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厚生労働科学研究費補助金
医療技術実用化総合研究事業

RET融合遺伝子陽性の
進行非小細胞肺癌に対する
新規治療法の確立に関する研究

平成24年度　総括研究報告書

研究代表者　後藤　功一

平成25（2013）年　4月

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目　　次

Ｉ．総括研究報告

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新規治療法の確立に関する研究 ---------------------- 1

後藤功一

II. 研究成果の刊行に関する一覧表 ----------------------- 5

III. 研究成果の刊行物・別刷 ---------------------------------- 7

Ⅰ．総括研究報告

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
(総括)研究報告書

RET 融合遺伝子陽性の進行非小細胞肺癌に対する新規治療法の確立に関する研究

研究代表者　後藤　功一
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研究要旨

2012 年に我が国で発見された肺癌の新規遺伝子異常である RET 融合遺伝子の臨床応用を目指した研究を実施した。RET 融合遺伝子陽性肺癌（RET 肺癌）は全肺癌の 1-2%と頻度が低いため、まず、全国規模の遺伝子診断ネットワーク（LC-SCRUM-Japan）を立ち上げ、実際に臨床検体を用いた遺伝子スクリーニングを平成 25 年 2 月より開始した。同時に、基礎研究で RET 肺癌に対する有効性が確認された Vandetanib の医師主導治験を世界で初めて開始した。平成 25 年 3 月 13 日現在、LC-SCRUM-Japan には 84 施設が参加し、約 30 検体の遺伝子解析が行われ、RET 肺癌が 1 例スクリーニングされている。

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A. 研究目的

希少疾患である RET 融合遺伝子陽性の進行非小細胞肺癌を対象に、国内未承認の医薬品である RET チロシンキナーゼ阻害薬 Vandetanib(治験成分記号：ZD6474）の薬事承認申請を目指した多施設共同非無作為化非盲検第Ⅱ相試験（医師主導治験）を実施する。

B. 研究方法

RET 融合遺伝子陽性の非小細胞肺癌の頻度は、肺癌全体の 1-2%と非常に低いため、患者のスクリーニングが重要となる。このため、日本全体から多施設が参加する遺伝子診断ネットワークを構築し、この中で「RET 融合遺伝子陽性肺癌の臨床病理学的、分子生物学的特徴を明らかにするための前向き観察研究」に基づいて遺伝子解析を行う。施設倫理委員会で本研究が承認された施設のみ、本研究へ

の参加が可能とした。RET融合遺伝子の診断は、国立がん研究センターで開発したRT-PCR法、FISH法を用いて行うこととし、この診断技術を株式会社エスアールエル（SRL）へ技術移管し、実際の臨床検体に基づいた遺伝子診解析はSRLが行った。遺伝子診断ネットワークでスクリーニングされたRET融合遺伝子陽性肺癌は、医師主導治験実施7施設（国立がん研究センター東・中央病院、がん研有明病院、静岡がんセンター、兵庫県立がんセンター、四国がんセンター、九州がんセンター）において、Vandetanibの投与が可能となるように、以下の臨床試験を計画した。

「RET融合遺伝子陽性の局所進行/転移性非扁平上皮非小細胞肺癌患者を対象としたVandetanib（ZD6474）の多施設共同非無作為化非盲検第Ⅱ相試験」のプライマリーエンドポイントは、奏効割合。セカンダリーエンドポイントは、無増悪生存期間、病勢制御割合、奏効期間、全生存期間、有害事象、前・後化学療法の有効性とした。予定登録数17例、登録期間2年、追跡期間1年であり、主な適格規準は、1)年齢20歳以上、2)扁平上皮癌以外の非小細胞肺癌、3)局所療法不能のIII期又はⅣ期、4)RET融合遺伝子陽性(RT-PCR法及びFISH法でいずれも陽性)、5)EGFR遺伝子変異陰性、ALK融合遺伝子陰性、6)1レジメン以上の化学療法を実施後、7)PS=0-2、8)測定可能病変あり、9)主要臓器機能が保持、10）患者本人から文書による同意が必要とした。治療方法は、21日を1コースとして、Vandetanib 300mgを1日1回朝食後に経口投与として、疾患の増悪、または許容できない毒性が認められるまで投与を継続することにした。Vandetanib（治験成分記号：ZD6474）は、治験薬提供者であるアストラゼネカ株式会社から無償提供され、治験薬の製造の記録、品質保証の記録も併せて提供される。医師主導治験への登録、モニタリング、安全性情報の管理、データセンター、統計解析については、国立がん研究センター東病院 臨床開発センター 臨床試験支援室において行うこととした。

倫理面への配慮としては、患者の人権保護のため、本医師主導治験に関係するすべての研究者は、ヘルシンキ宣言、ICH Harmonized Tripartite Guidelines for Good Clinical Practice、「医薬品の臨床試験の実施の基準に関する省令」（平成9年厚生省令第28号）およびその改正、関連通知を遵守して本治験を実施する。医師主導治験を実施するにあたり、治験実施計画書、説明同意文書等の関連文書は事前に、「医薬品の臨床試験の実施の基準に関する省令」（平成9年厚生省令第28号）に規定する治験審査委員会の承認を取得した。患者への説明は、治験審査委員会で承認が得られた研究の内容、費用及び補償の有無、利益相反の有無等について記載された説明文書を用いて行い、登録前に充分な説明と理解に基づく自発的同意を本人より文書で得ると規定した。また、本医師主導治験が適正に行われていることを確保するため、中央モニタリングに加えて原資料との照合を行う施設訪問モニタリングをサンプリングにて実施することを規定した。監査は、アストラゼネカ社が行う。データマネージメントはデータセンターで行い、データの取り扱い上、患者氏名等直接個人が識別できる情報を用いず、かつデータベースのセキュリティを確保し、個人情報保護を厳守した。

更に、薬事承認後の実地診療における確実な患者選択のために、RET融合遺伝子のコンパニオン診断薬の開発も同時に行うこととした。本研究では、RT-PCR法、FISH法を用いて遺伝子診断を行い、患者のスクリーニングを行うが、新鮮凍結検体からRNAの抽出が必要となるRT-PCR法は、臨床現場では実施困難な場合が多いと想定されるため、ターゲットキャプチャー法を応用したゲノムDNAからのRET融合遺伝子診断法の開発も同時に行った。このため、スクリーニングのために全国から収集した検体は保存し、今後のコンパニオン診断薬の開発のために二次利用することにした。

C. 研究結果

平成24年10月に「RET融合遺伝子陽性肺癌の臨床病理学的、分子生物学的特徴を明らかにするための前向き観察研究」の研究計画書を作成し、本研究に基づいたRET融合遺伝子陽性肺癌のスクリーニング組織として、Lung Cancer Genomic Screening Project for Individualized Medicine in Japan (LC-SCRUM-Japan)を組織し、平成25年1月19日にキックオフミーティングを開き、2月7日より遺伝子スクリーニングが開始となった。平成24年11月よりLC-SCRUM-Japanへの参加希望を募ったところ、84施設の参加希望があり（平成25年3月13日現在)、予定通り全国規模の遺伝子診断ネットワークが構築されている。本研究計画が倫理審査委員会で承認された35施設(平成25年3月13日現在）において、2月下旬より本格的に臨床検体を用いた遺伝子スクリーニングが開始となり、平成25年3月13日現在、29例の登録が完了し、5例が登録予定である。初回のRT-PCR法の結果では、RET融合遺伝子1名、ROS1融合遺伝子1名が陽性と診断され、現在FISH法による確認が行われている。

医師主導治験に関しては、平成24年4月にプロトコールコンセプトがアストラゼネカ株式会社で承認され、治験薬の無償提供が決定した。4月16日にPMDAの事前面談を受けた後、Vandetanibの薬事承認を目指した医師主導治験が実施可能な7施設（国立がん研究センター東・中央病院、がん研有明病院、静岡がんセンター、兵庫県立がんセン

ター、四国がんセンター、九州がんセンター）で治験実施組織を構築した。9月に英文プロトコール、10月に日本語プロトコールを作成し、アストラゼネカ社の承認、医師主導治験参加施設の承認を経て、12月に「RET融合遺伝子陽性の局所進行/転移性非扁平上皮非小細胞肺癌患者を対象としたVandetanib（ZD6474）の多施設共同非無作為化非盲検第II相試験」の治験実施計画書が完成した。11月19日にPMDAの薬事戦略相談を受け、12月から治験実施各施設の倫理審査委員会において審査が開始となり、平成25年3月13日現在、4施設で本治験の承認が得られている。平成25年1月29日に治験計画届けを厚生労働省へ提出し、2月より治験薬の搬入が開始となり、2月21日に患者登録を開始した。

D. 考察

肺癌はがん死亡原因第一位の難治性がんであり、2010年の年間死亡者数は約7万人で、がん死亡の約2割を占めている。非小細胞肺癌（主に腺癌、扁平上皮癌、大細胞癌）は、肺癌全体の約85%を占めるが、約2/3は発見時にすでに切除不能の進行癌であり、これらの患者に対しては化学療法が行われる。しかし、非小細胞肺癌は一般に化学療法の感受性が低く、現在の化学療法による治療成績は1年生存率が約40%と不良であり、非小細胞肺癌の治療成績の向上のためには優れた分子標的薬による個別化治療の開発が不可欠である。

近年、非小細胞肺癌における個別化治療の標的となる遺伝子異常（EGFR遺伝子変異、ALK融合遺伝子）が同定され、EGFR遺伝子変異例に対するEGFRチロシンキナーゼ阻害薬（ゲフィチニブ、エルロチニブ）や、ALK融合遺伝子陽性例に対するALKチロシンキナーゼ阻害薬（クリゾチニブ）の臨床応用によって、従来の化学療法と比較して、著しい治療成績の改善が認められている。

RET融合遺伝子は、2012年に報告された非小細胞肺癌の新しい遺伝子異常であり、新規の治療標的となることが期待される。RET融合遺伝子陽性の非小細胞肺癌は、肺癌全体の1-2%と頻度は低いが、基礎研究においてRETチロシンキナーゼ阻害薬であるVandetanibの有効性が確認されており、臨床試験に基づいたVandetanibの有効性の確認、早期臨床応用が期待されている。

Vandetanibはアストラゼネカ株式会社が開発中の国内未承認薬である。既に米国FDAでは、2011年4月に切除不能または進行性の甲状腺髄様癌に対する治療薬として承認されている。国内では、肺癌を含む固形がんを対象に第I相試験が行われ、推奨用量は海外と同じ300mg/日と設定され、更に、進行非小細胞肺癌に対する第II相試験において、主な有害事象は下痢、皮疹、高血圧、頭痛などで、これまでの報告とほぼ同じであり、安全性の確認は完了している。

RET融合遺伝子陽性の非小細胞肺癌は肺癌全体の1-2%という稀少疾患であるため、早期の治療開発は医師主導治験以外では困難であり、Vandetanibの有効性を評価して薬事承認申請を目指す本医師主導治験の実施が必須となる。このVandetanibの有効性を評価する本試験は、世界初の試みであると同時に、今後も明らかになる新たな遺伝子異常を伴う希少がんに対する分子標的治療薬の開発方法を考える上で、非常に重要な意味を持つと考えられる。

更に、本研究において、我が国初の全国規模の遺伝子診断ネットワークLC-SCRUM-Japanが構築されたことは非常に意義が大きい。少数施設で1-2%の頻度の肺癌をスクリーニングして、新規治療法の開発を実施するのは不可能なため、希少肺癌においては、従来とは異なる開発方法が模索されてきた。また、希少肺癌の治療開発においては、正確な診断、スクリーニングが可能となるコンパニオン診断薬の同時開発が必須とされている。今後、LC-SCRUM-Japanは、遺伝子異常に基づいた他の希少肺癌のスクリーニングにも応用可能であり、また、全国から集めた多くの検体を利用し、コンパニオン診断薬の開発が実施可能な組織として、更に必要性が高まっていくと予測される。

E. 結論

本研究では、2012年に我が国で発見された肺癌の新規遺伝子異常であるRET融合遺伝子の臨床応用を目指した研究を行った。今年度は、全国規模の遺伝子診断ネットワークであるLC-SCRUM-Japanを立ち上げ、実際に臨床検体を用いた遺伝子解析を開始した。同時に、RET融合遺伝子陽性肺癌の治療薬であるVandetanibの医師主導治験を世界で初めて開始した。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   知財(特許出願)
   なし
2. 実用新案登録
   なし
3. その他
   なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Goto K, Satouchi M, Ishii G, Nishio K, Hagiwara K, Mitsudomi T, Whiteley J, Donald E, McCormack R, Todo T.	An evaluation study of EGFR mutation tests utilized for non-small-cell lung cancer in the diagnostic setting.	Ann Oncol.	23(11)	2914-9	2012
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Yoshida A, Kohno T, Tsuta K, Wakai S, Shimada Y, Arai Y, Asamura H, Furuta K, Shibata T, Tsuda H.	ROS1-rearranged lung cancer: clinicopathologic and molecular study of 15 surgical cases.	Am J Surg Pathol			2013 [Epub ahead of print]
Kohno T, Ichikawa H, Totoki Y, Yasuda K, Hiramoto M, Nammo T, Sakamoto H, Tsuta K, Furuta K, Shimada Y, Iwakawa R, Ogiwara H, Oike T, Enari M, Schetter AJ, Okayama H, Haugen A, Skaug V, Chiku S, Yamanaka I, Arai Y, Watanabe S, Sekine I, Ogawa S, Harris CC, Tsuda H, Yoshida T, Yokota J, Shibata T.	KIF5B-RET fusions in lung adenocarcinoma.	Nature Medicine	18	375-377	2012

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Ⅲ．研究成果の刊行物・別刷

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An evaluation study of *EGFR* mutation tests utilized for non-small-cell lung cancer in the diagnostic setting

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Background: Epidermal growth factor receptor (*EGFR*) mutation is predictive for the efficacy of EGFR tyrosine kinase inhibitors in advanced non-small-cell lung cancer (NSCLC) treatment. We evaluated the performance, sensitivity, and concordance between five *EGFR* tests.
Materials and methods: DNA admixtures ($n = 34$; 1%–50% mutant plasmid DNA) and samples from NSCLC patients [116 formalin-fixed paraffin-embedded (FFPE) tissue, 29 matched bronchofiberscopic brushing (BB) cytology, and 20 additional pleural effusion (PE) cytology samples] were analyzed. *EGFR* mutation tests were PCR-Invader®, peptide nucleic acid-locked nucleic acid PCR clamp, direct sequencing, Cycleave™, and Scorpion Amplification Refractory Mutation System (ARMS)®. Analysis success, mutation status, and concordance rates were assessed.
Results: All tests except direct sequencing detected four mutation types at ≥1% mutant DNA. Analysis success rates were 91.4%–100% (FFPE) and 100% (BB and PE cytology), respectively. Inter-assay concordance rates of successfully analyzed samples were 94.3%–100% (FFPE; kappa coefficients: 0.88–1.00), 93.1%–100% (BB cytology; 0.86–1.00), and 85.0%–100% (PE cytology; 0.70–1.00), and 93.1%–96.6% (0.86–0.93) between BB cytology and matched FFPE.
Conclusions: All *EGFR* assays carried out comparably in the analysis of FFPE and cytology samples. Cytology-derived DNA is a viable alternative to FFPE samples for analyzing *EGFR* mutations.
Key words: cytology, EGFR mutation, FFPE, NSCLC, PCR

introduction

Epidermal growth factor receptor (*EGFR*) mutation is a key predictive factor for the efficacy of EGFR tyrosine kinase inhibitors in the treatment of patients with advanced non-small-cell lung cancer (NSCLC) [1–3]. *EGFR* mutation testing is necessary to enable the physician to offer the most suitable therapy for a patient with advanced NSCLC.

Four *EGFR* mutation tests, PCR-Invader® [4], peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp [5], PCR direct sequencing [6], and Cycleave PCR™ [7] are used commercially in Japan, with testing generally carried out by centralized contracted laboratories. The Scorpion Amplification Refractory Mutation System (ARMS)® [8] is another sensitive globally available method and in particular was used in the phase III Iressa Pan-Asia Study (IPASS) to determine *EGFR* mutation status [1, 9]. A variety of methods, including direct sequencing, PCR-Invader, PNA-LNA PCR clamp, fragment analysis, and Cycleave PCR, were used in the WJTOG3405 phase III study to select *EGFR* mutation-positive patients [2], and the PNA-LNA PCR clamp method was used in the NEJ002 study [3]. To date, a study to compare the sensitivity and concordance of methods for *EGFR* mutation testing in Japan has not been conducted.

Diagnostic practices, and therefore, samples available for *EGFR* mutation analysis, differ between laboratories and countries. Large surgical samples are optimal for *EGFR* mutation analysis but small tissue from a tumor biopsy is the most commonly used and preferred sample type for diagnosis by clinicians [10, 11]. In clinical practice, tissue samples are not always available for diagnosis, and cytology samples, including bronchofiberscopic brushing (BB) cytology and pleural effusion cytology samples, are used in Japan and increasingly globally.

The aim of this study was to evaluate the sensitivity and performance of different *EGFR* mutation tests using artificial DNA admixtures, and clinical samples including formalin-fixed

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paraffin-embedded (FFPE) tissue, BB cytology, and pleural effusion cytology samples from patients with NSCLC.

materials and methods

This was an observational study using control DNA admixtures and clinical samples. Patients provided written informed consent for samples to be used in research. The study was conducted as a collaborative research of AstraZeneca KK with National Cancer Center Hospital East (NCCHE) and Hyogo Cancer Center (HCC) after protocol approval by each Institutional Review Board and was conducted in accordance with ethical guidelines for epidemiological studies.

samples and DNA extraction

DNA admixtures

Four types of mutant plasmids were prepared including the *EGFR* mutation L858R, T790M, G719S, and E746-A750 deletion (nt del 2234-2249) in the Blue Heron pUC plasmid by Invitrogen Inc. (Tokyo, Japan). The sequence inserted into each plasmid corresponded with the longest sequence requirements spanning the exons across all of the methods to be evaluated, from −300 to +220 bp for exon 18 (for G719S) and from −200 to +200 bp for exons 19, 20, and 21 (for E746-A750 deletion, T790M, and L858R, respectively). Admixtures were prepared at Saitama Medical University Hospital. The plasmid preparations (5.4 ng/μl) were diluted with water and whole-human genomic DNA (12.5 ng/μl) (Promega Inc., Madison, WI) to prepare an admixture containing a 1 : 1 ratio (confirmed by Sanger sequencing) of copies of mutated and wild-type *EGFR* (5.4 fg/μl plasmid DNA, 10 ng/μl genomic DNA; referred to here as a 100% admixture). The 100% admixture solution was then diluted with genomic DNA to provide DNA solutions simulating those isolated from a clinical sample containing *EGFR*-mutated and wild-type cells at ratios of 50 : 50 (50% admixture), 25 : 75 (25%), 10 : 90 (10%), 5 : 95 (5%), 2 : 98 (2%), and 1 : 99 (1%). The samples were divided into aliquots for each laboratory, randomized and assigned an identification code, and 20 μl of each sample sent to the laboratories for mutation testing in a blinded manner. Ten wild-type control samples (from a single stock of genomic DNA) (10 ng/μl) were also distributed for testing.

formalin-fixed paraffin-embedded samples

In total, 120 FFPE NSCLC samples collected at NCCHE ($n = 100$) and HCC ($n = 20$) between December 2005 and October 2009 were used. Twelve consecutive sections (5-μm thickness), prepared by Sanritsu Co. Ltd (Tokyo, Japan) from each FFPE tissue block, were allocated as follows: sections 1 and 12, hematoxylin–eosin (H&E) staining; sections 2 and 7, PCR direct sequencing; sections 3 and 8, Cycleave PCR; sections 4 and 9, PCR-Invader; sections 5 and 10, PNA-LNA PCR clamp; sections 6 and 11, Scorpion ARMS. Samples were randomly assigned an identification code by Sanritsu Co. Ltd, with separate identification codes for the samples for PCR direct sequencing and Cycleave PCR (as they were to be analyzed by the same laboratory). A table of corresponding randomized identification codes was retained by AstraZeneca KK until analysis. H&E-stained sections (Sanritsu Co. Ltd) were reviewed by a single pathologist at NCCHE for histological type, tumor cell content, and tumor dimension in a blinded manner. DNA was extracted at each testing laboratory using their own standard operating procedures (SOPs), all of which utilized the QIAamp kit (QIAGEN Japan, Tokyo, Japan) (see supplemental Methods, available at *Annals of Oncology* online).

bronchofiberscopic brushing cytology samples

Thirty BB cytology samples (with matched FFPE samples available) obtained at NCCHE ($n = 10$) and HCC ($n = 20$) between 2006 and 2009 were used. Samples were collected by exfoliative cytodiagnosis brushing or curette washing in saline solution, without anticoagulant, and stored frozen. The BB cytology samples were randomized and assigned an identification code. The presence of tumor cells and histological type were confirmed by a pathologist at each center. DNA was extracted (QIAamp DNA Mini kit, QIAGEN Japan) at Kinki University of Medicine (Department of Genome Biology) and divided into 22 μl aliquots for analysis by the testing laboratories (direct sequencing was excluded due to the small amount of DNA anticipated, and for Scorpion ARMS, if the DNA concentration was <1 ng/μl, only exon 19 deletions, L858R, and T790M mutations were analyzed—see supplemental Methods, available at *Annals of Oncology* online).

pleural effusion cytology samples

Pleural effusion cytology samples were provided by NCCHE. Twenty pleural effusion cytology samples were collected from patients diagnosed with NSCLC (adenocarcinoma) between February 2009 and February 2010 and confirmed by a pathologist to contain tumor cells. Samples were frozen within 10 and 30 min of sampling and stored at −80°C. Frozen samples were thawed at 37°C and refrozen rapidly three times to disrupt the cells and ensure an even distribution and then divided into five equal aliquots that were sent to each of the testing laboratories. Samples were randomly assigned an identification code as for the FFPE samples. DNA was extracted at each laboratory using their own SOPs, all of which were based on the use of the QIAamp kit (see supplemental Methods, available at *Annals of Oncology* online).

EGFR mutation analysis

Samples were analyzed using five different *EGFR* mutation tests carried out by four different testing laboratories: PCR-Invader [4, 12] by BML Inc. (Tokyo, Japan); PNA-LNA PCR clamp [5] by Mitsubishi Chemical Medience Corp. (Tokyo, Japan); PCR direct sequencing (with the exception of the BB cytology samples, due to the anticipated tumor DNA yield based on published evidence regarding the detection limit of this method [13]) [6] by SRL Inc. (Tokyo, Japan), Cycleave PCR [7] also by SRL Inc., and Scorpion ARMS [14, 15] by Genzyme Analytical Services (Los Angeles, CA). Scorpion ARMS analysis employed the DxS *EGFR* Mutation Test Kit for research use only [QIAGEN Manchester (formerly DxS Ltd), UK] and was carried out according to the manufacturer's instructions with modifications described in the supplemental Methods (available at *Annals of Oncology* online). The other methods were carried out using each of the laboratories' experimental set up, with data analysis and quality control completed according to their own specific protocols (further details in the supplemental Methods, available at *Annals of Oncology* online). Samples were defined as mutation negative where sufficient material was present to generate a result but the presence of a mutation was not observed within the detection limit of the assay. The *EGFR* mutations detected by each *EGFR* mutation test are shown in supplemental Table S1 (available at *Annals of Oncology* online).

Analysis data (positive, negative, not detected, mutation type) and any supplemental information (e.g. failure of PCR amplification) were reported to AstraZeneca KK (Osaka, Japan).

statistical analysis

The correct determination rates (whether or not the positive/negative *EGFR* mutation assessment result was correct) and sensitivity (lowest percentage DNA admixture detected) by *EGFR* mutation type were assessed using DNA admixture samples for each *EGFR* mutation test.

The success and positive rates of each *EGFR* mutation test were determined using FFPE, and BB and pleural effusion cytology samples. The success rate was defined as the proportion of samples successfully analyzed

where it was possible to determine the mutation status. Samples were classified as unsuccessful where it was not possible to determine the mutation status, the PCR amplification failed, or if values of samples exceeded the cut-off value of Scorpion ARMS. The positive rate was defined as the number of samples analyzed as positive by each method as a proportion of the number of samples successfully analyzed. False-positive and false-negative rates were not determined, as no reference or 'gold standard' has been defined for *EGFR* mutation analysis.

The concordance rates and Cohen's kappa coefficients were determined between different methods of detection and between FFPE versus BB cytology sample types for mutation types commonly detectable by all assessed methods. Cohen's kappa coefficient was calculated as: kappa = (Po–Pe)/(1–Pe), where Po is the observed concordance rate and Pe is the expected probability of chance agreement.

results

patient samples

In total, 116 FFPE samples were evaluable for analysis, as four samples were confirmed not to contain NSCLC cells. The majority of samples were of adenocarcinoma histology and had a tumor cell content of at least 50%. Both tissue and tumor dimensions were ≤19 mm in most samples.

Of the 30 BB cytology samples (24 adenocarcinoma, four squamous, one adenosquamous, one large cell), one sample was excluded from the analysis because its matching FFPE sample was not judged as NSCLC. The samples were taken at a mean of 39 days (range 20–70 days) before operation and the mean DNA concentration was 8.73 ng/µl (range 0.2–40.3 ng/µl). All 20 pleural effusion cytology samples were assessable for analysis. Volumes of pleural effusion cytology samples used for each test method were 0.7–0.8 ml.

comparability of five *EGFR* mutation tests

DNA admixtures

PCR-Invader, PNA-LNA PCR clamp, Cycleave PCR, and Scorpion ARMS methods detected each of the *EGFR* mutation types L858R, T790M, G719S, and the in-frame deletion E746-A750 type I at ratios ranging from 1% to 50% of mutant/wild-type allele. PCR direct sequencing detected all types of mutations in samples containing 5%–50% of plasmid DNA but could not detect any of the mutations in the 1% mutant DNA admixture, nor exon 19 deletion or L858R in the 2% mutant DNA admixture. There were no false positives in wild-type samples.

formalin-fixed paraffin-embedded samples

Success rates of all five *EGFR* mutation tests were over 90% in FFPE samples (Table 1). Concordance rates between any two methods ranged from 85.3% to 99.1% including samples unsuccessfully analyzed and from 94.3% to 100% excluding samples unsuccessfully analyzed (supplemental Table S2, available at *Annals of Oncology* online). The rate of type 1 discordance (different mutations detected between the methods) was 3.4% (4/116 samples) and the rate of type 2 discordance (mismatch of mutation status between the methods) was 6.9% (8/116 samples) in FFPE samples (supplemental Table S3, available at *Annals of Oncology* online).

Unsuccessful rates of mutation analyses and discordance rates by tumor/sample characteristics for FFPE samples are shown in supplemental Figure S1 (available at *Annals of Oncology* online). Higher unsuccessful rates were associated with histological subtype [squamous cell carcinoma, 4/7 (57.1%)], older sample age [year of surgery 2006, 9/10 (90.0%)], and larger tumor dimension [20–29 mm, 3/15

Table 1. Success rate and *EGFR* mutation status determined by different *EGFR* mutation tests in FFPE, BB, and pleural effusion samples

Sample type and method	No. of samples successfully analyzed (%)	No. of mutation-positive samples (%)[a]	No. of mutation-negative samples (%)[a]
FFPE samples (*n* = 116)			
Scorpion ARMS	115 (99.1)	65 (56.5)	50 (43.5)
PCR-Invader	116 (100.0)	65 (56.0)	51 (44.0)
PNA-LNA PCR clamp	106 (91.4)	61 (57.5)	45 (42.5)
PCR direct sequencing	110 (94.8)	64 (58.2)	46 (41.8)
Cycleave PCR	116 (100.0)	63 (54.3)	53 (45.7)
BB cytology samples (*n* = 29)			
Scorpion ARMS	29 (100.0)	15 (51.7)	14 (48.3)
PCR-Invader	29 (100.0)	17 (58.6)	12 (41.4)
PNA-LNA PCR clamp	29 (100.0)	17 (58.6)	12 (41.4)
Cycleave PCR	29 (100.0)	16 (55.2)	13 (44.8)
Pleural effusion cytology samples (*n* = 20)			
Scorpion ARMS	20 (100.0)	11 (55.0)	9 (45.0)
PCR-Invader	20 (100.0)	11 (55.0)	9 (45.0)
PNA-LNA PCR clamp	20 (100.0)	10 (50.0)	10 (50.0)
PCR direct sequencing	20 (100.0)	11 (55.0)	9 (45.0)
Cycleave PCR	20 (100.0)	11 (55.0)	9 (45.0)

[a]Percentage calculated based on the number of samples successfully analyzed; *EGFR* mutation status was determined before the study and samples were selected to allow for an ~1 : 1 ratio of mutation-positive : mutation-negative samples.

ARMS, Amplification Refractory Mutation System; BB, bronchofiberscopic brushing; FFPE, formalin-fixed paraffin-embedded; PNA-LNA, peptide nucleic acid-locked nucleic acid.

(20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online). Discordance rates tended to be higher in samples with low tumor cell content [0–20%, 2/10 (20.0%); 30–40%, 3/10 (30%)], smaller tumor dimension [0–9 mm, 11/53 (20.8%)], smaller tissue dimension [0–9 mm, 8/33 (24.2%)], and older sample age [year of surgery 2006, 2/10 (20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online).

Concordance rates between five methods for the two major mutation types in FFPE samples were 81.8% (18/22) for exon 19 deletions and 87.2% (34/39) for L858R.

PCR direct sequencing identified rare mutations in six patients that were not detected by any other methods [V689L and E690V, E709V, V834L, I706T D770_N771 (insSVD), H773_V774(insPH)].

bronchofiberscopic brushing cytology samples

Success rates of the four *EGFR* mutation tests utilized for analysis of BB cytology samples were 100% (Table 1) and concordance rates between two methods ranged from 93.1% to 100% (supplemental Table S2, available at *Annals of Oncology* online). Discordances between two methods were found in two (6.9%) samples (supplemental Table S4, available at *Annals of Oncology* online). Both were type 2 discordances (mismatch of mutation status between the methods): in one sample, G719C detected by PCR-Invader and PNA-LNA PCR clamp was assessed as negative by Cycleave PCR (G719X not analyzed by Scorpion ARMS due to insufficient sample). In the remaining sample, L858R detected by PCR-Invader, PNA-LNA PCR clamp, and Cycleave PCR was assessed as negative by Scorpion ARMS.

Concordance rates between analysis of BB cytology and FFPE samples ranged from 65.5% to 96.6% including samples unsuccessfully analyzed and 93.1%–96.6% excluding samples unsuccessfully analyzed (supplemental Table S5, available at *Annals of Oncology* online). Discordances in analysis of BB cytology samples versus FFPE samples by the same detection method (excluding discordances due to unsuccessful mutation analysis of FFPE samples) were observed in three (10.3%) samples (supplemental Table S4, available at *Annals of Oncology* online); all were type 2 discordances (mismatch of mutation status between the methods).

pleural effusion cytology samples

Success rates of all five *EGFR* mutation tests were 100% (Table 1) and concordance rates between two methods ranged from 85.0% to 100% in the pleural effusion samples (supplemental Table S2, available at *Annals of Oncology* online). Discordances between five methods were found in three (15.0%) samples (supplemental Table S6, available at *Annals of Oncology* online). All were type 2 discordances (mismatch of mutation status between the methods): in one sample, an exon 19 deletion was detected by all methods except PCR direct sequencing; in another, an exon 19 deletion was only detected by Scorpion ARMS, Cycleave PCR, and PCR direct sequencing; and in the third sample, L858R was only detected by PCR-Invader. In one of the other 17 samples, PCR direct sequencing detected an additional mutation [exon 18 deletion (E709_T710>D)], which the other methods were not designed to assess.

discussion

Analysis of the control DNA admixture samples showed that the *EGFR* mutation tests had comparable sensitivity, with the exception of direct sequencing. The sensitivity of direct sequencing, although higher than expected and reported elsewhere [15], was lower than the other techniques.

The results of this study showed that all five *EGFR* mutation tests had comparable success rates (over 90%) in FFPE samples. These were consistently high success rates given that the fixation of some of the samples was not ideal (e.g. long fixation times). The success rates of direct sequencing were higher than anticipated based on previous studies of clinical samples. For example, in a recent study, ARMS and direct sequencing were used to detect known *EGFR* mutations in NSCLC FFPE samples, and ARMS was found to be a more sensitive and robust technique [13]. However, it should be recognized that even when utilizing the same technologies, differences in reagents, DNA quality, software, and crucially, primer design and amplicon size have a huge influence on direct sequencing success rates and mutation detection potential. Our results show that the processes implemented by the laboratory in this study are highly optimized for the detection of *EGFR* mutations from tumor DNA using direct sequencing. As the testing laboratories also carried out the DNA extraction (with the exception of BB cytology samples), the slight differences in DNA extraction and processes across the different laboratories could also impact on the overall performance of the test methods.

All the FFPE samples were examined by a pathologist and generally found to be of high quality and tumor content. The numbers of samples with different tumor/sample characteristics were too low to make any definitive conclusions regarding unsuccessful and discordance rates by these characteristics. However, sample unsuccessful rates appear to be associated with squamous cell carcinoma, older samples, and samples with long tumor dimension, all of which can make it difficult to extract DNA. In addition, discordance rates appeared higher in older samples or samples of low tumor cell content, short tumor dimension or short tissue dimension, where the quantities of DNA are small.

Concordance rates were generally over 85% (>94%, excluding samples unsuccessfully analyzed) between any two *EGFR* mutation tests in FFPE samples. The lowest concordance rates between the five methods were in comparison with the PNA-LNA PCR clamp method. As the PNA-LNA PCR clamp method also had a higher unsuccessful rate than the other methods, concordance rates were lower in comparison with other methods when including samples unsuccessfully analyzed. However, all kappa statistics were ≥0.70, indicating a high concordance of analysis results. Concordance rates for the two major *EGFR* mutation types, exon 19 deletions and L858R, across the five mutation tests in FFPE samples were also high (81.8% and 87.2%, respectively), illustrating the suitability of all

five methods for *EGFR* mutation analysis in clinical studies and diagnostic applications. However, as the concordance rates were not 100% for any one method, we would advocate the selection of a single method for consistent use during a clinical study. With regard to daily practice, the decision to select and adopt a particular technology is at the discretion of individual laboratories and may be influenced by the diagnostic environment in which they reside. Selection factors may include technical expertise of operators, cost, test status (*in vitro* diagnostic versus laboratory-developed test), or availability of instrumentation.

Several factors may have contributed to the discordances between the *EGFR* mutation tests. These factors may have included differences in sensitivity and specificity, different DNA extraction procedures between laboratories, variation in tumor cell content within and across samples, and tumor heterogeneity within an FFPE block [10, 11, 16].

The performance of all five *EGFR* mutation tests was comparable in the analysis of both BB and pleural effusion cytology samples, with 100% success rates. BB cytology samples showed high concordance rates (>93%, excluding samples unsuccessfully analyzed) between pairs of *EGFR* mutation tests and versus FFPE samples by each detection method. Using the PNA-LNA PCR clamp method, analysis of BB cytology samples was successful where the matched FFPE sample failed analysis. Some mutations were detected in cytology samples of low DNA concentrations where matching FFPE samples were assessed as mutation negative. This result suggests that cytology samples can be useful in mutation analysis when tissue samples cannot be used, are in a small quantity, or degradation of FFPE samples is suspected. Pleural effusion cytology samples may be particularly suitable for analysis as they can be obtained easily, non-invasively and repeatedly, and generally contain plenty of cancer cells, relative to other sample types.

To our knowledge, this is the first high-quality comparison study of *EGFR* mutation tests in both FFPE and cytology samples. The results of the current study indicate that cytology-derived DNA is a suitable alternative to FFPE samples for the analysis of *EGFR* mutations and may be useful when FFPE samples are unavailable for molecular analysis. Other studies have also shown that ARMS can be used to detect *EGFR* mutations in cytology samples from transbronchial needle aspirates [17] or pleural effusion [18] and that this technique appeared to be more sensitive than direct sequencing in this sample type. Other methods for *EGFR* mutation testing, including pyrosequencing [19] and high-resolution melting analysis [20], also exist.

In summary, the performance of all five *EGFR* mutation tests was comparable in the analysis of FFPE and cytology samples. Where *EGFR* mutation tests and standard operating procedures are used in a reliable robust way, with trained operators, in a well-developed diagnostic setting, comparable results are obtained across mutation tests and sample types. FFPE specimens are currently the sample of choice for determining *EGFR* mutation status [11]. However, the ability to use cytology samples allows additional patients to be tested for *EGFR* mutations, and therefore, more appropriate treatment of their disease.

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disclosure

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Epidermal Growth Factor Receptor Mutation Status in Circulating Free DNA in Serum

From IPASS, a Phase III Study of Gefitinib or Carboplatin/Paclitaxel in Non-small Cell Lung Cancer

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Introduction: In IPASS (IRESSA Pan-Asia Study), clinically selected patients with pulmonary adenocarcinoma received first-line gefitinib or carboplatin/paclitaxel. This preplanned, exploratory analysis was conducted to increase understanding of the use of surrogate samples, such as serum, versus tumor biopsy samples for determining *EGFR* mutation status in the Japanese cohort ($n = 233$).

Methods: *EGFR* mutations were assessed using tumor tissue-derived DNA ($n = 91$) and circulating free (cf) DNA from pretreatment serum samples ($n = 194$).

Results: Fewer patients were *EGFR* mutation positive when assessed using pretreatment cfDNA (23.7%) versus tumor tissue-derived DNA (61.5%). cfDNA results identified no false positives but a high rate of false negatives (56.9%). There was a significant interaction between cfDNA *EGFR* mutation status and treatment for progression-free survival (PFS) ($p = 0.045$). PFS was significantly longer and objective response rate (ORR) higher with gefitinib than carboplatin/paclitaxel in the cfDNA *EGFR* mutation-positive subgroup (PFS: hazard ratio [HR], 0.29; 95% confidence interval [CI], 0.14–0.60; $p < 0.001$; ORR: odds ratio [OR], 1.71; 95% CI, 0.48–6.09; 75.0% versus 63.6%; $p = 0.40$). There was a slight numerical advantage in PFS and ORR for gefitinib over carboplatin/paclitaxel in the cfDNA *EGFR* mutation-negative subgroup, likely due to the high rate of false negatives within this subgroup.

Conclusions: These results merit further investigation to determine whether alternative sources of tumor DNA, such as cfDNA in serum, could be used for determining *EGFR* mutation status in future; currently, where a sample is available, analysis of tumor material is recommended.

Key Words: EGFR, Mutation, Gefitinib, NSCLC, Serum.

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The epidermal growth factor receptor (EGFR) superfamily has been implicated in the regulation of tumor cell biology and, as such, has emerged as a therapeutic target.[1] In 2004, mutations in the *EGFR* were reported to be associated with sensitivity to EGFR tyrosine kinase inhibitors (EGFR-TKIs).[2–4] The presence of such mutations in tumor tissue is associated with a number of clinical factors including Asian origin, female sex, adenocarcinoma histology, and a never-smoking history, and these factors have additionally been correlated with response to gefitinib (IRESSA, AstraZeneca, Macclesfield, UK), an EGFR-TKI.[5]

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The IRESSA Pan-Asia Study (IPASS) compared gefitinib with carboplatin/paclitaxel as first-line treatment in 1217 never-smokers/light ex-smokers with advanced adenocarcinoma of the lung in East Asia.[6] Subgroup analysis of patients with *EGFR* mutations ($n = 261$) detected in DNA derived from tumor tissue samples demonstrated significantly longer progression-free survival (PFS) with gefitinib versus carboplatin/paclitaxel (hazard ratio [HR], 0.48; 95% confidence interval [CI], 0.36–0.64; $p < 0.001$).[6] In the *EGFR* mutation-negative (M−) subgroup ($n = 176$), PFS was significantly longer with carboplatin/paclitaxel versus gefitinib (HR, 2.85; 95% CI, 2.05–3.98; $p < 0.001$). Objective response rates (ORR) were 71.2% versus 47.3% ($p < 0.001$) and 1.1% versus 23.5% ($p = 0.001$) with gefitinib versus carboplatin/paclitaxel in *EGFR* M+ and M− patients, respectively.

The difficulties of collecting sufficient tumor tissue for biomarker analyses have stimulated interest in analyses using surrogate samples, such as serum and plasma samples, which frequently contain circulating free (cf) DNA derived from tumor tissues. Previous studies in relatively few patients had detected *EGFR* mutations in cfDNA in serum or plasma samples and suggested that using such methodology to predict response to gefitinib was worthy of further evaluation.[7–12] However, most of these studies were retrospective.

Herein, we report the evaluation of *EGFR* mutations in cfDNA from serum samples of patients in the IPASS study recruited in Japan. This preplanned, exploratory analysis was conducted to increase the understanding of the use of surrogate samples, such as serum, versus tumor biopsy samples for determining *EGFR* mutation status.

MATERIALS AND METHODS

Study Design and Patients

Full details of the IPASS study design (ClinicalTrials.gov identifier NCT00322452) have been published previously.[6] Planned objectives of this substudy of IPASS were evaluations of efficacy between the gefitinib and carboplatin/paclitaxel treatment groups by cfDNA *EGFR* mutation status from pretreatment serum samples and evaluation of the concordance between *EGFR* mutation status in pretreatment cfDNA versus tumor. Comparison of *EGFR* mutation status in pretreatment versus postprogression serum samples was also performed; however, not all patients with a pretreatment sample had a postprogression sample, which limited the comparison. In addition, comparisons with postprogression serum and pretreatment pleural effusion samples are reported in Supplemental Digital Content 1 (Methods http://links.lww.com/JTO/A152). Preplanned analysis of the Japanese subset of the IPASS population was performed to meet Japanese regulatory requirements.

All patients provided written informed consent. Provision of samples for biomarker research was optional and involved separate consent procedures for tumor and serum sampling. An independent ethics committee at each participating institution approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Guidelines for Good Clinical Practice, applicable regulatory requirements, and AstraZeneca's policy on bioethics.

Biomarker Analyses

Sample collection and DNA extraction are described in Supplemental Digital Content 1 (Methods http://links.lww.com/JTO/A152). *EGFR* mutations were detected using the DxS *EGFR* Mutation Test Kit for Research Use Only (DxS, Manchester, UK), which combines Amplification Refractory Mutation System (ARMS) (allele-specific polymerase chain reaction [PCR]) with the Scorpions real-time PCR technology.[13,14] Modified run conditions and cutoffs (delta Ct values [dCt]) used to define M+ samples for cfDNA derived from serum and pleural effusion samples were as follows: 50 cycles of PCR were carried out and the dCt for exon 19 deletions was 12, L858R was 14, and T790M was 8 (for tumor DNA, 40 cycles of PCR were carried out and the dCt cutoffs were 9, 11, and 8, respectively). In analyses of tumor DNA, all 29 mutations detected by the kit were assayed (19 deletions in exon 19, L858R, T790M, L861Q, G719X [S, A, or C], S768I, and 3 insertions in exon 20); whereas for serum and pleural effusion samples, the 21 most common mutations (19 deletions in exon 19, L858R, and T790M) were assayed (to make the best use of limited cfDNA yield). Samples were tested in duplicate, and only if both replicates were positive for at least one of the mutations was the sample defined as M+. Patients without a tumor sample evaluable for mutation analysis and samples which were not successfully analyzed were classified as *EGFR* mutation unknown. Biomarker samples were assayed blinded to clinical outcome and randomized treatment.

Statistical Analyses

Serum samples were collected for patients recruited in Japan and who consented to this optional analysis. Analyses of efficacy end points comparing treatment groups in the Japanese subset (intent-to-treat [ITT] population) were assessed as described previously for the overall IPASS population.[6] However, for the analyses in the cfDNA M+ and M− subgroups, the prespecified covariates of World Health Organization (WHO) performance status (PS), smoking history, and sex could not be included as covariates because of the small number of patients who had a WHO PS 2, were ex-smokers, or were males; therefore, models without covariates were used. Because of the lack of power to detect treatment differences, the result of the Japanese subset should be interpreted with caution, taking into account the associated variability and overlap in plausible range of effects (CIs). Analyses comparing treatment groups were performed for PFS (by Cox proportional hazards model) and ORR (by logistic regression model) in subgroups defined by cfDNA *EGFR* mutation status. A test for interaction between cfDNA *EGFR* mutation status (M+ or M−) and treatment was used to assess whether the PFS treatment effect was statistically different between subgroups.

Comparison of pretreatment cfDNA versus tumor *EGFR* mutations was based on the 21 mutations analyzed for cfDNA using patients with known mutation status (M+ or M−) in both samples. The sensitivity, specificity, positive

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