

201409013B

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24～26年度 総合研究報告書

研究代表者 後藤 功一

平成 27 (2015) 年 4 月

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24～26年度 総合研究報告書

研究代表者 後藤 功一

平成 27 (2015) 年 4 月

目 次

I. 総合研究報告		
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	-----	1
後藤功一		
II. 研究成果の刊行に関する一覧表	-----	6
III. 研究成果の刊行物・別刷	-----	8

I .総合研究報告書

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）
（総合）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

独立行政法人国立がん研究センター東病院
呼吸器内科長

研究要旨

2012年に発見された肺癌の新規ドライバー遺伝子である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、全国規模の遺伝子診断ネットワーク（LC-SCRUM-Japan）を立ち上げ、平成25年2月より遺伝子スクリーニングを開始した。同時に、RET肺癌に対するVandetanibの医師主導治験（LURET study）を世界で初めて開始した。平成27年1月31日現在、LC-SCRUM-Japanには190施設が参加し、1438例が登録された結果、RET肺癌が32例スクリーニングされ、このうち18例がLURET studyに登録されている。

研究分担者

葉 清隆	国立がん研究センター東病院	医長	田村友秀	国立がん研究センター中央病院	科長
河野隆志	国立がん研究センター研究所	分野長	(平成24年度～平成25年度)		
蔦 幸治	国立がん研究センター中央病院	医長	軒原 浩	国立がん研究センター中央病院	医長
土原一哉	国立がん研究センター	分野長	(平成26年度～)		
	早期・探索臨床研究センター		大江裕一郎	国立がん研究センター東病院	副院長
石井源一郎	国立がん研究センター東病院	ユニット長	(平成24年度～平成25年度)		
松本慎吾	国立がん研究センター	医員	山本信之	静岡がんセンター	副院長
	早期・探索臨床研究センター		(平成24年度)		
大津 敦	国立がん研究センター	センター長	村上晴泰	静岡がんセンター	医長
	早期・探索臨床研究センター		(平成25年度～)		
山中竹春	国立がん研究センター	室長	瀬戸貴司	九州がんセンター	医師
	早期・探索臨床研究センター		西尾誠人	がん研究会有明病院	部長
(平成26年9月より横浜市立大学大学院医学研究科 教授)			里内美弥子	兵庫県立がんセンター	部長
佐藤暁洋	国立がん研究センター	部長	野上尚之	四国がんセンター	医長
	研究支援センター				

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 (RET 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 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 融合遺伝子等の低頻度の遺伝子変化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 RET 肺癌のスクリーニングを行う。施設倫理委員会で本研究が承認された施設のみ、遺伝子スクリーニング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、実際の臨床検体の遺伝子解析は SRL が行った。同時に Vandetanib の医師主導治験を 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) で開始し、スクリーニングされた RET 肺癌は、医師主導治験へ登録して、Vandetanib の有効性を検討する方針とした。

医師主導治験である「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」(LURET study) 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 早期・探索臨床研究センター 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、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、登録前に十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ことを規定した。また、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、国立がん研究センター 研究支援センター 研究管理部が行う。データマネ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予想されるため、ターゲットキャプチャー法を応用したゲノム DNA から 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

RET 肺癌のスクリーニングのため、全国規模の遺伝子診断ネットワークとして、Lung Cancer Genomic Screening Project for Individualized Medicine in Japan (LC-SCRUM-Japan) を組織し、平成 25 年 2 月 7 日より遺伝子スクリーニングを開始した。平成 27 年 1 月 31 日現在、LC-SCRUM-Japan には 190 施設が参加し、施設倫理審査委員会で研究が承認された 169 施設で順調に遺伝子スクリーニングが進行中である。平成 27 年 1 月 31 日までに LC-SCRUM-Japan には 1438

例の登録があり、既に 32 例 (3%) の RET 肺癌がスクリーニングされている。更に、希少な ROS1 融合遺伝子陽性肺癌 57 例 (4%)、ALK 融合遺伝子陽性肺癌 22 例 (2%) も同時にスクリーニングされている。

LURET study は、平成 24 年 11 月 19 日に PMDA の薬事戦略相談を受け、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 21 日より患者登録を開始した。平成 27 年 1 月 31 日までに LC-SCRUM-Japan でスクリーニングされた RET 肺癌 32 例のうち 18 例 (内 2 例は不適格例) が LURET study に登録され、Vandetanib による治療が進行中であり、予想通りの治療効果が認められている。あと 1 例で目標症例数に到達するため、平成 26 年度で予定通りの登録完了を目指している。

これらと並行して、RET 融合遺伝子を含む複数の肺癌ドライバー変異を一度にかつ迅速に検出できる multiplex 遺伝子診断法の開発も行っており、微量のゲノム DNA (50ng~) から、ターゲットキャプチャーを用いて目的ゲノム領域を濃縮し、次世代シーケンサーで変異を検出するキットの開発が進行中である。

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推進が不可欠である。

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ドライバー遺伝子であり、新規の治療標的となることが期待される。RET 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 300 mg/day 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 肺癌は、頻度が 1-2% という稀少疾患であるため、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、希少肺癌のスクリーニングが成功したことは非常に重要である。少数施設で 1-2% の頻度の希少な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、希少肺癌では、従来とは異なる治療開発の方法が模索されてきた。本研究の中で LC-SCRUM-Japan という希少肺癌の遺伝子スクリーニング基盤が実際に構築されたことは大きな意義を持つと考えられる。また、希少肺癌の治療開発においては、正確な診断及び、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、遺伝子変化を伴うその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、multiplex 診断薬を含めたコンパニオン診断薬の開発を担える組織として、更に存在意義が高まっていくと予想される。

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ドライバー遺伝子である RET 融合遺伝子の臨床応用を目指した研究を行っている。RET 肺癌は希少頻度の肺癌であるが、全国規模の遺伝子診断ネットワークである LC-SCRUM-Japan において順調にスクリーニングが進行している。更に、スクリーニングされた RET 肺癌は、Vandetanib の医師主導治験に登録され、治療開発も同時に進行している。このような民間企業の研究と公的資金に基づく医師主導治験との連携による治療開発システムの構築は、今後も明らかになる遺伝子異常を伴う希少がんに対する分子標的治療薬の効率的な治療開発のモデルケースとして注目されており、個別化治療の発展への大きな貢献が期待される。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

1. 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.* 2012, 23(11):2914-2919.
2. Goto K, Ichinose Y, Ohe Y, Yamamoto N, Negoro S, Nishio K, Itoh Y, Jiang H, Duffield E, McCormack R, Saijo N, Mok T, Fukuoka M. 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. *J Thorac Oncol*, 2012.7(1):115-121.
3. 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*, 2012.18:375-377.
4. Okayama H, Kohno T, Ishii Y, Shimada Y, Shiraishi K, Iwakawa R, Furuta K, Tsuta K, Shibata T, Yamamoto S, Watanabe S, Sakamoto H, Kumamoto K, Takenoshita S, Gotoh N, Mizuno H, Sarai A, Kawano S, Yamaguchi R, Miyano S, Yokota J. Identification of genes up-regulated in ALK-positive and EGFR/KRAS/ALK-negative lung adenocarcinomas. *Cancer Res*, 2012.72(1):100-111.
5. Suzuki T, Shibata T, Takaya K, Shiraishi K, Kohno T, Kunitoh H, Tsuta K, Furuta K, Goto K, Hosoda F, Sakamoto H, Motohashi H, Yamamoto M. Regulatory nexus of synthesis and degradation deciphers cellular Nrf2 expression levels. *Mol Cell Biol*, 2013, 33(12):2402-2412.
6. Suzuki M, Makinoshima H, Matsumoto S, Suzuki A, Mimaki S, Matsushima K, Yoh K, Goto K, Suzuki Y, Ishii G, Ochiai A, Tsuta K, Shibata T, Kohno T, Esumi H, Tsuchihara K. Identification of a lung adenocarcinoma cell line with CCDC6-RET fusion gene and the effect of RET inhibitors in vitro and in vivo. *Cancer Sci*, 2013, 104(7):896-903.
7. Suzuki A, Mimaki S, Yamane Y, Kawase A, Matsushima K, Suzuki M, Goto K, Sugano S, Esumi H, Suzuki Y, Tsuchihara K. Identification and characterization of cancer mutations in Japanese lung adenocarcinoma without sequencing of normal tissue counterparts. *PLoS One*, 2013, 8(9):e73484.
8. Kohno T, Tsuta K, Tsuchihara K, Nakaoku T, Yoh K, Goto K. RET fusion gene: translation to personalized lung cancer therapy. *Cancer Sci.* 2013, 104 (11): 1396-1400.
9. Arai Y, Totoki Y, Takahashi H, Nakamura H, Hama N, Kohno T, Tsuta K, Yoshida A, Asamura H, Mutoh M, Hosoda F, Tsuda H, Shibata T. Mouse model for ROS1-rearranged lung cancer. *PLoS One.* 2013.8(2):e56010.
10. Tsuta K, Kawago M, Inoue E, Yoshida A, Takahashi F, Sakurai H, Watanabe S, Takeuchi M, Furuta K, Asamura H, Tsuda H. The utility of the proposed IASLC/ATS/ERS lung adenocarcinoma subtypes for disease prognosis and correlation of driver gene alterations. *Lung Cancer.* 2013.81(3):371-376.
11. Ichinokawa H, Ishii G, Nagai K, Kawase A, Yoshida J, Nishimura M, Hishida T, Ogasawara N, Tsuchihara K, Ochiai A. Distinct clinicopathologic characteristics of lung mucinous adenocarcinoma with KRAS mutation. *Hum Pathol.* 2013,44:2636-2642.
12. Bando H, Yoshino T, Shinozaki E, Nishina T, Yamazaki K, Yamaguchi K, Yuki S, Kajira S, Fujii S, Yamanaka T, Tsuchihara K, Ohtsu A. Simultaneous identification of 36 mutations in KRAS codons 61 and 146, BRAF, NRAS, and PIK3CA in a single reaction by multiplex assay kit. *BMC Cancer.* 2013,13:405.
13. Yoshida A, Kohno T, Tsuta K, Wakai S, Arai Y, Shimada Y, Asamura H, Furuta K, Shibata T, Tsuda H. ROS1-Rearranged Lung Cancer: A Clinicopathologic and Molecular Study of 15 Surgical Cases. *Am J Surg Pathol* 2013, 37:(4)554-562.
14. Kohno T, Tsuchihara K, Ogiwara H, Ichikawa H. RET and other genes: therapeutic targets in lung adenocarcinoma. *Lung Cancer Manag.* 2014, 3:219-226.
15. Tsuta K, Kohno T, Yoshida A, Shimada Y, Asamura H, Furuta K, Kushima R. RET-rearranged non-small-cell lung carcinoma: a clinicopathological and molecular analysis. *Br J Cancer.* 2014, 110(6):1571-1578.

16. Yoshida A, Tsuta K, Wakai S, Arai Y, Asamura H, Shibata T, Furuta K, Kohno T, Kushima R. Immunohistochemical detection of ROS1 is useful for identifying ROS1 rearrangements in lung cancers. *Modern Pathol.* 2014, 27(5):711-720.
17. Mizukami T, Shiraishi K, Shimada Y, Ogiwara H, Tsuta K, Ichikawa H, Sakamoto H, Kato M, Shibata T, Nakano T, Kohno T. Molecular mechanisms underlying oncogenic RET fusion in lung adenocarcinoma. *J Thoracic Oncol*, 2014, 9(5):622-630.

H. 知的財産権の出願・登録状況

1. 特許取得

知財(特許出願)

公告番号 WO2013018882 A1

公開タイプ 出願

出願番号 PCT/JP2012/069799

発明の名称: K i f 5 b 遺伝子と r e t 遺伝子との融合遺伝子、並びに該融合遺伝子を標的としたがん治療の有効性を判定する方法

公開日 2013年2月7日

出願日 2012年8月3日

優先日 2011年8月4日

発明者: 河野隆志, 葛 幸治

特許出願人: 独立行政法人国立がん研究センター

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)	2914-2919	2012
Goto K, Ichinose Y, Ohe Y, Yamamoto N, Negoro S, Nishio K, Itoh Y, Jiang H, Duffield E, McCormack R, Saijo N, Mok T, Fukuoka M.	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.	J Thorac Oncol	7(1)	115-121	2012
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
Okayama H, Kohno T, Ishii Y, Shimada Y, Shiraishi K, Iwakawa R, Furuta K, Tsuta K, Shibata T, Yamamoto S, Watanabe S, Sakamoto H, Kumamoto K, Takenoshita S, Gotoh N, Mizuno H, Sarai A, Kawano S, Yamaguchi R, Miyano S, Yokota J.	Identification of genes up-regulated in ALK-positive and EGFR/KRAS/ALK-negative lung adenocarcinomas.	Cancer Res	72(1)	100-111	2012
Suzuki T, Shibata T, Takaya K, Shiraishi K, Kohno T, Kunitoh H, Tsuta K, Furuta K, Goto K, Hosoda F, Sakamoto H, Motohashi H, Yamamoto M.	Regulatory nexus of synthesis and degradation deciphers cellular Nrf2 expression levels.	Mol Cell Biol	33(12)	2402-2412	2013
Suzuki M, Makinoshima H, Matsumoto S, Suzuki A, Mimaki S, Matsushima K, Yoh K, Goto K, Suzuki Y, Ishii G, Ochiai A, Tsuta K, Shibata T, Kohno T, Esumi H, Tsuchihara K.	Identification of a lung adenocarcinoma cell line with CCDC6-RET fusion gene and the effect of RET inhibitors in vitro and in vivo.	Cancer Sci	104(7)	896-903	2013
Suzuki A, Mimaki S, Yamane Y, Kawase A, Matsushima K, Suzuki M, Goto K, Sugano S, Esumi H, Suzuki Y, Tsuchihara K.	Identification and characterization of cancer mutations in Japanese lung adenocarcinoma without sequencing of normal tissue counterparts.	PLoS One	8(9)	e7344	2013

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kohno T, Tsuta K, Tsuchihara K, Nakaoku T, Yoh K, Goto K.	RET fusion gene: translation to personalized lung cancer therapy.	Cancer Sci	104 (11)	1396- 1400	2013
Arai Y, Totoki Y, Takahashi H, Nakamura H, Hama N, Kohno T, Tsuta K, Yoshida A, Asamura H, Mutoh M, Hosoda F, Tsuda H, Shibata T.	Mouse model for ROS1-rearranged lung cancer.	PLoS One	8(2)	e56010	2013
Tsuta K, Kawago M, Inoue E, Yoshida A, Takahashi F, Sakurai H, Watanabe S, Takeuchi M, Furuta K, Asamura H, Tsuda H.	The utility of the proposed IASLC/ATS/ERS lung adenocarcinoma subtypes for disease prognosis and correlation of driver gene alterations.	Lung Cancer	81(3)	371-376	2013
Ichinokawa H, Ishii G, Nagai K, Kawase A, Yoshida J, Nishimura M, Hishida T, Ogasawara N, Tsuchihara K, Ochiai A.	Distinct clinicopathologic characteristics of lung mucinous adenocarcinoma with KRAS mutation.	Hum Pathol	44	2636- 2642	2013
Bando H, Yoshino T, Shinozaki E, Nishina T, Yamazaki K, Yamaguchi K, Yuki S, Kajiuura S, Fujii S, Yamanaka T, Tsuchihara K, Ohtsu A.	Simultaneous identification of 36 mutations in KRAS codons 61 and 146, BRAF, NRAS, and PIK3CA in a single reaction by multiplex assay kit.	BMC Cancer	13	405	2013
Yoshida A, Kohno T, Tsuta K, Wakai S, Arai Y, Shimada Y, Asamura H, Furuta K, Shibata T, Tsuda H.	ROS1-Rearranged Lung Cancer: A Clinicopathologic and Molecular Study of 15 Surgical Cases.	Am J Surg Pathol	37:(4)	554-562	2013
Kohno T, Tsuchihara K, Ogiwara H, Ichikawa H.	RET and other genes: therapeutic targets in lung adenocarcinoma.	Lung Cancer Manag	3	219-226	2014
Tsuta K, Kohno T, Yoshida A, Shimada Y, Asamura H, Furuta K, Kushima R.	RET-rearranged non-small-cell lung carcinoma: a clinicopathological and molecular analysis.	Br J Cancer	110(6)	1571- 1578	2014
Yoshida A, Tsuta K, Wakai S, Arai Y, Asamura H, Shibata T, Furuta K, Kohno T, Kushima R.	Immunohistochemical detection of ROS1 is useful for identifying ROS1 rearrangements in lung cancers.	Modern Pathol	27(5)	711-720	2014
Mizukami T, Shiraishi K, Shimada Y, Ogiwara H, Tsuta K, Ichikawa H, Sakamoto H, Kato M, Shibata T, Nakano T, Kohno T.	Molecular mechanisms underlying oncogenic RET fusion in lung adenocarcinoma.	J Thoracic Oncol	9(5)	622-630	2014

Ⅲ.研究成果の刊行物・別刷

研究代表者	後藤 功一	国立がん研究センター 東病院
研究分担者	葉 清隆	国立がん研究センター 東病院
	河野 隆志	国立がん研究センター 研究所
	蔦 幸治	国立がん研究センター 中央病院
	土原 一哉	国立がん研究センター 早期探索臨床研究センター
	石井源一郎	国立がん研究センター 東病院
	松本 慎吾	国立がん研究センター 早期探索臨床研究センター
	大津 敦	国立がん研究センター 早期探索臨床研究センター
	山中 竹春	国立がん研究センター 早期探索臨床研究センター (平成 26 年 9 月より横浜市立大学大学院医学研究科教授)
	佐藤 暁洋	国立がん研究センター 研究支援センター
	田村 友秀	国立がん研究センター中央病院 (平成 24 年度～平成 25 年度)
	大江裕一郎	国立がん研究センター 東病院 (平成 24 年度～平成 25 年度)
	軒原 浩	国立がん研究センター 中央病院 (平成 26 年度～)
	山本 信之	静岡がんセンター (平成 24 年度)
	村上 晴泰	静岡がんセンター (平成 25 年度～)
	瀬戸 貴司	九州がんセンター
	西尾 誠人	がん研究会有明病院
	里内美弥子	兵庫県立がんセンター
	野上 尚之	四国がんセンター

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

K. Goto^{1*}, M. Satouchi², G. Ishii³, K. Nishio⁴, K. Hagiwara⁵, T. Mitsudomi⁶, J. Whiteley⁷, E. Donald⁷, R. McCormack⁷ & T. Todo⁸

¹Division of Thoracic Oncology, National Cancer Center Hospital East, Chiba; ²Department of Thoracic Oncology, Hyogo Cancer Center, Hyogo; ³Pathology Division, Innovative Medical Research Center, National Cancer Center Hospital East, Chiba; ⁴Department of Genome Biology, Kinki University School of Medicine, Osaka;

⁵Department of Respiratory Medicine, Saitama Medical University, Saitama; ⁶Department of Thoracic Surgery, Aichi Cancer Center Hospital, Aichi, Japan; ⁷Department of Personalised Healthcare and Biomarkers, AstraZeneca Pharmaceuticals, Macclesfield, UK; ⁸Department of Research and Development, AstraZeneca KK, Osaka, Japan

Received 19 December 2011; revised 2 March 2012; accepted 12 March 2012

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 $\geq 1\%$ 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

*Correspondence to: Dr K. Goto, Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwanoha, 6-5-1, Kashiwa, Chiba, 277-8577, Japan.
Tel: +81-4-7133-1111; Fax: +81-1-7131-4724; E-mail: kgoto@east.ncc.go.jp

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/ μ l) were diluted with water and whole-human genomic DNA (12.5 ng/ μ l) (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/ μ l plasmid DNA, 10 ng/ μ l 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 μ l 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/ μ l) 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- μ m 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 μ l 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/ μ l, 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/ μ l (range 0.2–40.3 ng/ μ l). 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)

^aPercentage 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, bronchoscopic 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.

acknowledgements

We thank Annette Smith, PhD, from Complete Medical Communications, who provided medical writing support funded by AstraZeneca.

funding

This work was supported by AstraZeneca. AstraZeneca employees participated in the conception and design of the study, collection and assembly of data, data analysis, and interpretation.

disclosure

KG has received honoraria from Ono Pharmaceutical and Chugai Pharmaceutical and fees for consultancy/advisory boards from Ono Pharmaceutical. MS has received honoraria from Chugai Pharmaceutical and AstraZeneca. KN has received research grants from Daiichi Sankyo, Chugai Pharmaceutical, AstraZeneca, Glaxo SmithKline, and Solasia Pharma KK, research support from Chugai Pharmaceutical, and honoraria from Kyowa Hakkō Kirin, Sumitomo Bakelite, Taiho Pharmaceutical, and Qiagen. KH has received patent fees from Mitsubishi Chemical Medience. TM has received honoraria from AstraZeneca and Chugai Pharmaceutical. JW, ED, RM, and TT are employees of AstraZeneca and hold stock in AstraZeneca. GI has declared no conflicts of interest.

references

- Mok TS, Wu Y-L, Thongprasert S et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009; 361: 947–957.
- Mitsudomi T, Morita S, Yatabe Y et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010; 11: 121–128.
- Maemondo M, Inoue A, Kobayashi K et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010; 362: 2380–2388.
- Hall JG, Eis PS, Law SM et al. Sensitive detection of DNA polymorphisms by the serial invasive signal amplification reaction. *Proc Natl Acad Sci U S A* 2000; 97: 8272–8277.
- Nagai Y, Miyazawa H, Huqun et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res* 2005; 65: 7276–7282.
- Lynch TJ, Bell DW, Sordella R et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; 350: 2129–2139.
- Yatabe Y, Hida T, Horio Y et al. A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer. *J Mol Diagn* 2006; 8: 335–341.
- Kimura H, Kasahara K, Kawaiishi M et al. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. *Clin Cancer Res* 2006; 12: 3915–3921.
- Yamamoto N, Ichinose Y, Nishiwaki Y et al. EGFR mutations based on circulating free DNA in the subset of Japanese patients from IPASS (IRESSA Pan Asia Study), a phase III study of first-line gefitinib vs carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer. Poster 78 presented at EORTC-NCI-ASCO. Brussels, Belgium 2009; 15–17 October.
- Eberhard DA, Giaccone G, Johnson BE. Biomarkers of response to epidermal growth factor receptor inhibitors in non-small-cell lung cancer working group:

- standardization for use in the clinical trial setting. *J Clin Oncol* 2008; 26: 983–994.
11. Pirker R, Herth FJ, Kerr KM et al. Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. *J Thorac Oncol* 2010; 5: 1706–1713.
 12. Naoki K, Soejima K, Okamoto H et al. The PCR-invader method (structure-specific 5' nuclease-based method), a sensitive method for detecting EGFR gene mutations in lung cancer specimens; comparison with direct sequencing. *Int J Clin Oncol* 2011; 16: 335–344.
 13. Ellison G, Donald E, McWalter G et al. A comparison of ARMS and DNA sequencing for mutation analysis in clinical biopsy samples. *J Exp Clin Cancer Res* 2010; 29: 132.
 14. Newton CR, Graham A, Heptinstall LE et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res* 1989; 17: 2503–2516.
 15. Whitcombe D, Theaker J, Guy SP et al. Detection of PCR products using self-probing amplicons and fluorescence. *Nat Biotechnol* 1999; 17: 804–807.
 16. Oliner K, Juan T, Suggs S et al. A comparability study of 5 commercial KRAS tests. *Diagn Pathol* 2010; 5: 23.
 17. Horiike A, Kimura H, Nishio K et al. Detection of epidermal growth factor receptor mutation in transbronchial needle aspirates of non-small cell lung cancer. *Chest* 2007; 131: 1628–1634.
 18. Kimura H, Fujiwara Y, Sone T et al. High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. *Cancer Sci* 2006; 97: 642–648.
 19. Takano T, Ohe Y, Sakamoto H et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 2005; 23: 6829–6837.
 20. Willmore-Payne C, Holden JA, Layfield LJ. Detection of epidermal growth factor receptor and human epidermal growth factor receptor 2 activating mutations in lung adenocarcinoma by high-resolution melting amplicon analysis: correlation with gene copy number, protein expression, and hormone receptor expression. *Hum Pathol* 2006; 37: 755–763.

Annals of Oncology 23: 2919–2924, 2012
doi:10.1093/annonc/mds123
Published online 10 July 2012

Prophylactic cranial irradiation in small-cell lung cancer: Findings from a North Central Cancer Treatment Group Pooled Analysis

S. E. Schild^{1*}, N. R. Foster², J. P. Meyers², H. J. Ross³, P. J. Stella⁴, Y. I. Garces⁵, K. R. Olivier⁵, J. R. Molina⁶, L. R. Past⁷ & A. A. Adjei⁵ on behalf of North Central Cancer Treatment Group

¹Department of Radiation Oncology, Mayo Clinic, Scottsdale; ²Section of Biomedical Statistics and Informatics, Mayo Clinic, Rochester; ³Division of Medical Oncology, Mayo Clinic; ⁴Michigan Cancer Research Consortium, Ann Arbor; ⁵Department of Radiation Oncology, Mayo Clinic, Rochester; ⁶Department of Medical Oncology, Mayo Clinic, Rochester; ⁷Department of Radiation Oncology, Luther Hospital Eau Claire; ⁸Department of Medicine, Roswell Park Cancer Institute, Buffalo, USA

Received 20 January 2012; revised 12 March 2012; accepted 14 March 2012

Background: This pooled analysis evaluated the outcomes of prophylactic cranial irradiation (PCI) in 739 small-cell lung cancer (SCLC) patients with stable disease (SD) or better following chemotherapy ± thoracic radiation therapy (TRT) to examine the potential advantage of PCI in a wider spectrum of patients than generally participate in PCI trials.

Patients and methods: Three hundred eighteen patients with extensive SCLC (ESCLC) and 421 patients with limited SCLC (LSCLC) participated in four phase II or III trials. Four hundred fifty-nine patients received PCI (30 Gy/15 or 25 Gy/10) and 280 did not. Survival and adverse events (AEs) were compared.

Results: PCI patients survived significantly longer than non-PCI patients {hazard ratio [HR] = 0.61 [95% confidence interval (CI): 0.52–0.72]; $P < 0.0001$ }. The 1- and 3-year survival rates were 56% and 18% for PCI patients versus 32% and 5% for non-PCI patients. PCI was still significant after adjusting for age, performance status, gender, stage, complete response, and number of metastatic sites (HR = 0.82, $P = 0.04$). PCI patients had significantly more grade 3+ AEs (64%) compared with non-PCI patients (50%) ($P = 0.0004$). AEs associated with PCI included alopecia and lethargy. Dose fractionation could be compared only for LSCLC patients and 25 Gy/10 was associated with significantly better survival compared with 30 Gy/15 (HR = 0.67, $P = 0.018$).

Conclusions: PCI was associated with a significant survival benefit for both ESCLC and LSCLC patients who had SD or a better response to chemotherapy ± TRT. Dose fractionation appears important. PCI was associated with an increase in overall and specific grade 3+ AE rates.

Key words: PCI, prophylactic cranial irradiation, radiotherapy, small-cell lung cancer, survival

*Corresponding author: Dr S. E. Schild, Department of Radiation Oncology, Mayo Clinic, 5777 East Mayo Boulevard, Phoenix, AZ 85255, USA. Tel: +1-480-301-8133; Fax: +1-480-301-7687; E-mail: sschild@mayo.edu

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

Koichi Goto, MD, PhD,* Yukito Ichinose, MD,† Yuichiro Ohe, MD, PhD,‡
 Nobuyuki Yamamoto, MD, PhD,§ Shunichi Negoro, MD,|| Kazuto Nishio, MD, PhD,¶
 Yohji Itoh, PhD,# Haiyi Jiang, MD,# Emma Duffield, MSc,** Rose McCormack, PhD,**
 Nagahiro Saijo, MD, PhD,¶¶ Tony Mok, MD,†† and Masahiro Fukuoka, MD, PhD¶¶

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.

(*J Thorac Oncol.* 2012;7: 115–121)

*National Cancer Center Hospital East, Chiba; †National Kyushu Cancer Center, Fukuoka; ‡National Cancer Center Hospital, Tokyo; §Shizuoka Cancer Center, Shizuoka; ||Hyogo Cancer Center, Hyogo; ¶Kinki University School of Medicine, Osaka; #AstraZeneca, Osaka, Japan; **AstraZeneca, Macclesfield, United Kingdom; and ††The Chinese University of Hong Kong, Shatin, Hong Kong.

Disclosure: K. Goto has received honoraria from AstraZeneca, Chugai Pharmaceutical Co., Ltd, and Ono Pharmaceutical Co., Ltd; S. Negoro and K. Nishio have received honoraria from AstraZeneca; Y. Itoh, H. Jiang, E. Duffield, and R. McCormack are employees of AstraZeneca and hold stock in the company; M. Fukuoka has received honoraria from AstraZeneca, Chugai Pharmaceutical Co., Ltd, Daiichi-Sankyo, and Ono Pharmaceutical Co., Ltd; T. Mok has acted as a consultant to AstraZeneca, Roche, Eli Lilly, Taiho Pharmaceutical Co., Ltd, Merck Serono, Boehringer Ingelheim, AVEO Pharmaceuticals Ltd, and Pfizer and has received honoraria from AstraZeneca, Roche, Eli Lilly, Boehringer Ingelheim, and Merck Serono and research funding from AstraZeneca.

Address for correspondence: Koichi Goto, Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwanoha, 6-5-1, Kashiwa, Chiba 277-8577, Japan. E-mail: kgoto@east.ncc.go.jp

Yuichiro Ohe is currently at National Cancer Center Hospital East, Chiba, Japan.

Presented in part at the 50th Annual Meeting of the Japan Lung Cancer Society, Tokyo, Japan, November 12–13, 2009; the European Organization for Research and Treatment of Cancer, the National Cancer Institute, and the American Society of Clinical Oncology (EORTC-NCI-ASCO) Annual Meeting, Brussels, Belgium, October 15–17, 2009; the 13th World Conference on Lung Cancer, July 31–August 4, 2009, San Francisco, CA, USA; the 49th Annual Meeting of the Japanese Respiratory Society, June 12–14, 2009; and the 45th Annual Meeting of the American Society of Clinical Oncology, May 29–June 2, 2009, Orlando, FL, USA.

Copyright © 2011 by the International Association for the Study of Lung Cancer
 ISSN: 1556-0864/12/0701-0115

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