 ng/ml TGF-β1 for the indicated time. Small interference RNA (siRNA) targeting Smad2/3 was purchased from Dharmacon Research Inc. (Lafayette, CO, USA) and the homologous negative control was obtained from Invitrogen. MiR-23a inhibitor, its negative control, miR-23a precursor (Pre-miR-23a) and its cognate negative control (Pre-miR-ctl) were synthesized by Ambion (Ambion). Pre-miR-23a and miR-23a inhibitor were transfected using Lipofectamine™ 2000 reagent 24 h after seeding, as per the manufacturer's instructions (Invitrogen). Transfections of precursor and inhibitor complexes were added to cells at a final concentration of 40 nM. Six hours after the transfection was performed, the transfection medium was replaced, and after 24 h, 5 ng/ml TGF-β1 was added to the medium which was then incubated at 37°C for 48 h.

Growth inhibition assay. Gefitinib was purchased from Seleck Chemicals (Houston, TX, USA). A549 cells (5,000 cells/well) were seeded into 96-well plates for 24 h. After being treated with Pre-miR-ctl or Pre-miR-23a, at a final concentration of 40 nM for 24 h, the cells were incubated in the various concentrations of gefitinib for 72 h at 37°C. Then, MTS was added to each well and the cells were incubated for a further 2 h at 37°C, after which absorbance was measured using a microplate reader with a test wavelength of 450 nm. The IC_{50} value was defined as the concentration needed for 50% reduction of the growth by treatment with gefitinib.

Results

MiR-23a, miR-24 and miR-27a expression in lung cancer cells. We first investigated miR-23a, miR-24 and miR-27a expression levels in NSCLC cell lines, including 6 AC cell lines and 4 SCC cell lines. Expression levels of these mature miRNAs were examined by qRT-PCR (Fig. 1). Although these miRNAs belonged to the same miRNA cluster, their expression levels varied among the 10 cell lines.

MiR-23a expression is directly induced by TGF-β1 in a Smad-dependent manner. A recent study reported that miR-23a/24/27a was induced by TGF-β in a Smad-dependent manner in HCC cells (26). Smad pathway is known as a major transducer of TGF-β signaling (11). We evaluated the correlation between miR-23a, miR-24 and miR-27a expression levels and Smad expression. High expression of miR-23a was observed in those NSCLC cells which also overexpressed Smad2/3. Thus, 5 cell lines (A549, LC-2/ad, ABC1, PC1 and SQ5) showed high expression of miR-23a and Smad2/3 (Fig. 2A), while low expression of miR-23a and Smad2/3 was found in the other 5 cell lines (PC9,

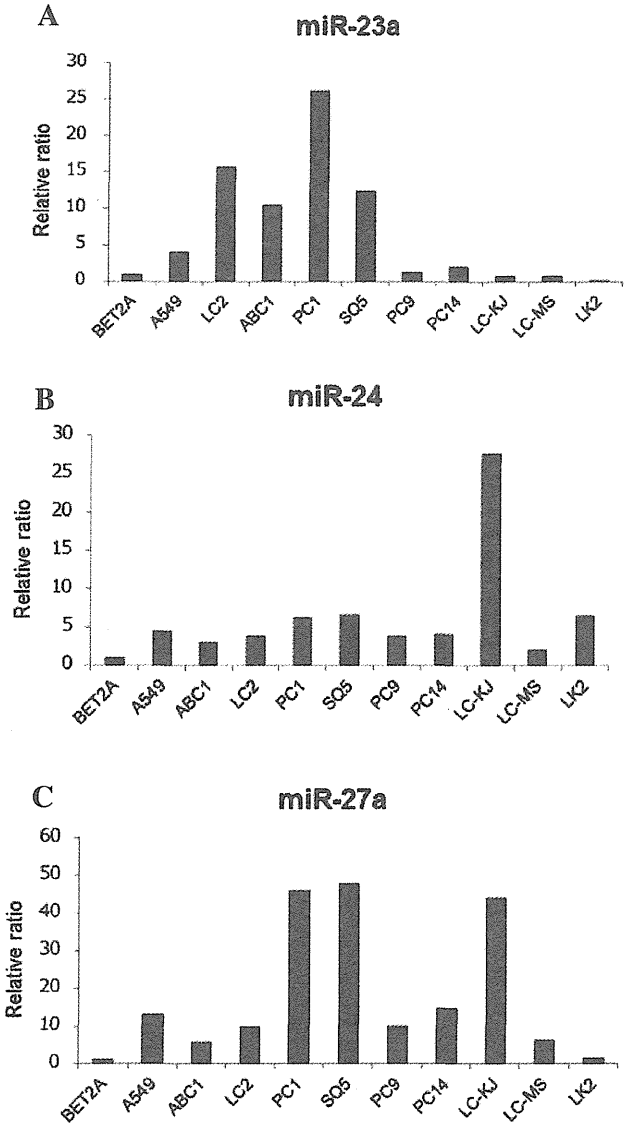

Figure 1. MiR-23a/24/27a expression in NSCLC cells. Expression levels of miR-23a/24/27a were analyzed in 10 NSCLC cells and BET2A cells by TaqMan® MicroRNA assay. MiRNA expression was quantified as $2^{-\Delta\Delta Ct}$ value. Relative expressions of miR-23a/24/27 against BET2A cells are shown.

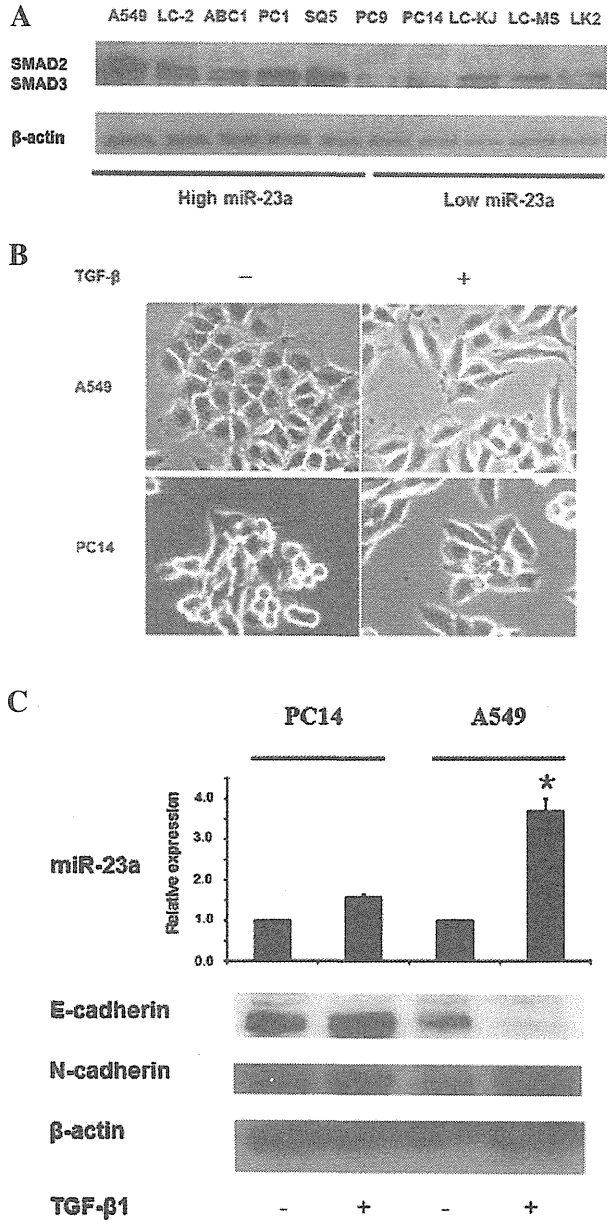

Figure 2. MiR-23a is directly induced by TGF-β1 in a SMAD-dependent manner and involved in EMT of lung cancer cells. (A) Five cell lines (A549, LC-2, ABC1, PC1 and SQ5) showed high expression of both miR-23a and Smad2/3. In contrast, low expression of miR-23a and Smad2/3 were found in the other five cell lines (PC9, PC14, LC-KJ, LC-MS and LK2). (B) A549 and PC14 cells were treated with 5 ng/ml of TGF-β1 for 48 h. The cells were observed under a light microscope. A549 cells exhibited a classic epithelial morphology. In contrast, A549 cells after the TGF-β1 exposure appeared to be less uniformly epithelial. (C) MiR-23a and protein expression of EMT markers in A549 and PC14 cells after TGF-β1 stimulation. Compared with PC14 cells, high expression of miR-23a, lost E-cadherin expression and enhanced N-cadherin expression were observed in A549 cells after TGF-β1 stimulation.

PC14, LC-KJ, LC-MS and LK2) (Fig. 2A). In contrast, no correlation was observed between miR-24 or miR-27a and Smad2/3.

It is well known that TGF-β1 stimulates the EMT of A549 lung cancer cells (10). In this study, A549 cells which overexpressed miR-23a and Smad2/3 was treated with 5 ng/ml of TGF-β1 for 48 h. We observed that, while the parent A549 cells exhibited a classic epithelial morphology (Fig. 2B), after TGF-β1 exposure they had a less uniform epithelial appearance (Fig. 2B). In contrast, PC14 cells with low expression of miR-23a and Smad2/3 retained their epithelial morphology after TGF-β1 treatment (Fig. 2B). Using western blot analysis, we evaluated the expression levels of EMT markers in A549 and PC14 cells treated with TGF-β1 in order to confirm the occurrence of EMT. A549 cells treated with TGF-β1 displayed reduced E-cadherin expression and increased N-cadherin expression when compared with A549 cells without TGF-β1 stimulation (Fig. 2C). We also examined whether TGF-β1 stimulated miR-23a expression in these two cells. MiR-23a expression level was significantly higher in A549 cells, which had shown the EMT phenomenon after the treatment of the respective parent cells with TGF-β1 (Fig. 2C). In contrast, miR-23a expression level in PC14 cells, which had not shown

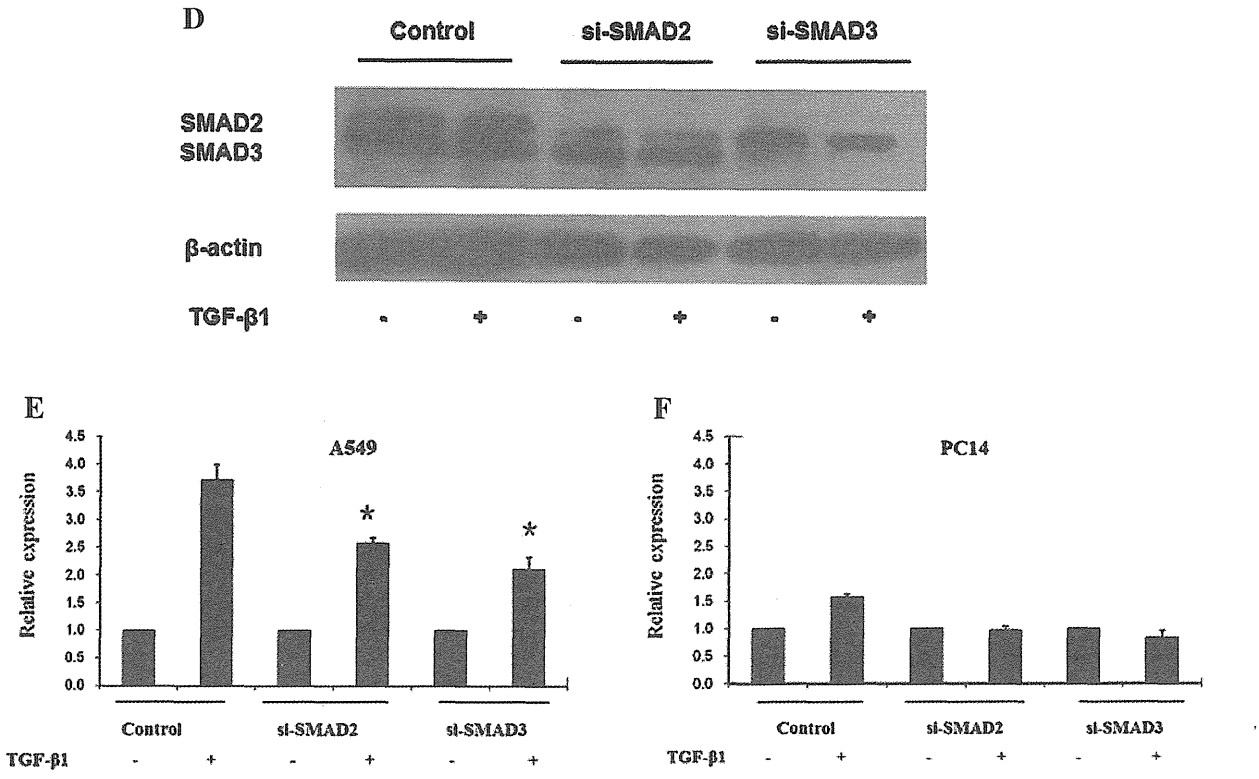

Figure 2. Continued. (D) Protein expression of Smad2/3 after treatment of siRNA in A549 cells. A549 cells were treated with control siRNA or siRNA of Smad2/3 with or without TGF-β1 stimulation. Smad2 or Smad3 specific siRNA completely diminished Smad2 or Smad3 expression. (E) Expression of miR-23a after the transfection of Smad2/3 siRNA was evaluated. MiR-23a expression was significantly decreased after the treatment of Smad2 or Smad3 siRNAs with TGF-β1 stimulation in A549 cells. (F) MiR-23a expression was unchanged after the transfection of Smad2 or Smad3 siRNAs with TGF-β1 stimulation in PC14 cells. Data are mean ± SD from 3 independent experiments. $^{*}p<0.05$ when compared with the respective parent cells.

the EMT phenomenon, was unaffected by exposure to TGF-β1 (Fig. 2C).

Next, we examined whether the Smad signal pathway directly regulated miR-23a expression. A549 and PC14 cells were treated with siRNAs of control or Smad2/3 (Fig. 2D). Knockdown of Smad2/3 significantly decreased TGF-β1-induced miR-23a expression in A549 cells in which miR-23a was overexpressed (Fig. 2E). On the other hand, in PC14 cells with low miR-23a, miR23a expression was unaffected by treatment with Smad siRNAs (Fig. 2F). These results suggested that, in A549 lung cancer cells, miR-23a was directly regulated by TGF-β1/Smad pathway and contributed to the EMT phenomenon.

MiR-23a regulates TGF-β1-induced EMT by targeting E-cadherin. Since miR-23a was significantly upregulated in A549 cells after the treatment with TGF-β1 and mediated EMT, we proceeded to identify potential targets known to play a role in EMT by using the Target Scan database. Among the candidate miRNAs for the E-cadherin gene (CDH1), we found that the region of 3' UTR of the CDH1 gene may serve as a binding site for miR-23a based on the prediction of Target Scan database (Fig. 3A).

To examine whether the CDH1 was a target of miR-23a, we knocked down miR-23a in A549 cells by using a specific inhibitor. Control or specific miR-23a inhibitor was transfected into A549 cells for 24 h, which were then treated with or without TGF-β1 for a further 48 h. We confirmed that miR-23a was effectively knocked down by miR-23a inhibitor in A549 cells (Fig. 3B). Using western blot analysis, we evaluated the expression of EMT markers after the treatment of miR-23a inhibitor to confirm the occurrence of EMT. After exposure to TGF-β1, E-cadherin expression in A549 cells was greatly diminished, resulting in TGF-β1-induced EMT (Fig. 3C). Interestingly, E-cadherin was still expressed in A549 cells transfected with miR-23a inhibitor after TGF-β1 exposure (Fig. 3C). N-cadherin expression was also weak after miR-23a treatment followed by TGF-β1 exposure (Fig. 3C). These findings demonstrated that miR-23a inhibition partially suppressed TGF-β-induced EMT phenomenon in A549 cells.

We transiently transfected A549 cells with the miR-23a precursor (Pre-miR-23a), and the control precursor miR (Pre miR-ctl). Mature miR-23a was remarkably induced by the miR-23a precursor in A549 cells between 24 to 72 h (Fig. 3D). After the treatment of precursor miR-23a, decreased E-cadherin expression and increased levels of vimentin were observed in A549 cells at 48 h (Fig. 3E). Under a light microscope, overexpression of miR-23a enhanced the spindle integration, resulting in an additive effect with TGF-β1-induced EMT in A549 cells (Fig. 3F). These results suggested that miR-23a may affect EMT by targeting E-cadherin in lung cancer cells.

MiR-23a stimulated EMT and induced resistance to gefitinib. Finally, to evaluate whether EMT leads to resistance against

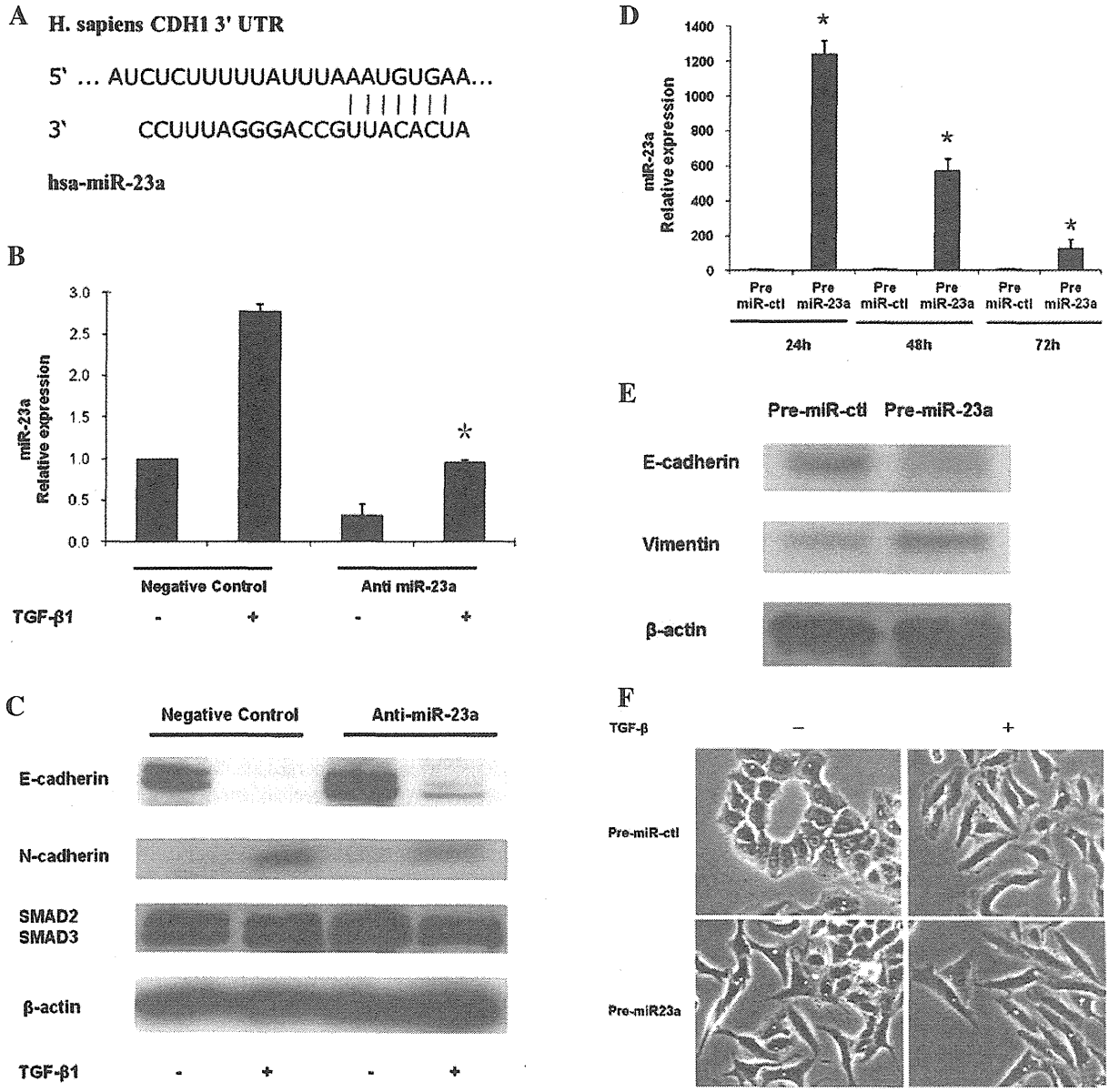

Figure 3. MiR-23a directly targets CDH1 and regulated TGF-β1-induced EMT. (A) Predicted duplex formation between human CDH1 3' UTR and miR-23a. (B) Control or specific miR-23a inhibitor was transfected into A549 cells for 24 h, which were then treated with or without TGF-β1 for a further 48 h. MiR-23a was significantly knocked down by miR-23a inhibitor in A549 cells. (C) EMT markers and SMAD2/3 expression were examined in A549 cells after the treatment with anti-miR-23a or control inhibitor. (D) Pre-miR-ctl or Pre-miR-23a was transfected into A549 cells for 72 h. The histograms show the relative expression of mature miR-23a in A549 cells after treatment with Pre-miR-23a or Pre-miR-ctl, by qRT-PCR analysis. (E) At 48 h, E-cadherin expression was reduced in A549 cells treated with Pre-miR-23a. In contrast, at 48 h, vimentin expression was increased in A549 cells transfected with Pre-miR-23a. (F) The cells were observed under a light microscope. A549 cells after the treatment of Pre-miR-23a appeared to have a spindle morphology.

the EGFR-TKI, gefitinib, we measured the response to gefitinib after the exposure of A549 cells to TGF-β1 or Pre-miR-23a. Consistent with previous studies, A549 cells treated with TGF-β1 were more resistant to gefitinib than were the parental cells (Fig. 4A). Interestingly, the resistance to gefitinib was also found in A549 cells after treatment with Pre-miR-23a only (Fig. 4A). The IC_{50} values of gefitinib with TGF-β1 and gefitinib after treatment with Pre-miR-23a were 32 and 24, respectively, whereas that of gefitinib monotherapy was 6.8 (Fig. 4B). These findings indicate that induction of EMT affects cellular response to gefitinib and that miR-23a contributes to the resistance as well as TGF-β1.

Discussion

TGF-β1 has been recognized as a regulator of EMT in advanced-stage human cancers, a phenomenon which promotes tumorigenesis, cancer progression and metastasis (10). Previous studies have demonstrated that EMT plays a key role in lung cancer progression (15,28,29). In addition, it has been demonstrated that cancer progression might stimulate EMT in lung cancer, resulting in resistance to anticancer drugs (17-19). Therefore, the early detection of EMT development or attenuation of the EMT phenotype in lung cancer cells may be useful, particularly in helping to improve the clinical treatment of lung cancer.

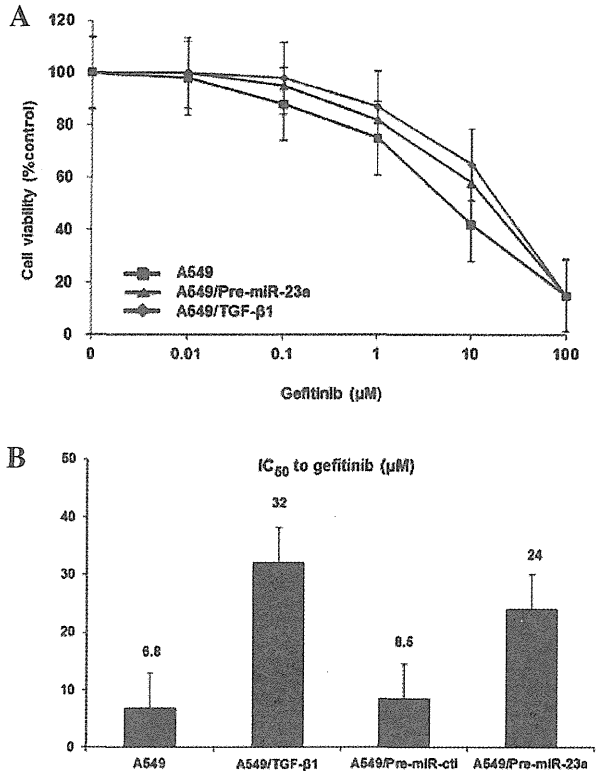

Figure 4. MiR-23a contributes to gefitinib drug resistance. (A) Gefitinib treatment with 5 ng TGF-β1 or Pre-miR-23a or Pre-miR-ctl for 24 h was examined in A549 cells. Then, the cells were incubated in the various concentrations of gefitinib for 72 h. (B) The IC_{50} values of gefitinib with TGF-β1 and gefitinib after treatment with Pre-miR-ctl or Pre-miR-23a. Each result is expressed as cell viability in the treated samples compared with the untreated sample (100%) for gefitinib therapy. Data are mean ± SD from 3 independent experiments.

Recent reports showed that several miRNAs play a crucial role in the regulation of EMT of several cancers (30-34). The miR-200 family and miR-205 have been shown to contribute to EMT in cancer cells by the direct targeting of transcriptional repressors of E-cadherin, ZEB1 and ZEB2 (30-32). More specifically, in breast cancer cells, miR-155 has been shown to facilitate TGF-β-induced EMT by targeting RhoA (33). Finally, miR-9 activated by MYC/MYCN mediated E-cadherin downregulation resulting in the activation of β-catenin, and VEGF, and metastases in human cancers, including neuroblastomas and breast tumors (34). However, in lung cancer, the mechanism by which miRNA contributes to TGF-β-induced EMT is largely unknown.

MiR-23a/24/27a is a miRNA cluster located in chromosome 19p13.12 and can be induced by TGF-β (26). This cluster functions as an oncogenic miRNA in several human cancers, and previous studies have reported that miR-23a/24/27a was upregulated in human cancers (26,35). Furthermore, miR-23a/24/27a functioned as a growth-promoting and anti-apoptotic factor in HCC cells (26), while miR-23a was also shown to promote the growth of gastric adenocarcinoma cells and downregulate interleukin-6 receptor (35). In addition, c-myc suppression of miR-23a enhances mitochondrial glutamine metabolism and glutaminase expression (36). The cognate of glutamine is the major component that catabolizes glutamine to generate energy and lactate. Plenty of large amounts of glutamine are aggressively transported into cells to promote cancer cell proliferation and act as a source of carbon in the carbon cycle. Taken together, miR-23a/24/27a can be induced by TGF-β and act as an oncogenic or tumor repressive miRNA in multiple human malignancies. However, the relation between miR-23a/24/27a and TGF-β/Smad pathway remains unclear in lung cancer cells.

In this study, we found that expression of miR-23a was directly induced by the TGF-β1/Smad pathway in A549 lung adenocarcinoma cells with the EMT phenomenon. In contrast, miR-24 and miR-27a belonging to the same cluster were induced in a Smad-independent manner in lung adenocarcinoma cells. We suggest that TGF-β1 mainly regulates the expression of miR-23a in lung cancer cells. Furthermore, overexpression of miR-23a decreased E-cadherin expression and increased levels of vimentin, resulting in the EMT phenomenon in A549 lung cancer cells; silencing of miR-23a partially restored E-cadherin expression. This is the first report showing that miR-23a regulated TGF-β1-induced EMT via E-cadherin suppression in lung cancer cells.

Molecular-targeted therapies have been recently developed for NSCLC treatment. NSCLC patients with EGFR gene mutations have shown a dramatic response to EGFR-TKIs such as gefitinib and erlotinib (4,5). We have recently reported that first-line gefitinib for advanced NSCLC patients with EGFR mutations improved progression-free survival with acceptable toxicity (5,37). However, it is recognized that, clinically, drug resistance eventually emerges and this limits the mean duration of response. Although mechanisms of acquired resistance, such as T790M secondary mutation and MET amplification, have recently been found, other mechanisms should be identified to widen the therapeutic strategy for NSCLC with EGFR mutations (38,39). EMT has been reported to be correlated with an unfavorable prognosis for NSCLC patients (40). Some studies showed that the mesenchymal phenotype was more resistant to EGFR-TKI than the epithelial phenotype in NSCLC (17-19). Similarly, restoration of E-cadherin increased the sensitivity to EGFR-TKI in lung cancer cell lines (41). Consistent with previous findings, induction of EMT by TGF-β1 in A549 cells led to the acquisition of resistance to gefitinib. Furthermore, overexpression of miR-23a induced EMT by suppressing E-cadherin expression and contributed to the reduced sensitivity to gefitinib in A549 cells. These findings demonstrated that suppression of EMT by miR-23a inhibition might overcome the resistance to EGFR-TKIs observed in NSCLC.

In conclusion, our study has provided evidence that miR-23a regulated TGF-β-induced EMT by suppression of E-cadherin and contributed to EGFR-TKI resistance in lung cancer cells. MiR-23a might be a potential prognostic marker and a new therapeutic target in NSCLC. Further studies should be performed to clarify the connection between miR-23a and TGF-β/Smad signaling during the EMT process in NSCLC.

Acknowledgements

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Enzastaurin has anti-tumour effects in lung cancers with overexpressed JAK pathway molecules

T Shimokawa[1], M Seike[*,1], C Soeno[1], H Uesaka[2], A Miyanaga[1], H Mizutani[1], K Kitamura[1], Y Minegishi[1], R Noro[1], T Okano[1], A Yoshimura[1] and A Gemma[1]

[1]Department of Internal Medicine, Division of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan; [2]MediBIC, Tokyo, Japan

BACKGROUND: Enzastaurin, an oral serine–threonine kinase inhibitor, was initially developed as an ATP-competitive selective inhibitor against protein kinase Cβ. However, the mechanism by which enzastaurin contributes to tumourigenesis remains unclear.

METHODS: We analysed the anti-tumour effects of enzastaurin in 22 lung cancer cell lines to ascertain the potential for enzastaurin-based treatment of lung cancer. To identify molecules or signalling pathways associated with this sensitivity, we conducted a gene, receptor tyrosine kinases phosphorylation and microRNA expression profiling study on the same set of cell lines.

RESULTS: We identified eight genes by pathway analysis of molecules having gene-drug sensitivity correlation, and used them to build a support vector machine algorithm model by which sensitive cell lines were distinguished from resistant cell lines. Pathway analysis revealed that the JAK/STAT signalling pathway was one of the main ones involved in sensitivity to enzastaurin. Overexpression of JAK1 was observed in the sensitive cells by western blotting. Simultaneous administration of enzastaurin and JAK inhibitor inhibited enzastaurin-induced cell growth-inhibitory effect. Furthermore, lentiviral-mediated JAK1-overexpressing cells were more sensitive to enzastaurin than control cells.

CONCLUSION: Our results suggested that the JAK1 pathway may be used as a single predictive biomarker for enzastaurin treatment. The anti-tumour effect of enzastaurin should be evaluated in lung cancer with overexpressed JAK pathway molecules.

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Translational Therapeutics

Non-small-cell lung cancer (NSCLC) patients are usually diagnosed with advanced disease, and their prognosis remains poor despite improvements in chemotherapies (Mountain, 1997; Schiller *et al*, 2002; Ohe *et al*, 2007; Jemal *et al*, 2009). Recently, molecular-targeted therapies have been developed for NSCLC treatment. For example, NSCLC patients with epidermal growth factor receptor (EGFR) mutations have shown a dramatic response to EGFR inhibitors such as gefitinib and erlotinib (Mok *et al*, 2009; Maemondo *et al*, 2010). However, there remain many other molecular abnormalities in lung cancer that are as yet unexplored (Salgia and Skarin, 1998).

The protein kinase C (PKC) family of serine–threonine protein kinases has been implicated in several important cellular functions including proliferation, motility, invasion and apoptosis (Livneh and Fishman, 1997). Among the PKC isoforms, PKCβ is known to be an important mediator of vascular endothelial growth factor (VEGF) (Xia *et al*, 1996; Yoshiji *et al*, 1999), the most potent angiogenic factor found in various tumours. Increased invasion and proliferation in tumours have also been associated with PKCβ (Zhang *et al*, 2004). Overexpression and increased activity of PKCβ have been implicated in transformation and tumourigenesis in lung cancer (Barr *et al*, 1997; Lahn *et al*, 2006). In several human cancers, PKCβ expression is linked to poor prognosis, most notably in B-cell lymphoma (Shipp *et al*, 2002; Li *et al*, 2007). Biochemical analysis demonstrated that PKCβ could target the phoshatidylinositol 3-kinase (PI3K)/AKT pathway and other signal transduction pathways (Graff *et al*, 2005; Rascoe *et al*, 2005). However, the mechanism by which PKCβ contributes to tumourigenesis remains unclear.

The PKCβ inhibitor enzastaurin, an oral serine–threonine kinase inhibitor, was initially developed as an ATP-competitive selective inhibitor against PKCβ (Faul *et al*, 2003). Enzastaurin is now being evaluated in several phase II studies across a variety of more common tumour types including: breast, ovarian colon and prostate cancers (Mina *et al*, 2009; Vergote *et al*, 2009; Dreicer *et al*, 2010; Glimelius *et al*, 2010). It has also been evaluated as second- or third-line therapy for NSCLC in a phase II study (Oh *et al*, 2008; Chiappori *et al*, 2010). *In vitro*, sequence-dependent, synergistic anti-proliferative and proapoptotic effects of the combination of cytotoxic drugs and enzastaurin have been found in NSCLC cells (Rademaker-Lakhai *et al*, 2007; Morgillo *et al*, 2008; Tekle *et al*, 2008). These studies suggest that enzastaurin may have an activity against lung cancer.

In this study, we analysed the anti-tumour effects of enzastaurin in a panel of 22 lung cancer cell lines to ascertain the potential for enzastaurin-based treatment of lung cancer. We also conducted gene, receptor tyrosine kinases (RTKs) phosphorylation and microRNA (miRNA) profiling on the same set of cell lines to identify the molecules associated with sensitivity of lung cancer to enzastaurin treatment. The correlation between the cytotoxic activity of enzastaurin and the corresponding gene, RTKs phosphorylation and miRNA expression patterns has been examined to clarify the

*Correspondence: Dr M Seike; E-mail: mseike@nms.ac.jp
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responsible mechanisms of the signalling pathway involved in the response of lung cancers to enzastaurin treatment.

MATERIALS AND METHODS

Cell lines

We used 22 lung cancer cell lines: A549, PC3, PC7, PC9, PC14, LC2/ad, ABC-1, RERF-LC-KJ, RERF-LC-MS, RERF-LC-AI adenocarcinoma (AC) cell lines and PC1, PC10, LK2, SQ5, QG56, EBC-1, LC1/sq squamous-cell carcinoma (SCC) cell lines and NCI-H69, NCI-N231, Lu135, SBC3, MS-1 small-cell lung carcinoma (SCLC) cell lines for this study. In addition, five cell lines comprising H1650, H1975, LC-1F, RERF-LC-OK and VMRC-LCD, were used as the test set for a validation study. A549, NCI-H69, NCI-N231, H1650 and H1975 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); RERF-LC-KJ, RERF-LC-AI, RERF-LC-OK, LC2-ad, SQ5, LC2/Ad, LC1/Sq, LC-1F and MS-1 were obtained from the RIKEN Cell Bank (Ibaraki, Japan) and PC1, PC3, PC7, PC9, PC10 and PC14 were obtained from Immuno-Biological Laboratories (Gunma, Japan); RERF-LC-MS, ABC-1, EBC-1, LK2, QG56 and VMRC-LCD were purchased from Health Science Research Resources Bank (Osaka, Japan). Lung cancer cell lines were maintained in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Drugs and growth-inhibition assay

Enzastaurin was kindly provided by Ely Lilly. Growth inhibition was assessed by MTS assay to examine the effect of enzastaurin on lung cancer cell lines. Cell suspensions (5000 cells per well) were seeded into 96-well plates and increasing concentrations of enzastaurin (0, 0.01, 0.1, 1.0, 10 and 100 μM) were added. After incubation for 72 h at 37 °C, MTS was added to each well and incubated for 2 h at 37 °C, after which absorbance was measured using a microplate reader with a test wavelength of 450 nm. The IC_{50} value was defined as the concentration needed for 50% reduction of the growth by treatment with enzastaurin.

JAK inhibitor (JAK inhibitor I, Cat. No 420099) was purchased from Calbiochem (San Diego, CA, USA). A549 and RERF-LC-KJ cells (5000 cells per well) were seeded into 96-well plates. After 24 h, the cells were incubated for 72 h in the various concentrations of enzastaurin (0, 0.01. 0.1, 1.0, 10 and 100 μM), with or without low-dose (1 μM) JAK inhibitor.

RNA isolation, cDNA array, RTKs phosphorylation antibody array and miRNA array

Total RNA was isolated from lung cancer cell lines with the use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. High-density oligonucleotide array analysis was carried out using Affymetrix HG-U133A (22 282 probe sets) expression array, as previously described (Gemma *et al*, 2006). Scanning was performed with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA), and GeneChip analysis was based on the Affymetrix GeneChip Manual with GeneChip Operating Software version 1.0 (Affymetrix), and Microarray Database software. We also performed human RTKs phosphorylation antibody array, including 71 antibodies (RayBiotech, Inc., Norcross, GA, USA). MicroRNA expression profiles were analysed by TaqMan MicroRNA Array set version 2.0 containing 667 miRNAs and validated by TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40 and 0.5% sodium deoxycholate. The lysates were kept on ice for 30 min, and then centrifuged at 13 000 g for 30 min. The supernatant was collected and 10 μg of protein were separated by gel electrophoresis on 10% gels, transferred to nitrocellulose membranes and detected by immunoblotting using a chemiluminescence system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The antibodies detecting JAK1, STAT3, phospho-STAT3 (p-STAT) and β-actin were purchased from Cell Signaling Technology (Beverley, MA, USA).

Lentiviral-mediated JAK1-overexpressing cells

Expression plasmid vector pEZ-Lv151 was used for lentiviral vector production (GeneCopoeia, Rockville, MD, USA). The coding sequence of human JAK1 or enhanced green fluorescent protein (EGFP) was inserted under the transcriptional control of the CMV promoter in pEZ-Lv151. The human JAK1 lentiviral expression plasmid (Ex-T8644-Lv151) or EGFP plasmid (Ex-EGFP-Lv151) was cotransfected into 293Ta cells with the Lenti-Pac HIV Packaging Mix (GeneCopoeia). Lentivirus-containing supernatants were harvested 48 h after transfection. The lentivirus particles were purified and stored at −80 °C in aliquots until use.

To establish stable JAK1-overexpressing cell lines, A549 cells were transduced with serial dilutions of lentiviral supernatant in the presence of 5 $\mu g\,ml^{-1}$ polybrene and selected by 0.8 $ng\,ml^{-1}$ geniticine. After antibiotic selection for 3 weeks, stable overexpressing JAK1 cells (LV-JAK1 A549 cells) were obtained.

Statistical analyses

Data analysis for the correlation coefficients that revealed the correlation between the drug activity patterns and the gene expression patterns was principally done by a modified National Cancer Institute programme (Miyanaga *et al*, 2008). We used pathway analysis to provide a viewpoint of the biological function of genes within the proposed classifier. Pathway analysis was done using the Pathway Architect software (Stratagene, La Jolla, CA, USA). The pathways showing the relationships among the genes on the list were drawn by selecting all molecules on the pathway edit window. All relationships among the molecules were retrieved from the database, with this information being derived from PubMed abstracts by natural language processing technology. The function was done by selecting the data of maximum reliability (MAX) by choosing all modes of interactions including 'Promoter Binding', 'Regulation', 'Protein Modification' and 'Expression' and by taking the relationships supported by three or more consistent data sources. Next, we picked out the incorporated genes from the imported gene list used at the onset of the pathway analysis, except the subunits of the target gene. Thus, a list of the genes associated with drug response was established with respect to not only gene expression profile data but also the biological functions of altered/associated genes. Data from the listed genes were used to build a support vector machine (SVM) model with ArrayAssist software (Stratagene) to predict the drug response (IC_{50}). The SVM algorithm model with Gaussian kernels was used to distinguish sensitive cells from resistant cells, using biomarkers identified by the gene expression-enzastaurin drug sensitivity correlation and pathway analysis. The classification ability of the genes was evaluated using leave-one-out cross-validation.

RESULTS

Effect of enzastaurin on the growth of lung cancer cells

Growth-inhibitory effects of enzastaurin on lung cancer cell lines were assessed by MTS assay.

Figure 1 shows the sensitivity to enzastaurin among the 22 lung cancer cells. Based on the IC_{50}, the 22 cell lines were classified into two groups, namely: enzastaurin sensitive and enzastaurin