おわりに

肺癌死亡の増加は著しく，日本人の健康増進のためには，肺癌死亡の減少を図ることが重要である．結核や脳血管障害の死亡が減少した経緯をみると，疾患の発生を抑える努力，早期発見の効果，画期的な治療法の開発の3つの力が結集すると効果が発揮されることが分かる．

肺癌はほかの臓器の癌に比べ，罹患数と死亡数が接近しているので，肺癌死亡を減少させるためには，禁煙の普及とさらなる発癌因子の発見と除去による1次予防，肺癌検診の精度向上と普及による早期発見・早期治療を行う2次予防，侵襲が少なく確実に治療が可能な3次予防の効果をそれぞれ確実に高めることが必要である．

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効果と展望を多視点から探る

CTによる肺がん検診の有用性
「東京から肺がんをなくす会」の活動の進展

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●Summary

The effectiveness of lung cancer screening has been proven, but it is not enough. Introduction of low dose CT is pushed forward to raise the effect. There are improvement of discovery rate and better survival rate of lung cancer, but it is not yet proved about an effect to decrease lung cancer death in the whole examinee.

要旨：現行の肺がん検診の効果は証明されたが、さらに精度を高めるべくCTの導入が進められ、発見率、早期がん率、発見肺がんの予後の向上はあるが、肺がん死亡減少効果については研究中である。また、肺気腫や内臓脂肪の診断効果も期待されている。

肺がん死亡を減少させるための、1次予防としての禁煙は、その効果発現までには長時間を要し、3次予防としての治療にも病期ごとの治療成績の向上はあまり期待できない。肺がんも早期であれば予後は期待できるが、症状が出現した時点では進行の可能性が極めて高く、2次予防としての早期発見に対する期待が大きい。

本稿では、現行の肺がん検診の方法とその効果と、さらにその精度を向上させるために開始された低線量CTでの検診の方法とその効果について紹介する。

現状の肺がん検診の方法と効果

肺がん検診は、集団検診では100㎜幅の高圧間接撮影が、個別検診では高圧直接撮影が行われ、いずれの方式でも50歳以上の喫煙指数600以上（喫煙指数＝喫煙年数×1日の本数）の高危険群、および半年以内に血痰を自覚例に対しては3日間の喀痰細胞診が行われている。[1]

肺がん検診の効果に関しては内外でも多くの研究が行われており、米国のメイヨークリニックで行われた無作為比較試験（RCT）では1万人の喫煙男性を2群に分け、検診群では年3回X線と細胞診を行い、対照群では口頭で検診受診を勧めるだけにとどめ経過を見たところ、検診群では肺がんの発見数が対照群よりも多かったが、肺がんの死亡数には差がなかったことから、肺がん検診は無効と判断された。[2]

本邦では結核予防法の下での検診がすでに全国的に行われていたために、非検診群を設定してのRCTは行えず、次善の策として症例対照研究が行われている。この方法は同一地域内の、肺がん死亡例（症例）と年齢性別喫煙歴の等しい健常者（対照）のペアを多数集め、それぞれの検診受診歴をさかのぼって調査する研究で、本邦の6つの症例対照研究では、すべてで検診による肺がん死亡の減少を示すオッズ比は1以下になった。そのうち、神奈川県、宮城県、新潟県、岡山県での研究では統計学的にも明らかな有意差を持って有効性が示された。[3,4]

これらの地域は元々非常に熱心に検診が行われているところで、全国の肺がん検診がすべて同じレベルで行われているわけではないが、定められた方法を遵守して行えば一定の効果があることは確かと考えられている。

有用性高いCTによる肺がん検診

現行の肺がん検診の効果は証明されたものの、他のがん検診に比べ精度が低いことは事実で、これを向上させるために、特に画像診断を中心に多くの方法が研究された。CTは開発当初から微小陰影の発見能に優れていることは知られていたが、低い処理能力、多い

施数は少ないが、導入している施設の数が多いので、全国的にはかなりの数の受診者になるものと思われる。

現在、日本CT検診学会（旧胸部CT検診研究会）で定期的行っている全国集計では、03年には、6万4723人が受診し、肺がんの発見率は10万対296・6人、病期もI期が72・9％になっている。これは通常のX線写真と喀痰での肺がん検診の発見率10万対50人、I期率50％前後に比べ明らかに高くなっている。しかし前述の「東京から肺がんをなくす会」の成績や初期の全国集計の成績と比較すると特に病期I期の率の低下傾向があり、また一部の施設では精検結果の把握ができていないところも散見するようになってきている。

CT検診では、通常の検診に比べ非常に多くの微小な結節が指摘されることも明らかになってきており、これらの診断基準や取り扱い基準も確立していない。CT検診はまだ研究段階であることを十分に意識して、確実な精度管理を行い、その結果を次の読影に生かしていかないと、いわゆる「やりっ放し検診」になってしまい、精度の低下を来し有効性も低下し効果がないという烙印を押されてしまう危険性も皆無ではない。

現在CT検診を導入している施設も、今後導入を計画している団体もこの点を十分に認識し、受診者の了解を得た上で検査を行うことが肝要である。

CT検診の肺がん以外の効果

CTを撮影することで、冠動脈の石灰化、肺気腫、骨粗鬆症、内蔵脂肪の多寡なども診断できることが明らかになった。冠動脈の石灰化と内蔵脂肪の増加は動脈硬化の大きなリスクファクターであり、これを見ることで生活習慣を改めたり早期に治療することで、心筋梗塞や脳梗塞の発症を予防できると考えられている。また肺気腫は喫煙との関連が強いので、本人自身のCT画像を示しながら禁煙指導を行うと達成率も高いといわれている。

CTの画像には極めて多くの情報が存在しており、そのすべてを利用することによりCT検診の効果はさらに高まるものと思われる。

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表1　東京から肺がんをなくす会の検診成績、CT導入前後の比較

	CT導入前(1975.9～1993.8)		CT導入後(1993.9～2005.2)	
	初回検診	複数回検診	初回検診	複数回検診
検査数	2,554	23,794	2,773	16,189
発見肺がん(10万対)	10(392)	33(139)	27(974)	58(358)
病期別				
ⅠA	2(20%)	16(48.5%)	16(59.3%)	49(84.5%)
ⅠB	2(20%)	3(9.1%)	2(7.4%)	2(3.4%)
ⅡA	0	3(9.1%)	1(3.7%)	3(5.2%)
ⅡB	1(10%)	2(6.1%)	0	0
ⅢA	2(20%)	6(18.2%)	3(11.1%)	2(3.4%)
ⅢB	0	1(3.0%)	2(7.4%)	1(1.7%)
Ⅳ	3(30%)	2(6.1%)	3(11.1%)	1(1.7%)

被曝量、高い費用等で検診への応用は不可能と思われていた。しかし、X線管球が連続的に、移動しながら撮影できる技術（ヘリカルCT）も開発され、撮影時間が著しく短縮し、また肺野の読影のためには被曝量を下げても可能なことが明らかになり、検診への導入が可能になった。

「東京から肺がんをなくす会」は1975年に設立した会員制の有料肺がん検診組織で、当初は会員に対して年に2回、胸部X線写真の正側2方向撮影と喀痰細胞診を行っていたが、前述のように90年代はじめからCT検診の可能性が出てきたことにより、93年から世界で最初に低線量による高速らせんCT撮影を定期的な検診に導入した。[5]

CTの撮影方法は当初はシングルスライスCT（SSCT）で、X線管電流は50mAs、毎秒2㎝移動、約17秒の息止めの間に全肺を撮影する方法で行われ、02年9月からは4列のマルチスライスCT（MSCT）撮影が行われている。読影に関してはSSCTでは、第1読影者がその回の画像だけを読影し、次に第2読影者が第1読影者の判定と、過去画像およびコンピュータによる診断支援（CAD）の結果を総合的に判断し最終判定を行い、要精検となった場合には再度呼び出して高分解能CT撮影（HRCT）撮影が行われた。MSCTの導入後は、第1読影者がはじめに過去画像を参照しながら10㎜の画像を読影し、さらにCADの結果を参照し判定する。第2読影者は第1読影者が要精検とした部分について、同時に再構成していた2㎜幅の画像を読影し最終的な判定としている。

CT導入前後での検診の成績を初回検査と複数回検査に分けて表1に示した。CT導入前においても複数回受診例での病期IA期の率は50%に近く、喫煙男性がほとんどを占める集団での検診成績としては決して悪くはないが、CT導入後はIA期が初回でも59・3%、複数回では84・5%とほとんどを占めている。複数回にもかかわらずIA期で発見できなかった9例の理由は、受診者が定期的に受診しなかったのが2例、精検機関での診断の遅れが1例、検診時の誤判定が3例、小細胞がんを含む非常に経過の早いがんが3例であった。読影能の向上でIA期の率はさらなる向上が期待できる。

「東京から肺がんをなくす会」での発見肺がんの予後調査では、CT導入前の5年生存率が50%であったのに対し、導入後には80%に上昇している。[6]しかし、発見肺がんの数も多いので、その集団全体の肺がん死亡数そのものの減少効果があるかどうかは不明であり、日本では大規模なコホート研究が行われ、米国でもCTと胸部X線を比較するRCTによる研究が進行中である。

集団検診へのCTの導入は95年に千葉県で試験的に開始され、その後、長野県、愛媛県、大阪府などでは全県的に行われた。その他でも市町村単位では広く行われ、企業検診でも日立製作所をはじめとして数社で行われている。

また、最近では人間ドックへの導入が盛んで、現在ほとんどの病院でドックの中に組み込まれたり、オプションとして選択できるようになっている。これらは個々の施設での実

EGFR Mutation Is Specific for Terminal Respiratory Unit Type Adenocarcinoma

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Abstract: We have previously reported that terminal-respiratory-unit (TRU) type adenocarcinoma is a distinct subset of lung adenocarcinoma in terms of molecular pathway for carcinogenesis and phenotypic profiles. This type of cancer shows TRU features, characterized by distinct cellular morphology and the expression of TTF-1 and surfactant proteins. Recently, two groups published novel mutations of the epidermal growth factor receptor (EGFR) that are closely associated with clinical response to gefitinib. The clinicopathologic features of gefitinib responders overlap with those of TRU-type adenocarcinoma, and the characteristics of TRU are likely to correspond to the bronchioloalveolar features reported as a predictor of gefitinib response. We therefore examined the characteristics of EGFR-mutated pulmonary adenocarcinomas with special reference to TRU-type adenocarcinoma. EGFR mutation was detected in 97 of 195 adenocarcinomas, 91 of 149 TRU-type adenocarcinomas and 6 of 46 tumors of other types. Conversely, 91 of 97 EGFR-mutated adenocarcinomas were categorized as TRU-type adenocarcinomas. This type-specific involvement was confirmed by logistic regression model. In addition, EGFR mutation was detected in some cases of atypical adenomatous hyperplasia, a preinvasive lesion of TRU-type adenocarcinoma. These findings further confirm that TRU-type-adenocarcinoma is a distinct adenocarcinoma subset in which a particular molecular pathway is involved.

Key Words: EGFR mutation, lung adenocarcinoma, atypical adenomatous hyperplasia, gefitinib, bronchioloalveolar features

(*Am J Surg Pathol* 2005;29:633–639)

Lung cancer is the leading cause of cancer deaths for both men and women in United States, Japan, and Western countries.[46] A significant number of clinical trials using chemotherapeutic strategy against this cancer have been attempted, but the effects on advanced lung cancer remain marginal. Recently, small molecules which inhibit receptor protein kinase activity, have been developed. Gefitinib is one of such drug, which targets epidermal growth factor receptor (EGFR) kinase. Although EGFR is expressed in more than 80% of non-small cell lung cancers (NSCLCs), as well as a wide range of epithelial cancers,[2,5,30] clinical trials have shown significant variability in the response to gefitinib: 10% to 20% of patients respond to gefitinib treatment, and in some of the patients the response is dramatic, whereas the remaining patients show no response.[10,13,15,24] Further analysis revealed that nonsmokers, female sex, and histologic subtype of adenocarcinoma, especially with bronchioloalveolar feature, are significantly prevalent in the responders.[10,31] These features overlap with the characteristics of terminal-respiratory-unit (TRU) type-adenocarcinoma, which we have noted previously.[54,55,57]

TRU is composed of alveolar cells and nonciliated bronchiolar epithelium, and its characteristics are highlighted by morphology and expression of thyroid transcription factor-1 (TTF-1) and surfactant proteins. The concept of the TRU is suggested by constant and uniform expression of TTF-1, appearing as a series of cells that represented a certain functional unit or common lineage. TTF-1 is a crucial transcription factor in the lung, required for lung development and the maintenance of lung function. TTF-1 regulates functional molecules of the lung, including surfactant proteins,[6,34,52] and TTF-1-deficient mice resulted in lung aplasia.[21,32] TRU-type adenocarcinoma, which is putatively derived from the TRU, demonstrates a different pattern of alteration of cancer-associated genes, suggesting a distinct molecular pathway of its carcinogenesis. Recently, two groups published novel mutations of the EGFR, which are closely associated with clinical response to gefitinib.[29,35] In this study, we attempted to explore the clinicopathologic significance of the mutations with special reference to the TRU-type adenocarcinoma, using a cohort of 241 patients with NSCLC, a part of which we previously reported on EGFR mutation.[23]

MATERIALS AND METHODS

Patients

A series of 241 consecutive patients with non-small cell carcinoma between 2001 and 2002, from whom frozen tissue was available, were selected for this study from a file held at the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan. This series included 195 patients with adenocarcinoma, including 5 bronchioloalveolar carcinomas, 34 patients with squamous cell carcinoma, 7 patients with large cell carcinoma, and 5 patients with adenosquamous carcinoma. The cohort was a part of a previous study, and their clinical details are described elsewhere.[23] In

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addition, atypical adenomatous hyperplasia (AAH) and corresponding primary tumors were examined in 5 patients to determine the mutation status in precursors of invasive adenocarcinoma.

Tissue Microarray and Immunohistochemistry

Expression status of TTF-1 and surfactant pro-protein B (SPPB) were addressed as described previously.[54,55] Briefly, using tissue microarray, immunohistochemical examination proceeded according to the standard avidin-biotin-peroxidase complex method. Antibodies used were TTF-1 (8G7G3, DAKO, Copenhagen Denmark) and SPPB (19H7, Novocastra, UK).

Mutation Status of EGFR, p53, and K-ras

All of the mutation data in this cohort have been published previously.[23,56,57] Briefly, frozen tumor specimens were grossly dissected to enrich the tumor cells, and total RNA was extracted using the RNaeasy kit (Qiagen, Valencia, CA). Using a standard RT-PCR procedure, p53 gene from exon 4 to exon 10 was amplified, and the products were directly sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). When no mutation signals were obtained, the result was confirmed using functional assay in yeast.[18,51] In the functional assay, when more than 10% red colonies or a significant deviation of a split assay were observed, RNA was reextracted from microdissected tumor cells using laser capture microdissection, and RT-PCR products were sequenced. Through this procedure, most molecules were derived from tumor cells. Using the same RNA as for the mutational analysis of p53, EGFR and K-*ras* genes were examined by direct sequencing.

For mutation analysis of AAH samples, DNA was extracted from paraffin-embedded sections,[53] followed by PCR amplification with the following primer sets (5′-3′): for mutations of codon 719, forward-GAGGTGACCCTTGTCTCTGTGT and reverse-CCCAAACACTCAGTGAAACAAA; for deletions in exon 19, forward-TGCCAGTTAACGTCTTCCTTCT and reverse-ATGTGGAGATGAGCAGGGTCTA; and for mutations of exon 21, forward-GAGCTTCTTCCCATGATGATCT and reverse-GAAAATGCTGGCTGACCTAAAG. The PCR products were directly sequenced.

Morphologic Definition of TRU Morphology

The details of the morphologic characteristics of TRU-type adenocarcinoma have been described previously.[54,55] Principally, TRU morphology is based on cellular morphology, and we categorized the tumor as TRU-type when morphologic differentiation to type II pneumocytes, Clara cells, and/or nonciliated bronchioles was seen. Differentiation to type II pneumocytes is characterized by a cuboidal or dome-shaped free cell contour, a clear to foamy cytoplasm with occasional fine vacuoles and occasional nuclear inclusions. Clara cell differentiation is recognized as a cuboidal to dome-shaped free cell contour, a pale eosinophilic cytoplasm, frequently with snouts, and an apical location of the nuclei. Transition between differentiation to type II pneumocytes and Clara cells is quite common. Differentiation to nonciliated bronchioles is difficult to distinguish from differentiation to bronchial surface epithelium, and thus we paid most attention to this point. Tumors with either differentiation contain columnar cells but differ in the features of the luminal, free cell border. Adenocarcinomas with bronchiole differentiation show dome-shaped protrusion of each luminal free cell border, but in cases of differentiation to bronchial surface epithelium, the luminal border gives a smooth line.

Statistical Analysis

The χ^2 test and Fisher exact test for independence were used to compare frequencies of clinicopathologic variables. To estimate the interaction of the clinicopathological variables, we generated a logistic regression model using SYSTAT (SYSTAT Software Inc, Richmond, CA). A P value below 0.05 was considered statistically significant.

RESULTS

EGFR Mutations Specific for TRU-Type Adenocarcinoma

EGFR mutations in kinase domain of EGFR gene were detected in 98 of 241 NSCLCs, all except one of which were adenocarcinomas (97 of 195 adenocarcinomas, including 3 of 5 bronchioloalveolar carcinomas). The typical morphologies of adenocarcinomas with and without EGFR mutation are shown in Figures 1 and 2, respectively. The profiles of patients with the mutation were similar to those of gefitinib responders reported previously,[10,23,24] and among the adenocarcinomas, the mutation was preferentially found in females (60 of 98, Fisher exact test, $P < 0.001$) and nonsmokers (65 of 98, Fisher exact test, $P < 0.001$). The mutation status was not associated with pathologic stage (χ^2 test, $P = 0.805$) and the extent of nodal metastasis (χ^2 test, $P = 0.407$), suggesting that the genetic alteration is not associated with tumor progression. Because similar characteristics were observed in TRU adenocarcinomas,[54,55] we compared the mutation with three features of TRU, including expression of TTF-1 and SPPB, and its morphologic characteristics (Table 1). All were significantly associated with EGFR mutation (Fisher exact test, $P < 0.001$). When TRU-type adenocarcinomas were defined as those showing at least two of the three features, the distinction of TRU or non-TRU-type demonstrated the smallest P value (7.0×10^{-9}). We therefore used the criteria to define TRU-type adenocarcinoma

Of 149 TRU-type adenocarcinomas, 91 (61%) harbored an EGFR mutation (Table 2), as did 6 of 46 (13%) non-TRU-type adenocarcinomas. Conversely, 91 of 97 (94%) adenocarcinomas with EGFR mutation were categorized as TRU-type adenocarcinoma, suggesting a specific involvement of EGFR mutation in TRU-type adenocarcinoma. We constructed a multivariate logistic regression model to determine factors that are significantly associated with EGFR mutation in this cohort. The model, using the variables listed in Table 3, revealed that cellular type and smoking status were independent factors that affected EGFR mutational status with high statistical significance ($P < 0.001$ and $P = 0.004$, respectively). This confirms the specific involvement of EGFR mutation in TRU-type adenocarcinoma.

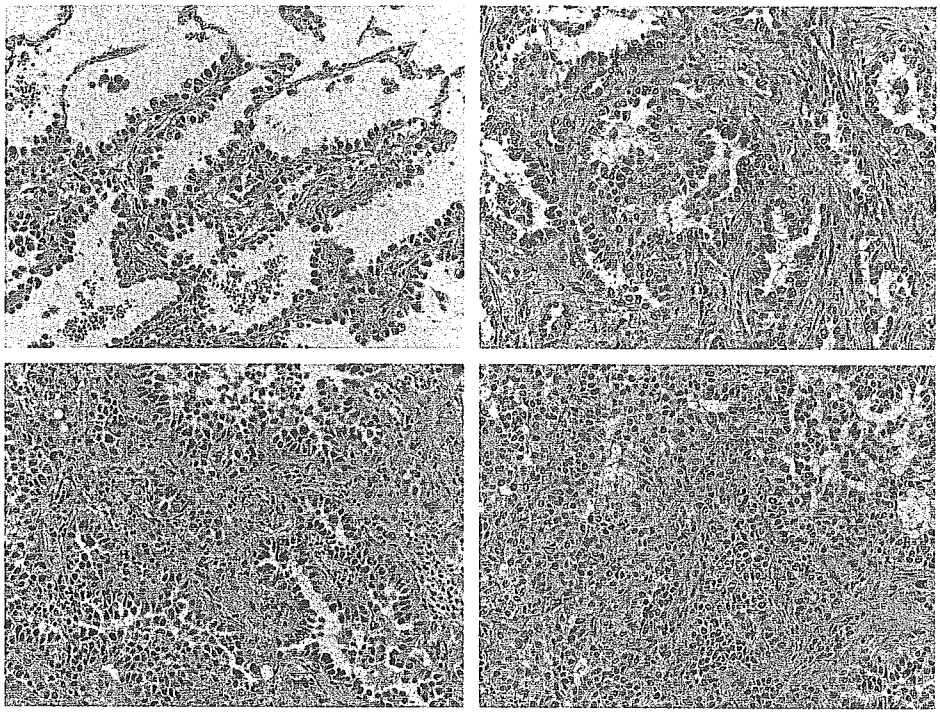

FIGURE 1. Typical feature of TRU-type adenocarcinoma with EGFR mutation. A tumor shows bronchioloalveolar feature in the periphery (left upper) and invasive portion in the center (left lower). In addition to bronchioloalveolar cell carcinomas, both moderately differentiated (right upper) and poorly differentiated adenocarcinomas (right lower) harbor EGFR mutation, while cellular features in both tumors are similar.

Clinicopathologic Features of TRU-Type Adenocarcinoma With Reference to EGFR Mutation

We then examined the clinicopathologic features of TRU-type adenocarcinoma. This type of adenocarcinoma, in which the EGFR gene is specifically mutated, was characterized by its prevalence in females and nonsmokers, and its infrequent mutations of K-*ras* and p53, compared with non-TRU-type adenocarcinoma (Table 2). The characteristics in this cohort were consistent with those we have reported previously,[54,55] which confirmed that they overlapped with EGFR mutation.

None of the tumors in this cohort harbored both EGFR and K-*ras* mutations, as reported.[23] This mutually exclusive relationship suggests a complementary role in the molecular carcinogenesis of lung adenocarcinomas, and either a mutation of EGFR or K-*ras* may contribute to the development of TRU-type adenocarcinoma. For K-*ras* mutation, a strong link with smoking habit has been reported.[1] This close correlation is observed in TRU-type adenocarcinomas in this study. Of the 15 TRU-type adenocarcinomas with K-*ras* mutation, 14 (93%) had developed in smokers, as did 29 of the 91 TRU-type adenocarcinomas with EGFR mutation (without K-*ras* mutation). This difference was statistically significant (Fisher exact test, $P < 0.001$). There were no other characteristics distinguishing TRU-type adenocarcinoma with EGFR mutation from those with K-*ras* mutation, including morphology, pathologic stage, presence or absence of nodal metastasis, and mutational status of p53.

EGFR Mutation in Preneoplastic Lesions

AAH is a preinvasive lesion that is recognized as the earliest lesion in the putative progression scheme of lung adenocarcinoma. Morphologic resemblance to either type II pneumocytes or Clara cells, and constant expression of TTF-1 and SPPB indicate that AAH corresponds to an edge of the spectrum of TRU adenocarcinomas. Because of difficulty obtaining frozen AAH tissues, we examined EGFR mutation status of exon 18, 19, and 21, using paraffin-embedded specimens. This set of examinations was considered to cover more than 90% of EGFR mutations.[23] EGFR mutation was detected in 2 of 5 patients with AAH examined (Table 4). A representative result (patient no. 2) is displayed in Figure 3. It is of note that 1 patient showed EGFR mutation only in the lung adenocarcinomas but not in the AAH (patient no. 3).

DISCUSSION

New identification of a gene alteration often contributes, not only to the understanding of molecular pathogenesis but establishment of the disease entity as well. This is exemplified by specific translocation in soft tissue sarcomas and hematopoietic malignancies. Recent identification of c-kit mutations shed light on gastrointestinal stromal tumors, which are currently considered an entity separate from leiomyogenic tumors and other rare stromal tumors.[14,17,22] Similar clarification might be preceded by the description of EGFR mutations in lung adenocarcinomas. We have previously proposed that the TRU-type adenocarcinoma is distinct from other adenocarcinomas in terms of their molecular carcinogenesis pathways and phenotypic profiles.[54,55,57]

In this study, 91 of 97 (94%) adenocarcinomas with EGFR mutation were categorized as TRU-type adenocarcinoma. This specific involvement of EGFR mutations in TRU-type adenocarcinoma supports that this type is a distinct subset of lung adenocarcinomas. Furthermore, EGFR mutation was detected in AAH, a preinvasive lesion representative of TRU

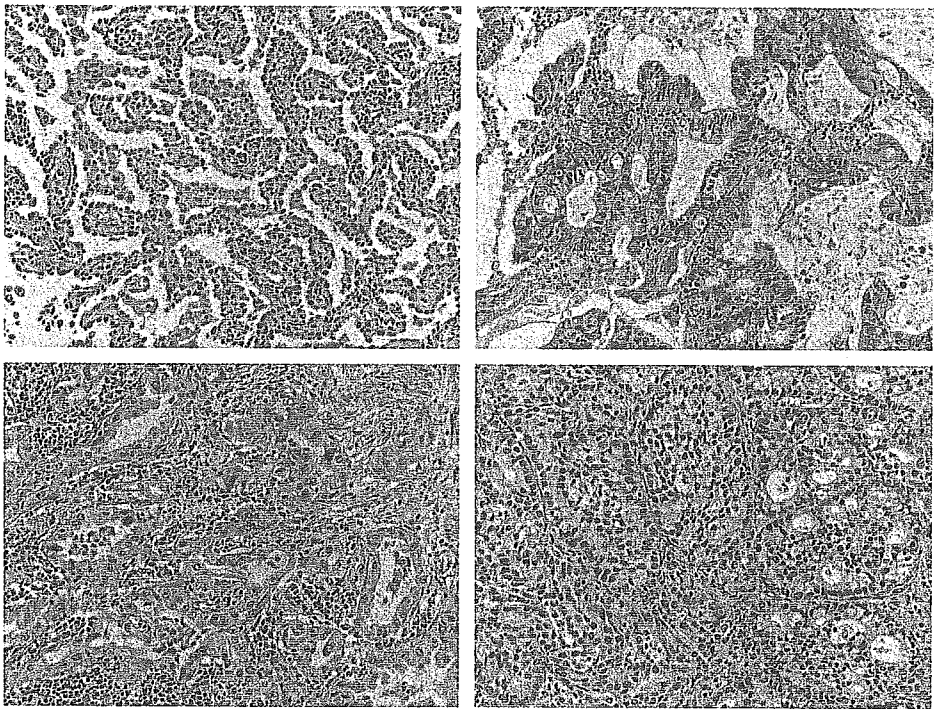

FIGURE 2. TRU-type and non-TRU-type adenocarcinomas that did not harbor EGFR mutations. Frequent mutation has been reported in bronchioloalveolar cell carcinomas, but two of five in our cohort did not harbor the mutation: the picture in the left upper corner shows such a case. Despite their TRU morphology, EGFR mutation was not detected in some TRU-type adenocarcinomas (left lower), which are indistinguishable from TRU-type adenocarcinoma with EGFR mutation. The two pictures on the right show the morphology of non-TRU-type adenocarcinomas without EGFR mutation: adenocarcinoma, resembling bronchial surface epithelium (right upper), and adenocarcinoma with abundant mucin in the cytoplasm (right lower).

neoplasia, suggesting an involvement of the EGFR at an early stage in the pathogenesis of TRU-type adenocarcinoma.

Because TRU is a new concept, it may be difficult to recognize TRU-type adenocarcinomas. TRU morphology is defined by its resemblance to type II pneumocytes, Clara cells, and/or nonciliated bronchiolar cells. This is quite similar to Shimosato's cytologic classification of lung adenocarcinoma.[25,38] The categories of Clara cell type, type II alveolar epithelial cell type, and mixed-cell or indeterminate cell type in the cytologic classification correspond to TRU-type adenocarcinoma. Similar attempts have been made by many others.[7,8,16,20,27,28,39–41] In the recently published WHO classification,[48] the morphologic characteristics of type II pneumocytes and Clara cells are well described, and the TRU-type adenocarcinoma corresponds in the classification schema to the majority of nonmucinous bronchioloalveolar carcinomas (BACs); most of adenocarcinoma mixed subtype with a BAC component; and a subset of papillary carcinoma. In addition, the majority of so-called sclerosing BACs are included as TRU-type adenocarcinomas. We have introduced TRU-type adenocarcinoma to allow better biologic understanding of pulmonary adenocarcinomas. For example, none of the EGFR mutations was detected in 635 nonpulmonary cancers.[26,29] This may be explained by specific involvement of the EGFR gene in TRU-type adenocarcinoma, of which its putative normal counterpart, ie, the TRU cell, is unique to the pulmonary parenchyma and is not contained in any organs. Furthermore, the distinction of TRU and non-TRU adenocarcinomas appears to be illustrated by hierarchical clustering according to genome-wide expression patterns, as discussed below.

In a practical sense, TTF-1 positivity is quite helpful and serves as the best available marker for identifying a TRU-type adenocarcinoma. The sensitivity of TTF-1 positivity for TRU-type adenocarcinomas reached 97% in this series, although specificity was not as high (76%, Table 2). In contrast, SPPB showed the lowest sensitivity (82%) and the highest specificity (98%) among the three factors examined. Therefore, an adenocarcinoma positive for TTF-1 and SPPB can be readily categorized as TRU-type. Regarding TRU morphology, the individual cell features of a dome-shaped protrusion with a luminal-free cell border are helpful for practical identification. For example, individual cancer cells of a TRU-type adenocarcinoma (Fig. 1; Fig. 2, left panel) appear to bulge out into the lumen or alveolar spaces and show dome-shaped luminal-free borders, whereas the luminal borders of non-TRU-type cancer cells (right two pictures in Fig. 2) appear as smooth lines. In addition to these cellular features, lepidic growth at the tumor periphery is a hallmark of TRU morphology.

Miller et al[31] has reported that adenocarcinoma with bronchioloalveolar features, as well as nonsmoker, serves as a predictor to respond to gefitinib treatment through multivariate analysis of 139 patients with NSCLCs. Because nonmucinous BAC is a prototype of TRU-type adenocarcinoma, it is likely that most of the "adenocarcinomas with non-mucinous BAC features" correspond to TRU-type carcinoma. Indeed, EGFR mutation, which has been reported to correlate with response to gefitinib treatment, is specifically observed in this characteristic subset. Therefore, gefitinib response is associated with EGFR mutation, which occurs in lung adenocarcinoma in a fashion specific for cellular lineage. It is of note that EGFR mutations can also occur in poorly differentiated adenocarcinomas, as long as the tumor belongs to the TRU cellular lineage, as shown Figure 1.

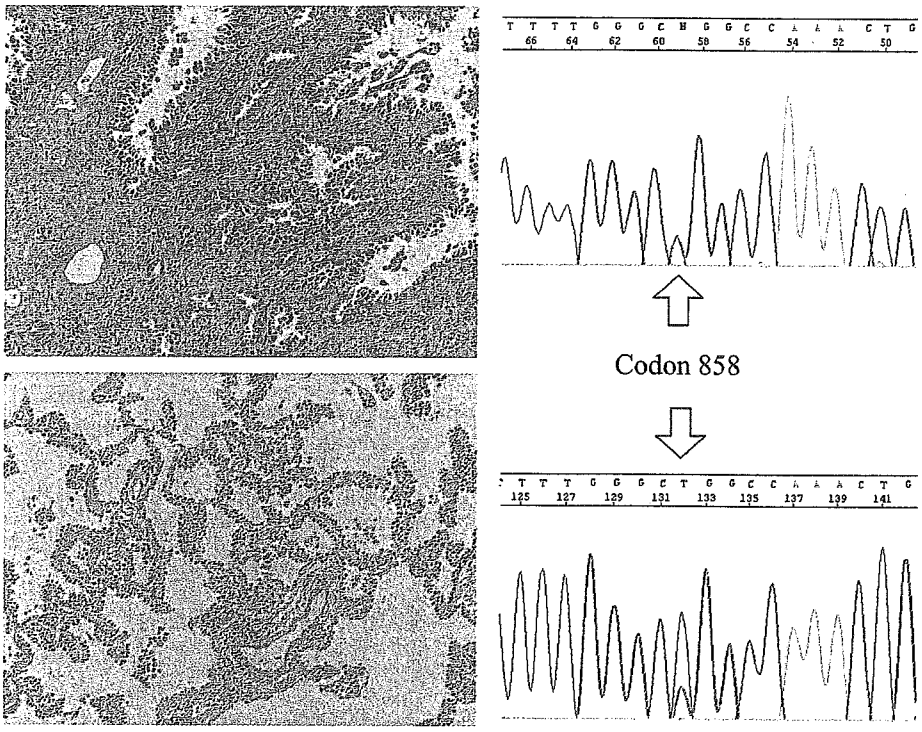

FIGURE 3. A representative result of AAH and its corresponding adenocarcinoma (patient no. 2). The left panel displays the morphologic features of adenocarcinoma (upper) and AAH (lower). Mutation at an identical position (right panel) was noted, although the AAH was found in a different lobe from the adenocarcinoma.

An important insight into the significance of cell lineage in cancers was provided by recent works on breast cancer research. Based on expression profiles, breast cancer is molecularly classified into basal cell-like, luminal cell-like, and HER2/*neu* amplified type,[36,43–45] the first two of which represent cell lineage, like the TRU-type in the lung. The classification was supported by the finding that BRCA1-related breast cancers are clustered specifically in the basal cell-like group[9,44] and that the genome-wide pattern of LOH is correlated with the classification scheme.[50] Recently, it was demonstrated that biologic response against chemotherapy differs between cellular types.[49] Regarding lung cancers, expression profiling analysis also revealed several subsets of adenocarcinoma. It is reasonable to speculate that some of them correspond to TRU-type adenocarcinoma because some subsets were characterized by high expression of TTF-1 and surfactant proteins, eg, adeno-group 1 and 2[12]; C3 and C4[4]; and AD2 to AD4.[47] Identification of properties based on cell lineage may have implications for understanding biologic nature of the tumors.

Recent advances in molecular oncology have revealed a crucial role for the involvement of pathways but not a particular molecule in the development if cancers. For example, about 80% of sporadic colon cancers harbor an alteration of APC, but mutation of β-catenin, a target molecule of APC, could substitute its role in carcinogenesis. Indeed, the mutation has been found in the subset of tumors without APC mutation.[33] Involvement of either c-kit or PDGFRA in gastrointestinal stromal tumors[14] also follows this fashion. Therefore, the mutually exclusive nature of EGFR and K-*ras* mutation

TABLE 1. Correlation of EGFR Mutation and TRU Features In Lung Adenocarcinoma

	EGFR		
	Wild type	Mutated	*P*†
TTF-1 (+/−)	65/33	90/7	<0.001
SPPB (+/−)	47/50	74/21	<0.001
TRU/non-TRU morphology	60/38	91/6	<0.001
TRU-type/non-TRU-type*	58/40	91/6	<0.001†

*TRU-type is defined by being positive for two or more of the three features above.
†*P*-value of this distinction was minimal (7.0×10^{-9}).

TABLE 2. Clinicopathologic Characteristics of TRU Adenocarcinomas

	TRU-type	Non-TRU-type	*P*
N	149	46	
Male/female	67/82	30/16	0.019
Smoker/nonsmoker	65/84	32/14	0.002
pStage (I/II/III/IV)	96/12/38/1	21/7/17/1	0.142
pN(0/1/2)	102/12/33	31/5/9	0.805
TTF-1 (+/−)	144/5	11/35	<0.001
SPPB (+/−)	120/26	1/45	<0.001
TRU/non-TRU morphology	143/6	8/38	<0.001
EGFR (wild-type/mutated)	58/91	40/6	<0.001
Deletion	42	4	
Point mutation	45	2	
Insertion	4	0	
K-ras (wild-type/mutated)	134/15	35/11	0.024
p53 (wild-type/mutated)	94/53	20/26	0.016

TABLE 3. Logistic Regression Model for Estimation of Significant Factors Related to EGFR Mutation

Variable	Category	Odds Ratio	95% CI	*P*
Age	≥median/<median	1.476	0.767–2.840	0.243
Sex	Female/male	0.783	0.300–2.048	0.618
Smoking status	Nonsmoker/smoker	4.352	1.618–1.710	0.004
Histology	BAC/non-BAC	0.541	0.082–3.546	0.522
Stage	Early-state (IA)/or more	0.769	0.386–1.533	0.455
Cell lineage	TRU-type/non-TRU-type	10.092	3.827–26.61	<0.001

indicates that impairment of this pathway plays an important role in the development of TRU-type adenocarcinomas. This idea suggests further two questions. The first question is what and how many pathways are crucial for the development of this tumor type. When EGFR mutation provides an effect equivalent to K-*ras* mutation, their mutually exclusive nature suggests a crucial role of the RAS-MAPK pathway. However, EGFR has several downstream targets other than the RAS-MAPK pathway, such as the PI3K/AKT and JAK/STAT pathways.[3,19] When EGFR mutation functions to impair the RAS-MAPK and other pathways, the K-*ras* mutation alone is not sufficient to develop TRU-type adenocarcinoma. Indeed, PIK3CA is shown to be mutated, despite low frequency in lung cancers,[37] and a recent study by Sordella et al[42] reported that mutated-EGFR targets the PI3K/AKT and JAK/STAT pathways rather than the RAS-MAPK pathway. Elucidation of these relationships may explain differences in the frequencies of gefitinib responder between Japan and the United States. It is of note that the incidence of K-*ras* mutation is different between the nations[1,11,56]; thus, an international comparative study is needed.

The second question is what is reflected in the preference of the involvement between EGFR and K-*ras* even though the resulted features are not different. The different frequency of smokers may provide a clue to the answer. K-*ras* mutation is known to be closely related with smoking,[1] and the frequency of smokers is quite different between EGFR and K-ras-mutated proportions in TRU-type adenocarcinoma. Smoking status may affect the mutation involved, although either mutation results in the development of the same type of adenocarcinoma.

In summary, we demonstrated a specific involvement of EGFR mutation in TRU-type adenocarcinoma. EGFR mutation was also detected in some AAHs, a preinvasive lesion of TRU-type adenocarcinoma. These findings confirmed that TRU-type adenocarcinoma is a distinct subset of lung adenocarcinomas.

TABLE 4. Mutational Status in Atypical Adenomatous Hyperplasia

Patient No.	Tumor	Feature	EGFR Gene
1	Metastatic cancer	Colon cancer metastases	Wild-type
	AAH	Same lobe, 4 mm	Mutation at codon 858
2	AD, poorly differentiated	25 mm in size, pT1N2	Mutation at codon 858
	AAH	6 mm, different lobe	Mutation at codon 858
3	AD, well differentiated	15 mm in size, pT1N0	Deletion at codon 746–750
	AAH	Different segment, 1.3 mm	No mutation
4	AD, moderately differentiated	33 mm in size, pT2N0	Wild-type
	AAH1	Different segment, 6 mm	No mutation
	AAH2	Different segment, 4 mm	No mutation
5	BAC, nonmucinous	9 mm in size, pT1N0	Wild-type
	AAH1	Same segment, 3 mm	No mutation
	AAH2	Different segment, 3 mm	No mutation

ACKNOWLEDGMENT

The authors thank Kaori Hayashi-Hirano for excellent technical assistance with the molecular genetic experiments, Hiroji Ishida for assistance in constructing the tissue array, and Ryuzo Ohno, President of Aichi Cancer Center, for special encouragement of this study.

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EGFR Mutation and Response of Lung Cancer to Gefitinib

TO THE EDITOR: Kobayashi et al. (Feb. 24 issue)[1] report that a second mutation in the gene encoding the epidermal growth factor receptor (*EGFR*), one resulting in a threonine-to-methionine substitution at amino acid position 790 (T790M), was associated with acquired resistance to gefitinib in their patient and that this mutant gene had been absent from the primary non–small-cell lung cancer. In a reanalysis of the data from the 397 subjects we have previously described,[2,3] we identified two women who had never smoked who had non–small-cell lung cancer and harbored two *EGFR* mutations — T790M and a leucine-to-arginine substitution at amino acid position 858 (L858R) — in resected tumor specimens before treatment with chemotherapy or radiotherapy. Both patients later had recurrent disease and eventually died — outcomes suggesting that tumors with both the L858R and T790M mutations are very aggressive. One patient was treated with gefitinib and had progression.

These findings indicate the existence of cases with inherent double mutations and provide evidence that the T790M mutant genotype is an important factor conferring resistance to gefitinib in non–small-cell lung cancers containing EGFR sensitivity mutations. In addition, detecting T790M may be useful for predicting pretreatment resistance to EGFR tyrosine kinase inhibitors. Our observation, together with data from recent reports,[1,4] may help clarify the role of *EGFR* mutations in the development of EGFR-related non–small-cell lung cancer and help establish effective strategies against specific subtypes of non–small-cell lung cancer.

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THE AUTHORS REPLY: Dr. Toyooka and colleagues describe two patients whose lung tumors harbored a T790M mutation before treatment with chemotherapy or radiotherapy was begun and suggest that this mutation might be a marker of tumor aggressiveness as well as resistance to gefitinib therapy. In the cases we and others[1] have described, the T790M mutation was not found in specimens from untreated patients. Nevertheless, the possibilities do exist that this second mutation might be present in some tumors at a low frequency at the time of diagnosis and that tumor cells harboring the mutation might be enriched over time during treatment with gefitinib or erlotinib. By analogy, imatinib-resistant *BCR-ABL* mutations have, on occasion, been detected in specimens from patients with untreated chronic myeloid leukemia.[2,3] We agree that such interesting findings should motivate further research to improve our understanding of the role of EGFR in non–small-cell lung cancers, to encourage the development of alternative EGFR inhibitors able to overcome such resistance mutations, and to incorporate the knowledge gained into clinical treatment.

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Review Article

Throwing new light on lung cancer pathogenesis: Updates on three recent topics

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(Received October 20, 2004/Accepted December 9, 2004/Online publication 17 February, 2005)

Lung cancers have become the leading cause of cancer deaths in Japan, claiming more than 55 000 lives annually. Unfortunately, substantial improvement in terms of cure rates has not been achieved over the last two decades, although during the same period of time in-depth basic knowledge of the molecular mechanisms, which underlies carcinogenesis and progression of this deadly group of neoplasms, has accumulated at an amazing pace. It has consequently become evident that they have many shared but also distinct features, when comparisons are made not only with other common epithelial cancers of adults, such as colon cancer, but also within the various histologic types of lung cancers themselves. This review article provides an up-date on cutting-edge research into the following three different topics, from which important new insights have been obtained. The first concerns genetic instability, especially chromosome instability, and checkpoint failure in lung cancers. Second, we deal with *EGFR* mutations, which shows revealing specificities in various aspects. Finally, advances in the expression profiling analysis of both transcriptomes and proteomes of lung cancers are summarized. (*Cancer Sci* 2005; 96: 63–68)

There are over one million deaths a year worldwide due to lung cancer, making it one of the most prevalent and deadly neoplasms. There is no doubt that smoking of tobacco is the most important causative factor in its development, the disease in fact apparently being rare before the widespread use of tobacco. Japan has been experiencing a steep increase in lung cancer cases, following the footsteps of the USA and other western countries, such as the UK, where increase in tobacco consumption was subsequently followed by an abrupt rise in lung cancer occurrence. Our current problem can be regarded as a ringing alarm bell for other Asian nations, such as China, in which tobacco consumption is now rapidly increasing. Lung cancer currently claims more than 55 000 lives annually in Japan, with a no more than 15% cure rate, meaning that the number of deaths remains unacceptably high. A better understanding of the molecular pathogenesis of this fatal disease is therefore an urgent issue in order to develop better diagnostic approaches and new targeted therapies, as well as effective means for its prevention.

Lung cancers can be classified into two major entities, based on their clinicopathologic characteristics, that is, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter being further divided into three major histologic types, the adenocarcinoma, squamous cell carcinoma and large cell carcinoma. In the development of squamous carcinoma, oncogenic triggering converts normal bronchial epithelium into hyperplastic, metaplastic and dysplastic lesions, leading to the subsequent emergence of carcinoma *in situ* (CIS) and then overt squamous cell carcinomas. Adenocarcinomas are also considered to develop, at least in part, through stepwise morphological changes, with atypical adenomatous hyperplasia (AAH) generally thought to be a premalignant precursor lesion. It has been clearly demonstrated that overt cancers carry multiple genetic and epigenetic alterations occurring during initiation of carcinogenesis and subsequent progression. Since cell-cycle regulation and checkpoint functions are crucial for maintaining genomic integrity, their abrogation is thought to contribute to genomic instability, playing important roles even in the early steps of cancer development.[1] Many of the tumor suppressor genes and oncogenes altered in lung cancer are known to contribute to the regulation of cell cycle progression in either a direct or an indirect manner, and a considerable proportion of the lung cancer-related gene products are components of vital checkpoint mechanisms. It is notable that exposure to carcinogens in tobacco smoke appears to leave fingerprints of the resulting insult to the genome, for example, as distinct mutational spectra of *p53* and *KRAS*.

In this review article on the molecular pathogenesis of lung cancers, we will confine ourselves to concisely summarizing recent advances in three different topics, rather than attempting a comprehensive coverage of all the related subjects (for this purpose, see other review articles by the authors[2–4]). The three topics dealt in this article are: (i) chromosome instability and alterations of cell cycle checkpoints; (ii) the clinicopathologic impact of recent identification of frequent *EGFR* mutations in a specific type of adenocarcinomas; and (iii) accumulating new insights obtained through expression profiling analysis with both genomic and proteomic approaches.

Chromosomal instability and alterations of cell cycle checkpoints

Non-random chromosomal deletion and loss of heterozygosity (LOH) are hallmarks for the involvement of tumor suppressor genes residing in the affected chromosomal regions, while oncogene amplification can be identified by cytogenetic findings, such as homogenously staining lesions and double minutes. In contrast to certain leukemias, lymphomas and sarcomas, specific balanced translocations are not present in lung cancers, whose karyotypes are frequently very complex.[5] Loss of chromosomal arm 3p is among the first non-random genomic aberrations identified in lung cancer. In addition to this common loss of 3p, chromosomal losses are also frequent on 4q, 5q, 8p, 10q, 13q and 17p in SCLC, and 8p, 9p, 13q, 17p in NSCLC. Chromosomal gains are frequent on 3q, 5p and 8q in SCLC, and 1q, 3q, 5p and 8q in NSCLC. Allelic losses and amplifications of multiple small chromosomal regions have also been documented through detailed LOH and comparative genomic hybridization (CGH)

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 doi: 10.1111/j.1349-7006.2005.00021.x

analyses. It is expected that systematic array CGH analysis will allow further refinement of the affected regions, leading to the identification of candidate genes for the responsible targets.

The frequent presence of complex chromosomal changes suggests significant roles in the development of lung cancer, and it is well known that both numerical and structural aberrations may occur. Failure in cell cycle checkpoint control and defects in the DNA double-strand break repair system are thought to play important roles in the development of chromosome instability (CIN). We have shown that persistent numerical CIN is present in human lung cancer cell lines with a notable association with aneuploid karyotypes, and demonstrated that aneuploidy is not the result of a few past catastrophic processes of missegregations, but rather a reflection of the persistence of an unstable karyotypic state.[6] This feature appears to be common in epithelial cancers in adults, which often show complex aneuploidy. As for the underlying mechanisms, it is notable that mice, deficient in the *Mad2* mitotic checkpoint gene, which is important for correct chromosome segregation, have been shown to specifically develop lung tumors characterized by CIN.[7] In human lung cancers, mitotic checkpoint impairment appears to be present at a considerably high frequency,[8] while mutations in the *MAD1* and *BUB1* mitotic checkpoint genes have been detected in human lung cancer cells, albeit relatively rarely.[9–11] In addition, *p53* inactivation appears to play an indirect role in the acquisition of the CIN phenotype.[6]

Double strand breaks (DSB) can be introduced into genome by intracellular stresses, such as oxidative damage, as well as by environmental factors, such as ionizing irradiation, and this highly detrimental form of DNA damage potentially serves as causes of erroneous rejoining of the broken genome. Cell-cycle checkpoints are built-in safeguards to prevent damaged cells from starting DNA replication (the G1/S checkpoint), from progressing with replication (the intra S checkpoint), or from going into mitosis (the G2 checkpoint).[12] Lung cancers are known to carry frequent G1/S checkpoint abrogation due to *p53* mutations, the most frequent genetic alterations found so far in this common epithelial cancer of adults,[13] and it has been shown that the G2 checkpoint is also frequently impaired in SCLC in a histological type-specific manner.[14] Failure of G2 checkpoint activation in the presence of DNA damage would generate broken chromosomes, the fates of which include: degradation, healing as truncated forms, and incorrect fusion to generate translocated chromosomes. Fusion between two centromere-containing chromosomes can trigger the highly unstable breakage-fusion-bridge cycle. Multiple components of the DNA damage G2 checkpoint mechanism appear to be involved in the development of lung cancers. CHK1 and CHK2 are preferentially activated by their respective upstream kinases, ATR and ATM, leading to phosphorylation of Cdc25C, a phosphatase that normally activates Cdc2, and resulting in its sequestration and inactivation of Cdc25C by 14-3-3 proteins. It is interesting that *14-3-3ε* was recently shown to be involved in a homozygous deletion identified in a SCLC cell line, and that reconstitution of *14-3-3ε* partially restored its G2 checkpoint impairment.[14] Another member of the 14-3-3 family, *14-3-3σ*, which is transactivated by p53 in response to DNA damage, has been shown to contribute to the maintenance of G2 arrest through nuclear exclusion of the Cdc2/cyclin B1.[15] Notably, epigenetic inactivation of *14-3-3σ*, due to aberrant DNA hypermethylation of its promoter region, has been shown to be frequent in SCLC[16,17] as well as in a few other types of human cancers, including, breast, gastric and hepatocellular carcinomas. We have also found that *CHK2* is somatically mutated in lung cancers at a low frequency.[18,19] It remains to be investigated whether there may be an, as yet, unidentified major target gene responsible for the observed checkpoint defects, or whether there might be a large number of affected genes each playing a role in a small proportion of cases.

In addition, our recent work by Nakagawa *et al.* provided direct evidence that decatenation at the G2 checkpoint,[20] which ensures sufficient chromatid decatenation by topoisomerase II before entering into mitosis,[21] is impaired in a proportion of human lung cancer cell lines. Therefore, the G2 checkpoint may also be important. This is clinically relevant, with considerable interest in the potential association between decatenation impairment and hypersensitivity to catalytic topoisomerase inhibitors, such as ICRF-193,[20] since this could ultimately lead to the development of an attractive strategy for lung cancer treatment, that is, selective killing of targeted cancer cells without causing major toxicity in normal cells. The *CHFR* gene, which has been postulated to play a key role in the prophase checkpoint, is also known to be inactivated by DNA hypermethylation in lung cancers.[22] The findings so far obtained, clearly indicate that multiple cell cycle checkpoints are impaired in lung cancers (Fig. 1), conceivably providing a driving force to acquisition of genetic instability, including CIN, and therefore contributing to the development of lung cancers.

Specific Involvement of *EGFR* and *ras* Mutations in Lung Adenocarcinomas

In the 1980s, various oncogenes were shown to be altered in lung cancers, and the *myc* and *ras* gene families are among the best studied in relation to their pathogenesis.[23] Gene amplification

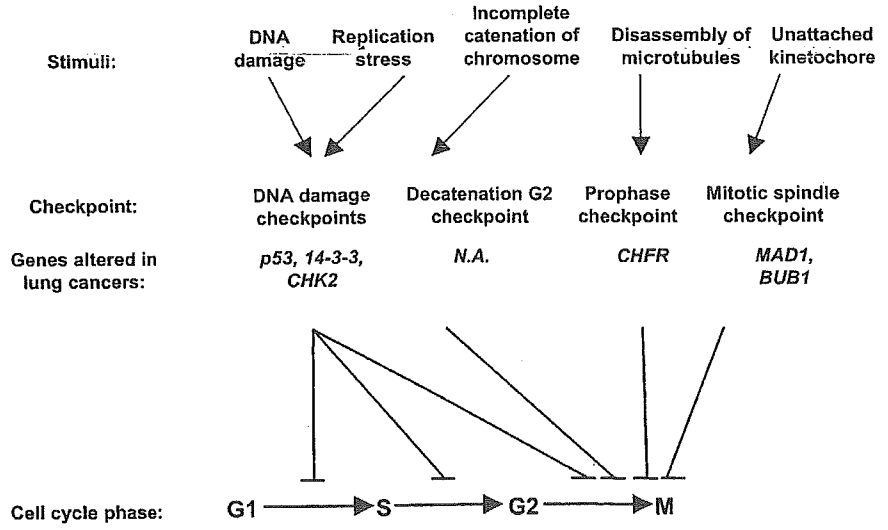

Fig. 1. Cell cycle checkpoints perturbed in human lung cancers. N.A., not available.

 doi: 10.1111/j.1349-7006.2005.00021.x

of one of the members of the *myc* family is detectable in about 25% of SCLC cell lines as well as in 5–15% of primary tumor specimens, while overexpression of one of the members is detectable in virtually all SCLC. Therefore, the vast majority of these lesions appear to have both *p53* mutations and MYC overexpression, consistent with the notion that inactivation of the ARF/MDM2/p53 pathway, which is nearly ubiquitous in SCLC, may be required for Myc-induced transformation. By contrast, *K-ras* mutations are detected almost exclusively in adenocarcinomas, usually at codon 12, in association with a poor prognosis of surgically treated cases,[24] whereas *H-ras* or *N-ras* are only very rarely mutated in any type of lung cancer. *K-ras* mutations are predominantly G to T transversions, as is also the case for *p53* mutations, implying their creation through DNA adduct formation from tobacco exposure. In addition, mutations in the *BRAF* gene are present in approximately 3% of adenocarcinomas without *K-ras* mutations,[25,26] consistent with *K-ras* and *BRAF* ultimately signaling through the same pathway.

The discovery of mutations of the two prototype tumor suppressor genes, namely *Rb* and *p53*, in 1989,[27,28] shifted much of the attention of investigators in this field, including ourselves, to the involvement of tumor suppressor inactivation, with development of candidate gene approaches and positional and functional cloning efforts. However, recent reports on *EGFR* mutations by two Boston groups have generated considerable interest because of the highly characteristic and clinically useful nature of this genetic change.[29,30] Therefore, the field of oncogene activation is being revisited in relation to lung carcinogenesis. *EGFR* mutations have been found to be more frequent in cases with an adenocarcinoma histology and female gender, and in Japanese patients (in comparison with American patients), and the presence of *EGFR* mutations was shown to have a potential predictive value for sensitivity to gefitinib (Iressa, ZD1839), a small molecule tyrosine kinase inhibitor that targets EGFR. Interestingly, Paez *et al.* observed a marked predominance of *EGFR* mutations in 26% (15 of 58) of specimens from Japanese patients, compared to only 2% (1 of 61) in a series from American patients.[30] In addition, a group at the Memorial Sloan-Kettering Cancer Center has recently confirmed the utility of detecting *EGFR* mutations as a marker for predicting responsiveness to gefitinib.[31] We have extended their findings by analyzing 277 Japanese NSCLC patients, and found *EGFR* mutations to be present exclusively in adenocarcinomas, with a single exception (an adenosquamous carcinoma case), at a frequency of 59% and 26% in female and male cases, respectively.[32] The vast majority of the mutations proved to be either deletions around codons 746–750 or a missense mutation substituting leucine with arginine at codon 858, generally affecting three functionally important structures (αC helix, activation loop, P-loop) flanking the ATP binding pocket of the tyrosine kinase domain (Fig. 2). In this connection, it is of interest that EGFR activates several downstream substrates in addition to the RAS-MAPK pathway, such as the PI3K/AKT and JAK/STAT pathways. *PIK3CA*, which encodes the p110α catalytic subunit of PI3K, is mutated in lung cancers,[33] though at low frequency, and a recent study by Sordella *et al.* showed that mutated-EGFR targets the PI3K/AKT and JAK/STAT pathways rather than the RAS-MAPK pathway.[34] The findings of a recent immunohistochemical investigation carried out by Cappuzzo *et al.* are consistent with this notion, that is, gefitinib-treated patients with P-Akt-positive tumors had a better response, disease control, and time to progression than those with P-Akt-negative tumors.[35] In addition, we have shown, for the first time, that patients with *EGFR* mutations survive for a longer period than those without *EGFR* mutations when treated with gefitinib for recurrent disease after surgery.[36] Although these findings must be confirmed in a prospective clinical trial, screening for *EGFR* mutations appears to be a promising means to target drugs to specific molecules. This may consequently become a common practice for treating pulmonary adenocarcinoma patients in the near future, making individualized therapy a reality.

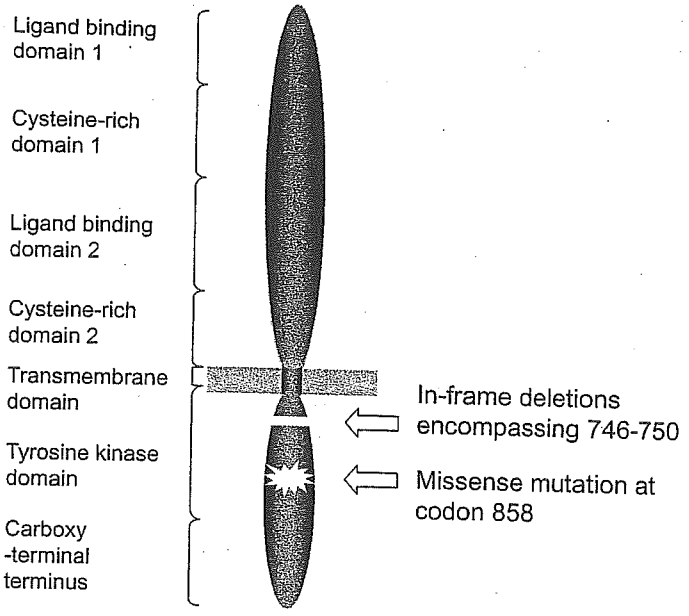

Fig. 2. Schematic diagram of the structure of EGFR as well as of the locations of two major types of mutations.

Notably, our multivariate logistic regression analysis demonstrated a non-smoking status and adenocarcinoma histology, but not female gender, to independently contribute to the occurrence of *EGFR* mutations, suggesting that the apparent difference in frequency observed between female and male may result from differences in lifestyle, including smoking habit, rather than involvement of the sex hormone-related environment, at least in the Japanese population. This is in a striking contrast to other genetic and epigenetic changes previously described in lung cancers. For instance, mutations in the *p53* and *K-ras* genes are known to be more frequent in smokers than in non-smokers.[37,38] Alterations of the *FHIT* gene encompassing a fragile site at 3p14.2, a chromosomal region frequently affected in lung cancers, are also more prevalent in smokers than in non-smokers.[39] The difference in the underlying mechanisms between *EGFR* mutations and the other genetic and epigenetic alterations reported previously, is also consistent with the occurrence of mutations in *EGFR* and *K-ras* in a mutually exclusive manner, as well as with the fact that *K-ras* mutations in adenocarcinomas without *EGFR* mutations are mostly G to T transversions, which may reflect exposure to carcinogens in tobacco smoke.[32] It is also worthy of mention that Yatabe *et al.* have found that even within adenocarcinomas, *EGFR* mutations appear to occur in a distinct subset, that is, terminal respiratory unit-type adenocarcinomas, which is a specific type of lesion conceivably arising from peripheral lung epithelial cells, including alveolar cells and non-ciliated bronchiolar epithelium.[40,41] It should be stressed that *EGFR* mutations appear to be extremely specific to lung cancers, mutations being reported to be present in none of 95 primary tumors and 108 cancer-derived cell lines, of diverse tumor types.[29] At present, it is not clear why the occurrence of *EGFR* mutations shows such specificity in terms of the smoking status and histology. In our opinion, there may be at least two possible explanations, which are not necessarily mutually exclusive. First, as yet unidentified carcinogens other than those in tobacco smoke might be involved in the development of adenocarcinomas in non-smoking individuals through the imposition of *EGFR* mutations in their genome. Cooking oil fumes or HPV 16/18

infection, both of which reportedly have associations with lung cancer occurring in non-smoking women in Taiwan, might be relevant. Alternatively, *EGFR* mutations might provide selective advantage only in peripheral lung epithelial cells. In this regard, it is interesting that EGF stimulates anchorage-independent growth of our HPL1D cell line, the only human peripheral lung epithelial line so far established.[42] These intriguing points await future clarification.

Genomic and Proteomic Profiling of Lung Cancers for Better Understanding and Future Applications

The recent rapid progress in microarray technology has made it possible to analyze gene expression profiles on a genome-wide basis in order to better understand molecular pathogenesis of human cancers, as well as to search for molecular markers for classification and prediction of outcome (Fig. 3). While lung cancers are known to be very heterogeneous in various aspects, recent expression profiling studies have clearly shown the presence of several distinct subclasses.[43–48] SCLC express a set of genes which are related to neuroendocrine differentiation, including *ASH1*, a key transcription factor that is indispensable for the development of neuroendocrine cells of the lung. Squamous cell carcinomas show marked elevation of a group of keratin isoforms related to squamous differentiation, as well as of *p63*, a *p53* homolog that is believed to play a role in squamous cell differentiation. Although these results are not unexpected, considering the characteristics evident even on routine pathology examination, it is certainly interesting that expression-profiling analysis has proven sufficiently powerful to detect the presence of distinct expression profiles, even within a subgroup of tumors with adenocarcinoma histology. Garber *et al.* identified three subgroups in adenocarcinomas through unsupervised hierarchical clustering analysis,[43] while Bhattacharjee *et al.* made a subclassification into four subgroups.[44] We have also reported the identification of four distinct subsets of adenocarcinomas based on unsupervised hierarchical clustering analysis.[48] Similarly, Virtanen *et al.* observed adenocarcinomas to form three distinct clusters.[46] Although the expression signatures identified in these studies do not show complete correspondence with each other, there is a high degree of consistence in the fact that all these studies have identified a subset of adenocarcinomas with high-level expression of genes related to differentiation of normal peripheral lung epithelial cells, such as *TTF-1* and *SP-C*. Notable features of this subclass are a significantly higher proportion in female non-/light smokers and the tumors are well-differentiated, suggesting that they may arise from cells committed to becoming peripheral lung epithelial cells, that is, terminal respiratory unit-type adenocarcinomas.[41] With these lesions, smoking presumably has no or only a weak influence. In fact, we note that these features correspond to those of adenocarcinoma cases with a high frequency of *EGFR* mutations and hence higher sensitivity to the gefitinib treatment (see above). Indeed, we observed *EGFR* mutations in 50% of the cases belonging to this cluster (unpublished observation). Therefore, it will be interesting to examine adenocarcinoma cases for expression profiles and the presence of *EGFR* mutations in a comprehensive manner in order to verify this intriguing possibility.

In addition, the advantages of expression profiling analysis have been shown through the identification of previously undefined subgroups in other types of lung cancers. The existence of two clinically relevant subsets of squamous cell carcinomas has been suggested, with distinct gene expression signatures and markedly different postoperative survival,[1,48] while two prognostically significant subtypes of high-grade neuroendocrine tumors, which appear to be independent of the currently employed pathologic subclassification, have been identified.[49] Based on the hypothesis that genes specifically expressed in various types of NSCLC may be associated with developmentally regulated genes and pathways, Borczuk *et al.* conducted an interesting exercise.[50] They found that the gene set specific for adenocarcinoma histology corresponded to those expressed in the late stage (terminal sac and alveolar stages) of murine lung development, whereas the large-cell carcinoma set was associated with genes expressed in the earlier pseudoglandular and canalicular stages. It is also interesting to note that a metastatic signature, obtained by comparing expression profiles of adenocarcinoma metastases of multiple tumor types to unmatched primary adenocarcinomas, was shown to be associated with a poor prognosis,[51] as to be expected from the fact that deaths from lung adenocarcinomas are in most cases attributable to metastasis.

Expression profiling analysis, aimed at individualized patient outcome prediction, is another important and much needed application, since survival or death is a matter of all or nothing, and currently available information regarding what percentage of those at a certain disease stage are likely to survive after a

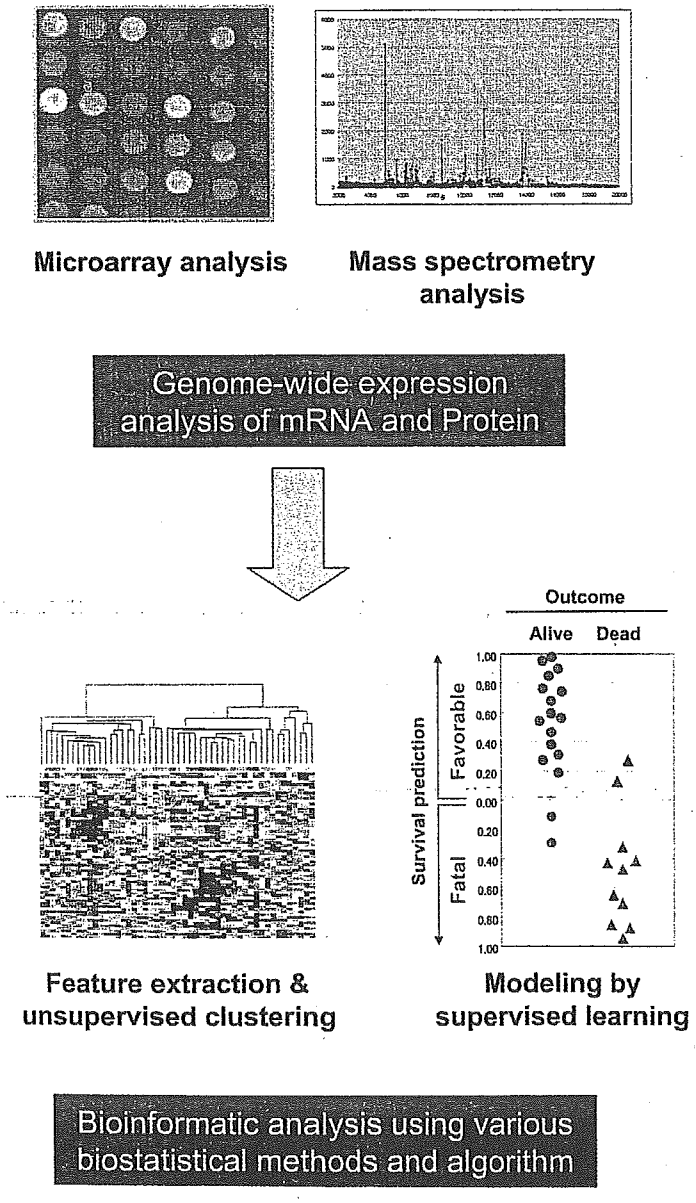

Fig. 3. Expression profiling analyzes of both transcriptome and proteome of lung cancers.

 doi: 10.1111/j.1349-7006.2005.00021.x

certain period of time is insufficient in many respects. In this regard, there are two good examples of obvious benefit. Beer *et al.* identified prognosis-related genes using the *t*-test, and constructed a prognosis prediction classifier of adenocarcinomas by defining a risk index as a linear combination of gene-expression values for selected genes weighted by their estimated regression coefficient in the preceding COX regression analysis.[45] We have also succeeded in constructing individualized outcome classifiers of NSCLC, using gene expression profiling data- and a weighted-voting algorithm-based approach with genes selected by the signal-to-noise metric.[48] Reasoning from potential differences in outcome-related expression signatures in two major histologic types of NSCLC, histologic type-specific outcome classifiers were further constructed, showing an accuracy of more than 90% for the prediction of 5-year survival or death. A study by Endoh *et al.*, in which they selected 44 candidate genes from those previously identified through exploratory expression profiling analysis, which was validated by real-time reverse transcriptase-polymerase chain reaction using a completely separate large cohort, points to a sensible direction for the next step aimed at translation into the clinical setting.[52] It can be envisaged that the establishment of new disease classifications and highly accurate, individualized outcome classifiers for identifying those who are at high risk of future failure, and therefore most eligible for intensive adjuvant therapy with the intention of eradicating undetectable micrometastases, sources of future recurrence, would be a realistic immediate goal. In addition, expression profiling employing sophisticated bioinformatic analyzes should soon transform current strategies for clinical evaluation of drug combinations for cancer treatment, making it possible to provide more sound treatment decisions than in current practice.

In addition to analyzing global changes in the transcriptome, recent advances in biomedical technology have enabled new proteomic approaches to be developed. Yanagisawa *et al.* used matrix-assisted laser desorption/ionization mass spectrometry to identify more than 1 600 protein peaks from 79 cases of lung tumors.[53] Biostatistical selection of differentially expressed peaks allowed discrimination between normal and malignant tissue, subclassification of primary tumors, identification of patients with nodal involvement, and classification according to their prognosis based on 15 distinct peaks on mass spectrometry. Protein profiling of serum or plasma using high throughput mass spectroscopy has also been reported for lung cancers, distinguishing cases and controls based on key protein patterns with 50–70% detection rates and a 10% false positive rate.[54] Although anonymous peaks or protein profile spectra might be useful for classification, identification and functional investigation of these proteins are essential for understanding the underlying molecular biology. In this regard, we should mention that candidate molecular markers have been identified,[53,55] and far more examples will be found with recently developed sophisticated technologies for proteomic analysis, such as multidimensional liquid chromatography combined with tandem mass spectrometry. This should make it possible to further extend appropriate means of generating and exploiting new biological insights and clinical applications for the benefit of patients.

Conclusions

Accumulating evidence clearly indicates that perturbation of the integrity of the genome leads to the genesis and progression of lung cancers. It is becoming evident that the sequential accumulation of various genetic and epigenetic alterations confers various capabilities on lung cancer cells, including escape from growth inhibitory signals as well as from excess shortening of telomeres, resistance to apoptosis, sensitivity to stimuli for proliferation and angiogenesis, and invasive and metastatic characteristics. In the coming decades, taking advantage of the ample information on the human genome sequence as well as emerging new technologies including sophisticated informatics, we should be able to acquire a complete picture of lung cancer biology and revolutionize the prevention, diagnosis and treatment strategies for this presently fatal disease.

Acknowledgment

We are grateful to all members of our lung cancer research group at Aichi Cancer Center, not only for helpful discussion and comments on the manuscript, but also for continuing fruitful collaborations for many years.

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 doi: 10.1111/j.1349-7006.2005.00021.x

VOLUME 23 · NUMBER 11 · APRIL 10 2005

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Mutations of the Epidermal Growth Factor Receptor Gene Predict Prolonged Survival After Gefitinib Treatment in Patients With Non–Small-Cell Lung Cancer With Postoperative Recurrence

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Submitted September 10, 2004; accepted January 11, 2005.

Supported in part by Grant-in-Aid (16591424) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Terms in blue are defined in the glossary, found at the end of this issue and online at www.jco.org.

Authors' disclosures of potential conflicts of interest are found at the end of this article.

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0732-183X/05/2311-2513/$20.00

DOI: 10.1200/JCO.2005.00.992

ABSTRACT

Purpose
To evaluate the relationship between mutations of the epidermal growth factor receptor (*EGFR*) gene and the effectiveness of gefitinib treatment in patients with recurrent lung cancer after pulmonary resection.

Patients and Methods
We sequenced exons 18-21 of the *EGFR* gene using total RNA extracted from 59 patients with lung cancer who were treated with gefitinib for recurrent lung cancer. Gefitinib effectiveness was evaluated by both imaging studies and change in serum carcinoembryonic antigen (CEA) levels.

Results
EGFR mutations were found in 33 patients (56%). Of these mutations, 17 were deletions around codons 746-750 and 15 were point mutations (12 at codon 858, three at other codons), and one was an insertion. *EGFR* mutations were significantly more prevalent in females, adenocarcinoma, and never-smokers. Gefitinib treatment resulted in tumor shrinkage and/or CEA decrease to less than half of the baseline level in 26 patients, tumor growth and/or CEA elevation in 24 patients, and gefitinib effect was not assessable in nine patients. Female, never-smoking patients with adenocarcinoma tended to respond better to gefitinib treatment. Gefitinib was effective in 24 of 29 patients with *EGFR* mutations, compared with two of 21 patients without mutations ($P < .0001$). Of note, del746-750 might be superior to L858R mutations for prediction of gefitinib response. Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment ($P = .0053$).

Conclusion
EGFR mutations were a good predictor of clinical benefit of gefitinib in this setting.

J Clin Oncol 23:2513-2520. © 2005 by American Society of Clinical Oncology

INTRODUCTION

Lung cancer has long been the leading cause of cancer death in North America. In 1998, it became the leading cause of cancer death in Japan, and now claims more than 55,000 lives annually.[1] Lung cancer is divided into two morphologic types: small-cell lung cancer and non–small-cell lung cancer (NSCLC). NSCLCs are further subdivided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Adenocarcinoma is the predominant histologic subtype, and is increasing among patients with lung cancer who are candidates for surgical treatment in Japan. In our institution, adenocarcinoma accounted for 76% of 407 patients who were operated on from 2001 through 2003. Adenocarcinomas are characterized by a high degree of morphologic heterogeneity. Analyses of various cancer-associated genes, including *K-ras*,[2] *p53*,[3,4] cyclin D1,[5] $p27^{Kip1}$,[6] and cyclooxygenase-2,[7]

suggests a different molecular pathway for carcinogenesis in lung adenocarcinomas at least partly accounts for this heterogeneity. In addition, the NSCLC frequently overexpresses receptors of the ErbB family, including the epidermal growth factor receptor (EGFR) encoded by ErbB1 (HER-1).[8,9]

EGFR is a 170 kd receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues upon binding of several specific ligands including epidermal growth factor and transforming growth factor alpha.[8] These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes.[8] Downstream pathways include ras-raf-MEK-ERK, phosphatidylinositol-3 kinase-Akt, and PAK-JNKK-JNK.[8]

Gefitinib is an orally bioavailable small molecule that specifically inhibits EGFR tyrosine phosphorylation.[10] Clinical trials revealed that there is significant variability in response to gefitinib. Good clinical responses have been observed most frequently in women, in nonsmokers, in patients with adenocarcinomas, and in Japanese patients.[11,12] However, it was not possible to predict gefitinib sensitivity by levels of EGFR overexpression as determined by immunohistochemistry[13] or immunoblotting.[14] The factors that determine gefitinib sensitivity have long been an enigma. Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib[15-17] or erlotinib, another EGFR TK inhibitor. Furthermore, the incidence of *EGFR* mutations is significantly higher in female, never-smoking, Japanese patients with adenocarcinoma.[15] These features coincide with those of good responders to gefitinib.

In this study, we studied patients who had recurrent disease after pulmonary resection for NSCLC and who were subsequently treated with gefitinib. We searched for mutations of the *EGFR* gene in tumor specimens taken at the time of surgery and we correlated *EGFR* mutations with gefitinib effectiveness, including tumor response and patient survival.

PATIENTS AND METHODS

Patients

Seventy-five patients were treated with gefitinib for their recurrent diseases after they had undergone surgery between 1999 and 2003. We studied 59 patients whose tumors were available for RNA extraction, which was a sole determinant of inclusion into the present study. There were 32 men and 27 women with ages ranging from 48 to 79 years. Fifty patients had adenocarcinomas, five had squamous cell carcinomas, three had large-cell carcinomas, and one had adenosquamous carcinoma. Eight patients had stage IA disease; seven stage IB; three stage IIA; five stage IIB; 24 stage IIIA; eight stage IIIB; and three stage IV at the time of surgery. Lobectomy had been performed in 57, and pneumonectomy and partial resection in one patient each. Four patients received postoperative adjuvant chemotherapy (two with oral uracil/tegafur and two with gemcitabine monotherapy). Forty patients had had chemotherapy before gefitinib treatment (23 patients, platinum doublet; 16 patients, monotherapy with vinorelbine or gemcitabine, one patient, oral uracil/tegafur). Gefitinib treatment with a daily dose of 250 mg was initiated between July 2002 and May 2004, with the median interval between operation and gefitinib treatment being 778 days (range, 107 to 1,931 days). Fifty patients had distant metastatic tumors, eight patients had pleural dissemination and malignant effusion, and one patient had hilar lymph-node metastasis at initiation of gefitinib treatment.

Molecular Analysis of Lung Cancer Specimens

After we obtained appropriate approval from the institution and written informed consent for comprehensive use of molecular and pathologic analysis from the patients, tumor samples were collected during surgery, rapidly frozen in liquid nitrogen and stored at −80°C. A surgical pathologist (Y.Y.) grossly dissected the frozen tumor specimens to enrich the tumor cell population as much as possible. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18-21) of the seven exons (exons 18-24) that code for TK domain of the *EGFR* gene (which includes all the mutations reported so far[15-17]) was amplified with primers F1 (5′-AGCTTGTGGAGCCTCTTACACC-3′) and R1 (5′-TAAAATTGATTCCAATGCCATCC-3′) in a one-step reverse transcription polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (Qiagen). The cDNA sequence of the *EGFR* gene was obtained from GenBank (accession number NM 005228). The RT-PCR conditions were: one cycle of 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 50 seconds, 62°C for 50 seconds, and 72°C for 60 seconds, followed by one cycle of 72°C for 10 minutes.

RT-PCR products were diluted and cycle-sequenced using the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST (basic local alignment search tool) and chromatograms by manual review. High-quality sequence variations found in both directions were scored as candidate mutations.

Definition of Effectiveness of Gefitinib

Because this study was a retrospective analysis of the daily clinical practice of oncology, the evaluation of tumor response could not be performed strictly according to predefined criteria, such as Response Evaluation Criteria in Solid Tumors (RECIST).[18] RECIST are not necessarily applicable or complete in such a context and the evaluation may instead be based on a subjective medical judgment that results from clinical and laboratory data.[18] Therefore, gefitinib treatment was judged as effective when the tumors showed at least a 30% decrease in tumor diameter in imaging studies. However, because of the nature of the study, confirmation of tumor response no less than 4 weeks apart, as in RECIST,[18] was not necessarily required.

As patients with recurrent lung cancer often do not have measurable disease, we also included change in serum carcinoembryonic antigen (CEA) level (cut off, 5 ng/mL) as an evaluation