厚生労働科学研究委託費

革新的がん医療実用化研究事業

がん疼痛へのオピオイド使用に対するバイオマーカーを用いたランダム化比較試験

平成２６年度　委託業務成果報告書

業務主任者　中川　和彦

平成２７（２０１５）年　３月

様式第１８

委託業務成果報告書への標記について

委託業務に係る成果報告書の表紙裏に、次の標記を行うものとする。

本報告書は、厚生労働省の厚生労働科学研究委託事業による委託業務として、学校法人近畿大学　理事長　清水由洋が実施した平成２６年度「がん疼痛へのオピオイド使用に対するバイオマーカーを用いたランダム化比較試験」の成果を取りまとめたものです。

別添１

厚生労働科学研究委託費委託業務成果報告書表紙

別添２

厚生労働科学研究委託費委託業務成果報告書目次

別添３

厚生労働科学研究委託費委託業務成果報告（総括）

別紙１

厚生労働科学研究委託費

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別紙３

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（総括）

がん疼痛へのオピオイド使用に対するバイオマーカーを用いたランダム化比較試験

業務主任者　中川　和彦
近畿大学医学部内科学腫瘍内科部門　教授

研究要旨　本研究の目的は、がん疼痛患者の COMT-SNPs 測定に基づいてモルヒネとオキシコドンの第三相ランダム化比較試験を行い、オピオイド個別化治療の有用性を検討、将来のオピオイドの個別化治療に繋げることである。また、治療効果や有害事象に関わる薬理学的バイオマーカー候補分子との相関より、実測可能な薬理学的バイオマーカーを得ることを目的とする。

倉田　宝保　（関西医科大学内科学第一講座　教授）
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A．研究目的

本研究の目的は、がん疼痛患者の COMT-SNPs 測定に基づいてモルヒネとオキシコドンの第三相ランダム化比較試験を行い、オピオイド個別化治療の有用性を検討、将来のオピオイドの個別化治療に繋げることである。また、治療効果や有害事象に関わる薬理学的バイオマーカー候補分子との相関より、実測可能な薬理学的バイオマーカーを得ることを目的とする。

B．研究方法

がん疼痛を有する者を対象とした多施設共同ランダム化第三相試験

＜対象＞一次登録：根治手術不能がん患者。二次登録：がん疼痛を有し、NSAIDs またはアセトアミノフェンを常用量投与中で、疼痛スケール Numerical Rating Scale（NRS）3 以上のオピオイド治療の対象となる症例。登録前 2 週間以内にオピオイドによる治療歴のない症例。化学療法後 2 週間以上経過している症例。本プロトコール中、化学療法施行のない症例。文書による同意が得られている症例。＜目標症例数＞200 例（二次登録）

＜症例数設定根拠＞　先行研究より、COMT-SNPs (rs4680) GG 症例において、モルヒネ高用量必要症例は 36.8%であり、オキシコドンの高用量必要症例は 5%程度と見積もった。以上のことから、GG 症例において、モルヒネ群、オキシコドン群のイベント発生割合をそれぞれ 35%、5%と仮定した。有意水準を片側 2.5%、検出力を 80%として、フィッシャーの直接確率検定にて算出すると、必要症例数は 1 群 31 例（両群計 62 例）となる。GG genotype 症例が全体の 1/3 程度と見込まれるため、必要登録数は 186 例となる。若干の解析不能例を見込んで、目標症例数を 200 例とした。

＜方法＞　前向き臨床試験において、がん患者の一次登録を行い、COMT-SNPs(rs4680)をライフテクノロジー社 Taqman SNP Genotyping Asssays を用いて測定する。がん疼痛の出現後、初回オピオイド治療の適応時に、COMT 遺伝子多型における GG アレル群、Non-GG アレル群の二群に層別化し、二次登録を行う。それぞれモルヒネ速放剤群、オキシコドン速放剤群の二群にランダム化を行い、NRS の 33%以上の減少かつ NRS3 以下になるまでタイトレーションを行う。Day1 投与時の高用量必要症例（モルヒネ力価換算量）の頻度を臨床的・定量的エンドポイントにして、GG アレル群、Non-GG アレル群での、モルヒネおよびオキシコドンの治療効果を比較検討する。また、オピオイドの有害事象発現に関わる各種バイオマーカー候補を測定し、両者の相関を統合的に検討する。オピオイド治療前・治療 1 日後、治療 8 日後に NRS、心理テストおよび QOL 評価尺度および採血（前 13.5ml, 1,8 日目各 5ml）を施行する。オピオイド治療は通常の治療指針に従って行う。

（倫理面への配慮）

本研究による身体的な危険性は採血のみでありきわめて少ない。本研究に用いるゲノム DNA 遺伝子多型の検出はモルヒネおよびオキシコドンの代謝およ

び薬理作用に関連した遺伝子に制限して解析を行う。本研究では、検体提供者に登録前に同意説明文書・同意書に基づき、本研究の意義、目的、方法、予測される結果や不利益について説明し、文書により自由意思による検体提供者の同意を得る。原則的に各施設は倫理委員会への承認を必要とする。個人情報は個人情報管理者により連結可能匿名化され、厳重に管理される。連結した遺伝子情報が第三者に渡ることはない。本研究では、3省合同「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守する。各臨床試験の実施にあたっては「ヘルシンキ宣言」「臨床試験に関する倫理指針」「個人情報保護法」「ヒトゲノム・遺伝子解析研究に関する倫理指針」など関連の指針や法律・省令・告示等に従う。

C．研究結果

56名の固形がん患者が1次登録され、全ての患者のCOMT遺伝子多型の解析を行っている。解析結果は遺伝子情報を含む個人情報管理データベースに入力され保管されている。これらの内、4名にNRS3を超える疼痛の出現が観察され、2次登録、無作為層別、オピオイドによる、タイトレーションが行われた。期間中8名に2次登録がなされないままオピオイド治療が施され、脱落例と考えられた。

D．考察

56例中12例にNRSが3以上の疼痛が出現し、オピオイドによる除痛が必要と判断された。このうち8例は何らかの理由により2次登録がなされずに、脱落症例となっており、原因の究明と改善策の提案が必要と考えられた。

E．結論

4か月でオピオイドが必要とされない疼痛あるいは無痛の56例が登録されたが、経過中に約25%にオピオイド治療が施された。がん患者の緩和ケアにおける疼痛コントロールは重要な課題であると考えられた。

今後、症例集積を重ね、COMT遺伝子多型に基づくオピオイド製剤選択の重要性を明らかにしてゆく。

F．研究発表

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4. H Matsuoka,C Makimura,A Koyama,K Sakai,R Sakamoto,Y Ohtake,M Murata,H Okumi,M Otsuka,K Nakagawa, Correlation between cancer patients' anticipated pain reduction and actual reduction after treatment. 6th Congress of Asian College of Psychosomatic Medicine [ACPM],2014年8月

G．知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　　なし

様式第１９

学　会　等　発　表　実　績

委託業務題目「がん疼痛へのオピオイド使用に対するバイオマーカーを用いたランダム化比較試験」
機関名　学校法人　近畿大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
全人的苦痛（トータルペイン）の評価を行い診療に臨んだ非定型顔面痛の一例	酒井清裕 阪本亮 松岡弘道 小山敦子	第5回日本プライマリケア連合学会学術大会	2014年5月	国内
治療前の患者自身の疼痛改善度予測が、　疼痛予後に与える影響について　～良くなると思う患者ほど良くなる～	松岡弘道 牧村ちひろ 大塚正友 酒井清裕 阪本亮 村田昌彦 大武陽一 奥見裕邦 小山敦子 中川和彦	第19回日本緩和医療学会	2014年6月	国内
The importance of psycho-oncology in primary care	A Koyama H Okumi H Matsuoka Y Ohtake C Makimura R Sakamoto K Sakai M Murata	16th Congress of Asian College of Psychosomatic Medicine [ACPM]	2014年8月	国外
Correlation between cancer patients ' anticipated pain reduction and actual reduction after treatment	H Matsuoka C Makimura A Koyama K Sakai R Sakamoto Y Ohtake M Murata H Okumi M Otsuka K Nakagawa	16th Congress of Asian College of Psychosomatic Medicine [ACPM]	2014年8月	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
Multiplex genomic profiling of non - small cell lung cancers from the LETS phase III trial of first-line S-1/carboplatin versus paclitaxel/carboplatin: results of a West Japan Oncology Group study	Okamoto I, Sakai K, Morita S, Yoshioka H, Kaneda H, Takeda K, Hirashima T, Kogure Y, Kimura T, Takahashi T, Atagi S, Seto T, Sawa T, Yamamoto M, Satouchi M, Okuno M, Nagase S, Takayama K, Tomii K, Maeda T, Oizumi S, Fujii S, Akashi Y, Nishino K, Ebi N, Nakagawa K, Nakanishi Y, Nishio K.	Oncotarget	2014年4月	国外
Chemotherapeutic drugs that penetrate the blood-brain barrier affect the development of hyperactive delirium in cancer patients	Matsuoka H, Yoshiuchi K, Koyama A, Otsuka M, Nakagawa K	Palliative Support Care	2014年6月26日	国外
Risk factors for cisplatin-induced nephrotoxicity and potential of magnesium supplementation for renal protection.	Kidera Y, Kawakami H, Sakiyama T, Okamoto K, Tanaka K, Takeda M, Kaneda H, Nishina S, Tsurutani J, Fujiwara K, Nomura M, Yamazoe Y, Chiba Y, Nishida S, Tamura T, Nakagawa K	PLoS One	2014月7月	国外

Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced non-squamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study.	Seto T, Kato T, Nishio M, Goto K, Atagi S, Hosomi Y, Yamamoto N, Hida T, Maemondo M, Nakagawa K, Nagase S, Okamoto I, Yamanaka T, Tajima K, Harada R, Fukuoka M, Yamamoto N.	Lancet Oncol	2014月8月	国外
Development of On-Chip Multi-Imaging Flow Cytometry for Identification of Imaging Biomarkers of Clustered Circulating Tumor Cells.	Kim H, Terazono H, Nakamura Y, Sakai K, Hattori A, Odaka M, Girault MA, Arao T, Nishio K, Miyagi K, Yasuda K.	PLOS ONE	2014年8月	国外
First-line crizotinib versus chemotherapy in ALK-positive lung cancer.	Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, Felip E, Cappuzzo F, Paolini J, Usari T, Iyer S, Reisman A, Wilner KD, Tursi J, Blackhall F; PROFILE 1014 Investigator	N Engl J Med	2014月12月	国外
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Randomized Phase III Trial Comparing Weekly Docetaxel Plus Cisplatin Versus Docetaxel Monotherapy Every 3 Weeks in Elderly Patients With Advanced Non-Small-Cell Lung Cancer: The Intergroup Trial JCOG0803/WJOG4307L.	Abe T, Takeda K, Ohe Y, Kudoh S, Ichinose Y, Okamoto H, Yamamoto N, Yoshioka H, Minato K, Sawa T, Iwamoto Y, Saka H, Mizusawa J, Shibata T, Nakamura S, Ando M, Yokoyama A, Nakagawa K, Saijo N, Tamura T.	J Clin Oncol	2015月2月	国外
Randomized phase II trial of nimotuzumab plus irinotecan versus irinotecan alone as second-line therapy for patients with advanced gastric cancer.	Satoh T, Lee KH, Rha SY, Sasaki Y, Park SH, Komatsu Y, Yasui H, Kim TY, Yamaguchi K, Fuse N, Yamada Y, Ura T, Kim SY, Munakata M, Saitoh S, Nishio K, Morita S, Yamamoto E, Zhang Q, Kim JM, Kim YH, Sakata Y.	Gastric Cancer	E-pub ahead of print	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Multiplex genomic profiling of non-small cell lung cancers from the LETS phase III trial of first-line S-1/carboplatin versus paclitaxel/carboplatin: results of a West Japan Oncology Group study

Isamu Okamoto[1], Kazuko Sakai[2], Satoshi Morita[3], Hiroshige Yoshioka[4], Hiroyasu Kaneda[5], Koji Takeda[6], Tomonori Hirashima[7], Yoshihito Kogure[8], Tatsuo Kimura[9], Toshiaki Takahashi[10], Shinji Atagi[11], Takashi Seto[12], Toshiyuki Sawa[13], Masashi Yamamoto[14], Miyako Satouchi[15], Motoyasu Okuno[16], Seisuke Nagase[17], Koichi Takayama[18], Keisuke Tomii[19], Tadashi Maeda[20], Satoshi Oizumi[21], Shinji Fujii[22], Yusaku Akashi[23], Kazumi Nishino[24], Noriyuki Ebi[25], Kazuhiko Nakagawa[5], Yoichi Nakanishi[1,18] and Kazuto Nishio[2]

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ABSTRACT:

Archival formalin-fixed, paraffin-embedded (FFPE) tumor specimens were collected from advanced NSCLC patients enrolled in LETS phase III trial comparing first-line S-1/carboplatin with paclitaxel/carboplatin and subjected to multiplex genotyping for 214 somatic hotspot mutations in 26 genes (LungCarta Panel) and 20 major variants of *ALK*, *RET*, and *ROS1* fusion genes (LungFusion Panel) with the Sequenom MassARRAY platform. *MET* amplification was evaluated by fluorescence in situ hybridization. A somatic mutation in at least one gene was identified in 48% of non–squamous cell carcinoma and 45% of squamous cell carcinoma specimens, with *EGFR* (17%), *TP53* (11%), *STK11* (9.8%), *MET* (7.6%), and *KRAS* (6.2%). Mutations in *EGFR* or *KRAS* were associated with a longer or shorter median overall survival, respectively. The LungFusion Panel identified *ALK* fusions in six cases (2.5%), *ROS1* fusions in five cases (2.1%), and a *RET* fusion in one case (0.4%), with these three types of rearrangement being mutually exclusive. Nine (3.9%) of 229 patients were found to be positive for de novo *MET* amplification. This first multiplex genotyping of NSCLC associated with a phase III trial shows that MassARRAY-based genetic testing for somatic mutations and fusion genes performs well with nucleic acid derived from FFPE specimens of NSCLC tissue.

INTRODUCTION

Lung cancer is the leading cause of death related to cancer worldwide,with non–small cell lung cancer (NSCLC) accounting for 85% of lung cancer cases (1). Advanced or metastatic NSCLC has been treated with platinum-based chemotherapies in a manner dependent on tumor histological features, with consideration given to the balance between the modest efficacy and side effects of such treatment. Over the last decade, however, substantial progress has been made in the development of genotype-based targeted therapies for advanced NSCLC. The success of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in the treatment of *EGFR* mutation–positive advanced NSCLC established a proof of concept that molecularly targeted agents are far more effective than conventional chemotherapy when administered to the appropriate genetically defined patient population (2-7). Somatic mutations in other genes including *KRAS*, *HER2*, *PIK3CA*, *BRAF*, and *DDR2* have also been investigated as potential targets for genotype-based treatment approaches in NSCLC (8). More recently, the anaplastic lymphoma kinase (ALK) TKI crizotinib was approved with a companion diagnostic test for the treatment of a relatively small (up to 3 to 5%) subset of patients with advanced NSCLC who harbor *ALK* rearrangements (9-11). The subsequent discovery of *ROS1* and *RET* rearrangements as potentially treatable targets suggested that several chromosomal translocations and corresponding gene fusions may serve as a driving force for NSCLC (12-16). These findings have highlighted the genetic diversity of NSCLC, which can no longer be considered a single disease. Furthermore, the coexistence of different genetic alterations and therapeutic targets in NSCLC patients can profoundly affect the response to therapy (17). The clinical implementation of genomic profiling for NSCLC with high-throughput and multiplex genotyping tests is thus warranted in order to prioritize appropriate therapies for individual patients (18).

We have previously presented the results of the Lung Cancer Evaluation of TS-1 (LETS) study (19, 20). This multicenter randomized phase III trial demonstrated the noninferiority of the combination of S-1 and carboplatin compared with that of paclitaxel and carboplatin in terms of overall survival (OS) for chemotherapy-naïve patients with advanced NSCLC. Our West Japan Oncology Group (WJOG) has now embarked on multiplex genomic analyses of the archival formalin-fixed, paraffin-embedded (FFPE) tumor specimens collected from the patients enrolled in the LETS study. The primary platform for genotyping of tumors adopted in the present study is the Sequenom MassARRAY system, which combines multiplex polymerase chain reaction (PCR) analysis with single-base primer extension, followed by analysis of the primer extension products by matrix-assisted laser desorption-ionization (MALDI)–time-of-flight (TOF) mass spectrometry. We thus conducted high-throughput genotyping of 214 somatic hotspot mutations in 26 genes (LungCarta Panel) (Supplementary Table S1) as well as of 20 major variants of *ALK*, *RET*, and *ROS1* fusion genes (LungFusion Panel). Given that recent preclinical and clinical studies have also implicated de novo *MET* amplification as an oncogenic driver (21-23), we also evaluated *MET* amplification in available tumor specimens by fluorescence in situ hybridization (FISH).

RESULTS

Patients and sample collection

FFPE specimens obtained at diagnosis were available for 304 (53.9%) of the 564 patients enrolled in the LETS study. Most (229 out of 304, 75.3%) of the specimens were obtained by transbronchial biopsy. Nine specimens contained no tumor cells and were excluded from further analysis. The remaining 295 specimens were subjected to extraction of DNA and RNA, yielding median amounts of 504 ng (range, 33 to 25,230 ng) and 516 ng (range, 6 to 32,795 ng), respectively. The numbers of evaluable patients were 275 for somatic gene mutations (LungCarta Panel), 240 for fusion gene characterization (LungFusion Panel), and 229 for *MET* amplification (FISH) (Figure 1). The characteristics of these groups of patients, including the efficacy results, were similar overall

Table 1. Characteritics and outcome for patients subjected to molecular analyses compared with those for the intention-to-treat (ITT) population of the LETS study

	Somatic mutation analysis (n = 275)	Fusion gene analysis (n = 240)	*MET* amplification analysis (n = 229)	ITT population (n = 564)
Characteristic				
CBDCA+PTX/CBDCA+S-1	136 (49%)/139 (51%)	117 (49%)/123 (51%)	113 (49%)/116 (51%)	282 (50%)/282 (50%)
Median age (range), years	63 (36–74)	64 (36–74)	63 (36–74)	64 (36–74)
Male/female	211 (77%)/64 (23%)	184 (77%)/56 (23%)	178 (78%)/51 (22%)	433 (77%)/131 (23%)
ECOG PS 0/1	76 (28%)/199 (72%)	63 (26%)/177 (74%)	62 (27%)/167 (73%)	177 (31%)/387 (69%)
Clinical stage IIIB/IV	68 (25%)/207 (75%)	59 (25%)/181 (75%)	60 (26%)/169 (74%)	136 (24%)/428 (76%)
Nonsmoker/smoker	49 (18%)/226 (82%)	44 (18%)/196 (82%)	38 (17%)/191 (83%)	104 (18%)/460 (82%)
Outcome				
PFS hazard ratio (95% CI)	0.88 (0.70–1.12)	0.95 (0.74–1.24)	0.83 (0.64–1.09)	1.04 (0.86–1.22)
OS hazard ratio (95% CI)	0.93 (0.71–1.21)	0.85 (0.64–1.13)	0.91 (0.68–1.21)	0.96 (0.79–1.15)

Abbreviations: CBDCA, carboplatin; PTX, paclitaxel; ECOG, Eastern Cooperative Oncology Group; PS, performance status; PFS, progression-free survival; CI, confidence interval; OS, overall survival.

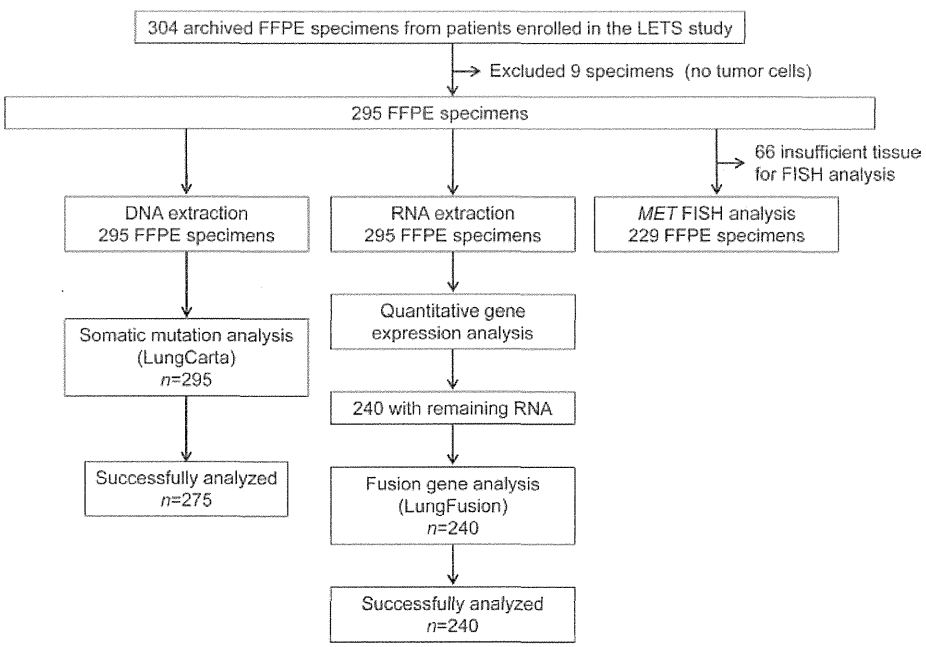

Figure 1: CONSORT diagram for the study. Of the FFPE specimens obtained from 304 advanced NSCLC patients (54%) enrolled in the LETS study, 9 specimens contained no tumor cells and the remaining 295 specimens were subjected to extraction of DNA and RNA. In addition, 229 FFPE specimens were analyzed for *MET* amplification by FISH.

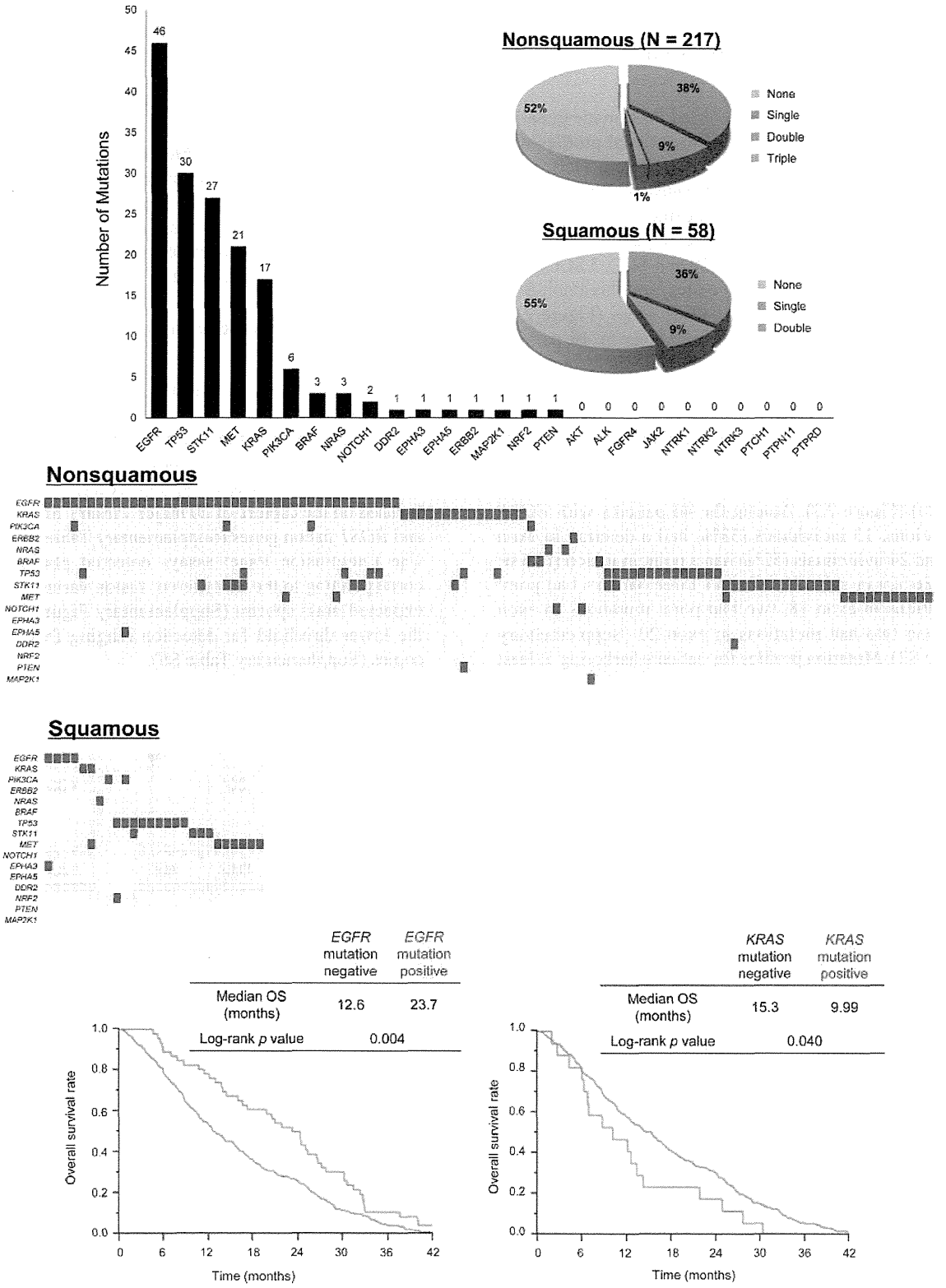

Figure 2: Analysis of somatic gene mutations in FFPE specimens from advanced NSCLC patients. A, The pie charts show the distribution for the number of mutations detected in specimens according to tumor histology. B, Number of mutations in each of the 26 analyzed genes for the 275 specimens that were successfully genotyped. C, Mutational profiles for the patients harboring at least one mutation. D, OS analysis for advanced NSCLC patients according to *EGFR* mutation and *KRAS* mutation status.

to those of the intention-to-treat population (Table 1).

Analysis of somatic gene mutations

Of the 295 specimens referred for somatic mutation analysis, 275 (93.2%) provided mutational profiles with a >90% success rate for genotyping (Figure 1). Somatic mutations in at least one gene were identified in 105 (48%) of the 217 patients with non–squamous cell carcinoma (non-SCC) and in 26 (45%) of the 58 patients with SCC. Twenty-five (9.1%) specimens (20 non-SCC, 5 SCC) were positive for mutations in two genes, and three non-SCC tumors each had mutations in three genes (Figure 2A). Overall, we identified *EGFR* mutations in 46 patients (17%), *TP53* mutations in 30 (11%), *STK11* mutations in 27 (9.8%), *MET* mutations in 21 (7.6%), *KRAS* mutations in 17 (6.2%), *PIK3CA* mutations in 6 (2.2%), *BRAF* and *NRAS* mutations in 3 each (1.1%), *NOTCH1* mutations in 2 (0.7%), and *DDR2*, *EPHA3*, *EPHA5*, *ERBB2*, *MAP2K1*, *NRF2*, and *PTEN* mutations in 1 each (0.4%) (Figure 2B). Among the 46 patients with *EGFR* mutations, 15 individuals (33%) had a deletion in exon 19 and 24 individuals (52%) had a point mutation (L858R or L861Q) in exon 21, whereas three patients had point mutations in exon 18, two had point mutations in exon 19, and two had mutations in exon 20 (Supplementary Table S2). Mutation profiles for patients harboring at least one mutation are shown in Figure 2C. *EGFR* and *KRAS* mutations were mutually exclusive. Of the 46 patients with *EGFR* mutations, three also harbored *PIK3CA* mutations. Four patients with *KRAS* mutations also had an additional mutation in *STK11*, in *TP53* and *PTEN*, in *TP53*, or in *MET*.

The median OS of *EGFR* mutation–positive patients was significantly longer than that of patients without *EGFR* mutations (23.7 vs. 12.6 months, $P = 0.004$) (Figure 2D). Conversely, patients with *KRAS* mutations had a significantly shorter median OS than did those with wild-type *KRAS* (9.99 vs. 15.3 months, $P = 0.040$) (Figure 2D).

Fusion gene characterization

We previously established an assay system based on the MassARRAY platform for detecting *EML4-ALK* in FFPE biopsy specimens of advanced NSCLC (24). In the present study, we further developed a new multiplex system for MassARRAY assays (LungFusion Panel) focused on the capture of 20 major variants of *ALK*, *RET*, and *ROS1* fusion genes (Supplementary Tables S3 to S5). The LungFusion Panel assays detected plasmid DNA corresponding to the 20 different fusion variants with the expected mass spectra (Supplementary Figure S1), with the lower threshold for detection ranging from 5 to 60 copies (Supplementary Table S6).

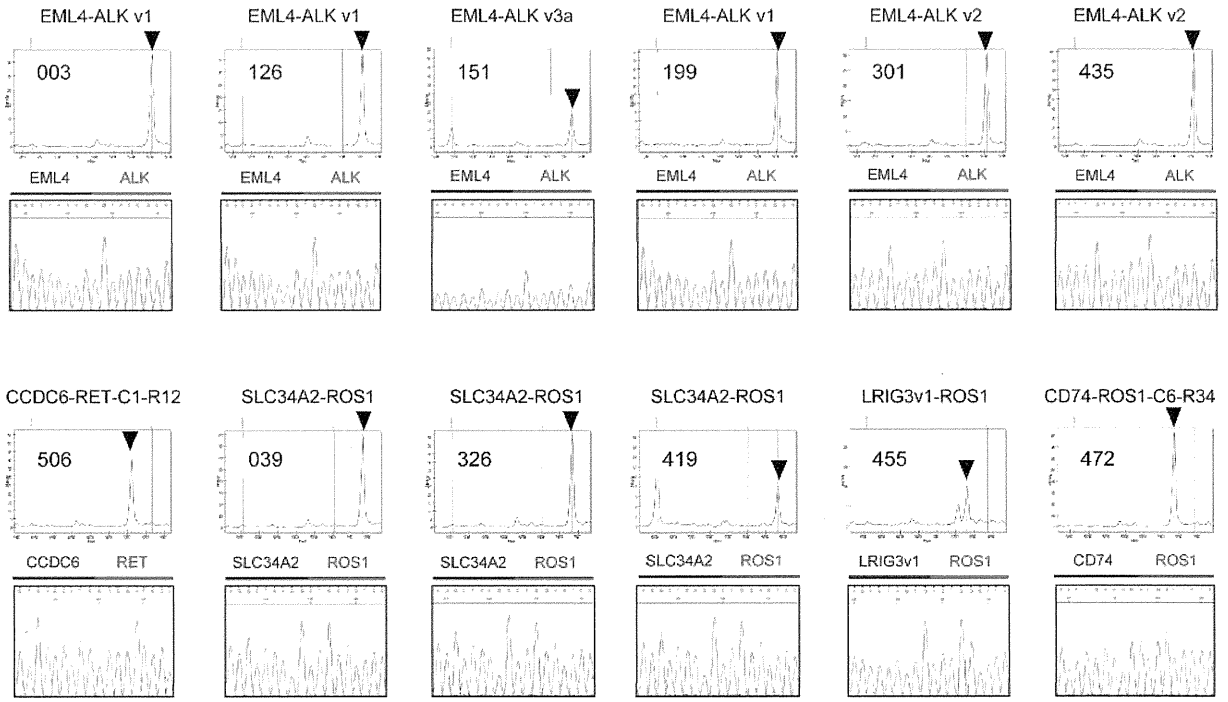

Figure 3: Detection of *ALK*, *RET*, and *ROS1* fusion genes in FFPE specimens of advanced NSCLC. Arrowheads indicate mass spectrometry peaks corresponding to the indicated fusion genes. The variants of these fusions identified with the LungFusion Panel were validated by direct sequencing.

Table 2. Clinicopathologic characteristics of the 12 patients with fusion gene–positive NSCLC
Ad: Adenocarcinoma, Sq: Squamous cell carcinoma

Fusion gene	Age (years)	Sex	Smoking history	Tumor histology	Clinical stage	Concomitant mutations
EML4-ALK v1	70	F	No	Ad	IV	*STK11* (F354L)
EML4-ALK v1	50	M	Yes	Ad	IV	*MET* (N375S)
EML4-ALK v3a	55	M	Yes	Sq	IIIB	None
EML4-ALK v1	56	M	Yes	Ad	IV	None
EML4-ALK v2	57	F	No	Sq	IIIB	None
EML4-ALK v2	50	F	Yes	Ad	IIIB	*STK11* (F354L)
CCDC6-RET	58	F	No	Ad	IV	None
SLC34A2-ROS1	74	M	Yes	Ad	IV	*KRAS* (G12V)
SLC34A2-ROS1	65	F	No	Ad	IV	*EGFR* (L858R), *PIK3CA* (E542K), *STK11*(F354L)
SLC34A2-ROS1	58	M	Yes	Ad	IV	*KRAS* (G12A)
LRIG3v1-ROS1	65	M	Yes	Other	IV	None
CD74-ROS1	53	M	Yes	Ad	IIIB	None

All 240 specimens referred for analysis with the LungFusion Panel were tested successfully. The LungFusion assay followed by direct sequencing identified *ALK* fusions in six cases (three *EML4-ALK* variant 1, two *EML4-ALK* variant 2, and one *EML4-ALK* variant 3a), a *CCDC6-RET* fusion in one case, and *ROS1* fusions in five cases (three *SLC34A2-ROS1*, one *LRIG3v1-ROS1*, and one *CD74-ROS1*) (Figure 3). The frequencies of *ALK*, *RET*, and *ROS1* rearrangements were 2.5%, 0.4%, and 2.1%, respectively, and these three types of rearrangement were mutually exclusive. Clinicopathologic characteristics of the 12 fusion-positive patients are shown in Table 2. Although these patients tended to be younger than the fusion-negative patients (median age of 58 vs. 64 years), there was no statistically significant difference in age, sex distribution, smoking history, tumor histological type, or

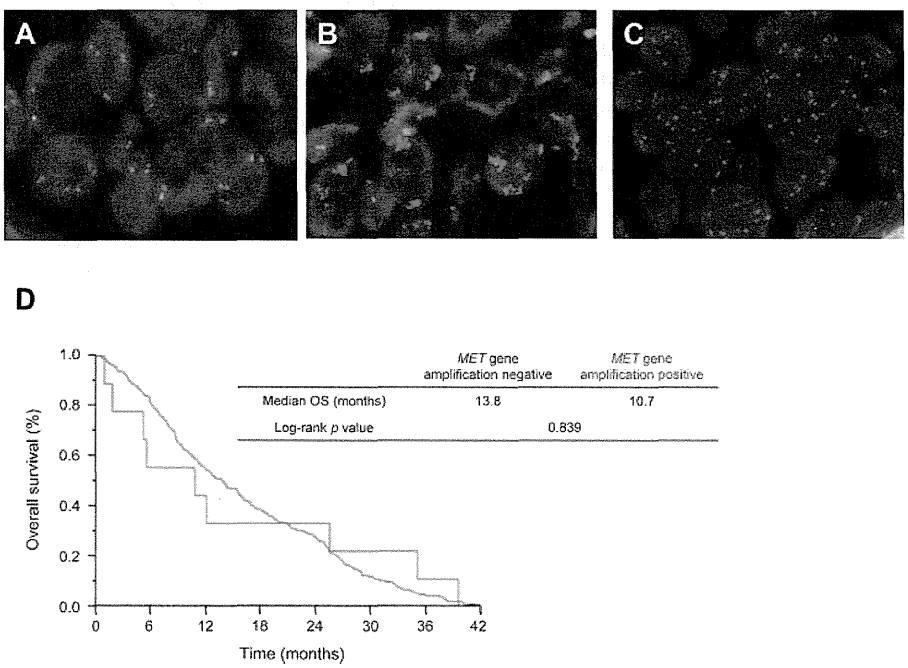

Figure 4: FISH analysis of de novo *MET* amplification in advanced NSCLC and survival analysis according to *MET* amplification status. A–C, Representative FISH images for specimens negative (A) or positive (B and C) for *MET* amplification. Green and red signals correspond to CEN7p and the *MET* locus, respectively. D, OS according to de novo *MET* amplification status in advanced NSCLC patients.

Table 3. Clinicopathologic characteristics of the nine patients with *MET* amplification–positive NSCLC

Age (years)	Sex	Smoking history	Tumor histology	Clinical stage	Concomitant mutations
54	M	Yes	Ad	IV	None
71	F	No	Ad-sq	IV	*TP53* (R248Q), *STK11* (F354L)
54	M	Yes	Ad	IV	*TP53* (R273L)
57	M	Yes	Ad	IV	None
59	M	No	Ad	IV	*EGFR* (E709A, G719S)
64	M	Yes	Ad	IV	None
46	M	Yes	Ad	IV	None
54	M	Yes	Ad	IV	None
72	M	Yes	Ad	IV	None

disease stage between these two groups. Among the *ALK* fusion–positive patients, two individuals had concurrent *STK11* (F354L) mutations and one had a *MET* (N375S) mutation (Table 2). Among the five *ROS1* fusion–positive patients, two individuals also had a *KRAS* mutation (G12V or G12A) and one had *EGFR* (L858R), *PIK3CA* (E542K), and *STK11* (F354L) mutations (Table 2). The median OS was 19.5 and 13.8 months ($P = 0.89$) for fusion-positive and fusion-negative patients, respectively.

MET amplification

MET copy number was evaluated by FISH in 229 cases and was detected in 9 cases (3.9%) (Figure 4A–C), among which the median gene copy number was 8.8 (range, 6.1 to 15.3). All *MET* amplification–positive patients had non-SCC (5.2%, 9 of 174 patients) and most were male and smokers (Table 3). Two of these patients had a *TP53* mutation, either alone or together with an *STK11* mutation, and one patient had two *EGFR* mutations (E709A + G719S) (Table 3). Although the median OS tended to be shorter for *MET* amplification–positive patients than for amplification-negative patients (10.7 vs. 13.8 months), this difference was not statistically significant (Figure 4D).

DISCUSSION

As the number of molecularly targeted therapies for molecularly defined subsets of patients with NSCLC increases, there is an increasing need for high-throughput genotyping tests to evaluate the corresponding genetic abnormalities. The successful clinical application of such tests will depend on attainment of robust performance with minute samples derived from the FFPE tumor material collected for pathological diagnosis. In the present study, we tested FFPE specimens of NSCLC tissue for multiple genetic abnormalities simultaneously with the use of multiplex assay panels based on Sequenom's MassARRAY platform. The LungCarta Panel encompasses 214 distinct mutations in 26 genes previously annotated in NSCLC. Although collection of tumor material was not mandatory in the LETS study, FFPE archival tumor specimens were obtained from more than half of the advanced NSCLC patients enrolled in the study. Although most of the collected specimens were obtained by transbronchial biopsy and were small in size, >90% were successfully genotyped, thus satisfying the dual requirements of pathological diagnosis and multiplex analysis of somatic mutations with a single biopsy sample. We detected mutations in at least one gene in about half of the tested subjects, consistent with previous studies performed with other platforms (25). The frequency of *EGFR* mutations in our study (17%) is lower than that previously determined for Japanese patients with NSCLC (26). Given that *EGFR* mutation tests have been commercially available with insurance coverage since 2007 in Japan, the reason for this difference is likely that many *EGFR* mutation–positive patients were not enrolled in the LETS study because EGFR-TKIs were available as a first-line treatment option. The bias toward a higher percentage of wild-type *EGFR* patients may also have affected the observed incidence of other somatic mutations, including both those that are nonoverlapping or associated with *EGFR* mutations. The 6% prevalence of *KRAS* mutations in our cohort is also lower than the frequency reported for Caucasian patients, consistent with the previously described ethnic differences in the incidence of *KRAS* mutations (26). We also retrospectively evaluated the influence of *EGFR* or *KRAS* genotype on survival outcome for the advanced NSCLC patients enrolled in the LETS study. *EGFR* mutation–positive patients had a significantly superior OS compared with individuals with wild-type *EGFR*, likely because most mutation-positive patients received EGFR-TKIs as second-line or later chemotherapy. On the other hand, patients who had tumors with wild-type *KRAS* had a significantly better survival compared with those who had

KRAS mutations. Given that some patients with wild-type *KRAS* had *EGFR* mutations or *ALK*, *RET*, or *ROS1* fusion genes, however, we also compared the survival outcome of *KRAS* mutation–positive patients with that of wild-type *KRAS* patients negative for these treatable targets. Although *KRAS* mutation–positive patients showed a trend toward a shorter survival compared with those negative for *KRAS* and *EGFR* mutations as well as for fusion genes (9.99 vs. 12.9 months, $P = 0.113$) (Supplementary Figure S2), the negative prognostic value of *KRAS* mutations remains uncertain on the basis of the data in the present study.

Several oncogenic gene fusions have recently been identified in NSCLC. *EML4-ALK* was the first such fusion detected in NSCLC, with its discovery in 2007 (9) being followed by the identification of *ROS1* and *RET* fusions in 2012 (12-15). Although the frequency of each of these types of fusion gene is only ~1 to 5% in unselected NSCLC patients, the affected patient subsets are treatable with corresponding kinase inhibitors. A break-apart FISH assay is the FDA-approved diagnostic test to screen for *ALK* rearrangement in NSCLC. FISH is thus currently considered the standard diagnostic technology for gene rearrangement, but its high cost and requirement for technical expertise limit its clinical application. Furthermore, timely acquisition of genotype information including oncogenic gene fusion status is required to guide rapid initiation of appropriate molecularly targeted therapies. The development of novel platforms that allow simultaneous screening for *ALK*, *ROS1*, and *RET* fusions is thus urgently needed. In the present study, we extended the MassARRAY technique to develop a multiplex screen (LungFusion Panel) designed to assess RNA isolated from FFPE biopsy specimens for *ALK*, *ROS1*, and *RET* fusion genes simultaneously. In this initial proof-of-concept effort, we confirmed robust performance of the LungFusion assay with 240 FFPE clinical samples obtained from advanced NSCLC patients, revealing a prevalence of 2.5%, 2.1%, and 0.4% for *ALK*, *ROS1*, and *RET* fusion genes, respectively. We also confirmed the mutual exclusivity of these three types of fusion gene. Of note, we found that three of five *ROS1* fusion–positive patients harbored concurrent actionable oncogenic somatic mutations of *EGFR*, *PIK3CA*, or *KRAS*. A 65-year-old woman who had never smoked had adenocarcinoma harboring *SLC34A2-ROS1* as well as *EGFR* (L858R) and *PIK3CA* (E542K) mutations. Two previous studies of Asian populations also detected coexistence of *EGFR* mutations and *ROS1* rearrangements in NSCLC patients (27, 28). Given that our cohort was also exclusively Japanese, the high prevalence of *EGFR* mutations in Asian patients with NSCLC may increase the chance for detection of coexistence of these two types of genetic alterations. As far as we are aware, the above-mentioned 65-year-old woman in our cohort is the first reported patient with both a *ROS1* fusion and a *PIK3CA* mutation. We also detected *KRAS* mutations (G12V or G12A) in two *SLC34A2-ROS1*–positive patients, with coexistence of *ROS1* rearrangement and *KRAS* mutation not having been previously described. Further studies are warranted to investigate whether the overlap between these oncogenes is clinically relevant and might affect the choice of optimal therapy.

We have previously shown that inhibition of MET signaling either with the small-molecule MET and ALK inhibitor crizotinib or by RNA interference targeted to MET mRNA resulted in marked antitumor effects in *MET* amplification–positive NSCLC cell lines both in vitro and in vivo (21). Furthermore, NSCLC patients with de novo *MET* amplification have shown a pronounced clinical response to crizotinib (22, 23), which was originally developed as a TKI for c-MET. These preclinical and clinical findings suggest that de novo *MET* amplification is an oncogenic driver for, and therefore a valid target for the treatment of, NSCLC. The clinicopathologic profile of advanced NSCLC patients with de novo *MET* amplification remains largely unknown, however. Several studies performed with different methods and different criteria for definition of gene amplification have reported a frequency of de novo *MET* amplification in NSCLC ranging from 2% to 20% (29). In the present study, we applied strict guidelines of the American Society of Clinical Oncology/College of American Pathologists for the definition of gene amplification and thereby identified 9 out of 229 advanced NSCLC patients (3.9%) as having de novo *MET* amplification. Eight of these nine patients had adenocarcinoma and one had adenosquamous carcinoma histology. Although most of the nine patients were male and smokers, no specific clinicopathologic feature was significantly associated with de novo *MET* amplification. The notion that tumors positive for de novo *MET* amplification, *EGFR* mutations, or oncogenic (*ALK*, *ROS1*, *RET*) fusions are distinct biological entities was supported by our finding that, with one exception, these genetic alterations were mutually exclusive.

There are several potential limitations to our study. First, although we detected significant survival differences between advanced NSCLC patients positive or negative for *EGFR* or *KRAS* mutations, the analysis did not take into account other prognostic factors and should be interpreted within the context of its retrospective nature. Second, although the LungCarta Panel encompasses >200 mutations across 26 cancer genes, important gene mutations may be present outside of the selected hotspot regions. Given that the MassARRAY system involves multiple primer sets for both PCR amplification and primer extension, the addition of new mutations to existing panels is straightforward but still requires effort. Lastly, we performed molecular testing with a single biopsy specimen, which may not be representative of all sites within a tumor.

In summary, the present study constitutes the