Cross-Validation Study for Epidermal Growth Factor Receptor and *KRAS* Mutation Detection in 74 Blinded Non-small Cell Lung Carcinoma Samples

A Total of 5550 Exons Sequenced by 15 Molecular French Laboratories (Evaluation of the EGFR Mutation Status for the Administration of EGFR-TKIs in Non-Small Cell Lung Carcinoma [ERMETIC] Project—Part 1)

Michèle Beau-Faller, MD, PhD,†‡ Armelle Degeorges, PhD,§ Estelle Rolland, MSc,‖ Mounia Mounawar, PhD,¶ Martine Antoine, MD,‡# Virginie Poulot, LabTec,** Audrey Mauguen, MSc,‖ Véronique Barbu, MD, PhD,†† Florence Coulet, PharmD, PhD,‡‡ Jean-Luc Prétet, PhD,§§ Ivan Bièche, PharmD, PhD,‖‖ Hélène Blons, PharmD, PhD,¶¶ Jean-Christophe Boyer, PharmD, PhD,## Marie-Pierre Buisine, PharmD, PhD,*** Florence de Fraipont, PharmD, PhD,††† Sarab Lizard, PhD,‡‡‡ Sylviane Olschwang, MD, PhD,§§§ Patrick Saulnier, PhD,‖‖‖ Delphine Prunier-Mirebeau, MD, PhD,¶¶¶ Nicolas Richard, PharmD, MSc,### Claire Danel, MD,‡**** Elisabeth Brambilla, MD, PhD,‡†††† Christos Chouaid, MD, PhD,‡,‡‡‡‡ Gérard Zalcman, MD, PhD,‡§§§§ Pierre Hainaut, PhD,¶ Stefan Michiels, PhD,‖ and Jacques Cadranel, MD, PhD‡#‖‖‖‖*

Introduction: The Evaluation of the epidermal growth factor receptor (EGFR) Mutation status for the administration of EGFR-Tyrosine Kinase Inhibitors in non-small cell lung Carcinoma (NSCLC) (ERMETIC) project part 1 assessed the accuracy of *EGFR* and *KRAS* mutations detection in NSCLC among 15 French centers.

Methods: The 15 ERMETIC centers selected 74 NSCLC surgical specimens from previously untreated patients. Paraffin and paired frozen DNA were sequenced for *EGFR* exons 18 to 21 and *KRAS* exon 2 by an external molecular laboratory, yielding a gold standard. The 74 blinded paraffin DNAs were redistributed to the 15 ERMETIC laboratories for sequencing of a total of 5550 exons. Results were compared with the gold standard and between centers by discordance rates and kappa statistics.

Results: The gold standard included 39 mutated samples with 22 *EGFR* and 17 *KRAS* mutated samples. Kappa statistics showed that 10, 6, and 6 of the 15 ERMETIC centers had a moderate to good kappa score, when compared with external laboratory for *EGFR* exon 19, *EGFR* exon 21, and *KRAS* exon 2, respectively. Kappa statistics showed moderate score between centers which increased to good for *EGFR* exon 19 mutation when removing 16 poor-quality samples with high nonamplificable rates.

Conclusions: Paraffin-embedded specimens may represent a suitable source of DNA for sequencing analyses in ERMETIC centers. *EGFR* exon 19 deletions were most accurately detected by ERMETIC centers. Ease and accuracy of results, depended more on the quality of sample than on the difference in molecular sequencing procedures between centers, emphasize the need of preanalytical quality control programs.

*Laboratoire de Biologie Moléculaire, Hôpital de Hautepierre, Strasbourg; †EA4438 Faculté de Médecine, Strasbourg; ‡IFCT, French Thoracic Intergroup, Paris; §Unité de Pharmacologie, Département de Biologie des Tumeurs, Institut Curie, Paris; ‖Service de Biostatistique et d'Epidémiologie, Institut Gustave-Roussy, Villejuif; ¶International Agency for Research on Cancer, Lyon; #Service d'Anatomie pathologique, Hôpital Tenon, Paris; **Equipe de Recherche 2 de la Faculté de Médecine Pierre et Marie Curie, Université Paris VI, Hôpital Tenon, Paris; ††Laboratoire Commun de Biologie et Génétique moléculaires, Pôle de Biologie Imagerie, Assistance Publique-Hôpitaux de Paris, Hôpital Saint Antoine, Paris; ‡‡Laboratoire d'Oncogénétique et d'Angiogénétique moléculaires, Groupe Hospitalier Pitié-Salpétrière, Paris; §§EA3181, IFR133, Université de Franche-Comté, Laboratoire de Biologie Cellulaire et Moléculaire, CHU Besançon, Boulevard Fleming, Besançon; ‖‖Laboratoire d'Oncogénétique, Plateforme HUVEGEN, Centre René Huguenin, Saint-Cloud; ¶¶Service de Biochimie, UF de Pharmacogénétique et Oncologie moléculaire, Hôpital Européen Georges Pompidou, Paris; ##Unité de Toxicologie, Laboratoire de Biochimie, CHU Carémeau, Nîmes; ***Laboratoire Oncologie et Génétique Moléculaires, Pôle de Biochimie et de Biologie Moléculaire, Centre de Biologie Pathologie, CHRU de Lille, Lille; †††Université Joseph Fourier, INSERM U823, UF Cancérologie biologique et biothérapie, CHU Grenoble, Grenoble; ‡‡‡Unité de Biologie Moléculaire, Centre GF Leclerc, Dijon; §§§Département de Biopathologie, Institut Paoli Calmettes, Marseille; ‖‖‖Laboratoire de

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Primary lung cancer accounts for the highest number of cancer deaths worldwide,[1] with, a 5-year survival between 10 and 15% in France.[2,3] More than 80% of lung cancers are non-small cell lung cancer (NSCLC), which are subdivided into squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell carcinoma.[4]

In ADC, epidermal growth factor receptor (*EGFR*) is found to be mutated in 10 to 15% tumors from white patients[5] and in more than 40% of tumors from Asian patients.[6] Inhibiting EGFR signaling using tyrosine kinase inhibitors (TKIs), gefitinib or erlotinib, is an effective treatment for patients with tumors expressing *EGFR*-sensitizing mutations.[7–10] Molecular selection should be performed because clinical characteristics were shown to be insufficient to accurately select patients harboring *EGFR* mutations and because EGFR-TKI resistance may be conferred by mutations in *KRAS* (~30% of ADC cases) or in *EGFR* exon 20.[11,12]

Many diagnostic methods are available for *EGFR* and *KRAS* mutation analysis, but standardized procedures are lacking,[13] although gefitinib recently obtained restrictive European Medicines Agency/Food and Drug Administration approval for first-line treatment of patients with *EGFR* mutated NSCLC.[14] In 2005, the French National Cancer Institute granted a nationwide 2-years multicenter prospective project to address the standardization of mutation analysis. The project entitled Evaluation of the EGFR Mutation status for the administration of EGFR-TKIs in NSCLC (ERMETIC) involves 15 French clinical/pathological/biological centers. The project has three consecutive objectives: (i) validate the widespread use of sequencing as a screening method for *EGFR* and *KRAS* molecular diagnosis on fixed paraffin-embedded tissues; (ii) select and rank clinical, pathological, and biological predictors of EGFR-TKI response and clinical benefit in a large prospective clinical cohort; and (iii) determine the most cost-effective strategy to prescribe EGFR-TKIs, i.e., based on EGFR biomarkers. This study focuses on part 1 of the ERMETIC project.

PATIENTS AND METHODS

The ERMETIC project part 1 was subdivided into two phases, phase A and phase B. The phase A addressed the question of the discordances that could be observed between results of paraffin and frozen samples. It consisted in the comparison of direct sequencing analysis of paraffin-embedded samples from 74 patients with NSCLC and their snap-frozen counterparts by an external molecular laboratory (P. Hainaut, IARC, Lyon, France). The phase B addressed the cross-validation of paraffin-embedded samples analysis among the 15 ERMETIC French molecular laboratories.

Description of the ERMETIC Tumor Bank

Each of the 15 ERMETIC centers selected 1 to 10 NSCLC surgical specimens from previously untreated patients, according to French regulations. For each deidentified specimen, a fixed paraffin-embedded block containing more than 50% tumor cells and a snap-frozen counterpart was required. Samples were selected for clinical features linked to a high probability of *EGFR* mutation: female, ADC, and nonsmoker.[6] Each center also selected one patient with SCC, with a high probability of being wild type for *EGFR* and *KRAS*. Tumor paraffin blocks were sent to the coordination center for centralized review by three pathologists (M.A., E.B., and C.D.) based on 2004 World Health Organization classification and to determine the proportion of tumor cells assessed on a slide performed at the end of block sections and stained by hematoxylin-eosin-safran coloration.

DNA Preparation

Each ERMETIC centers prepared, without macrodissection, 16 × 3 sections (15 μm thick) from their own paraffin blocks to perform 16 extractions for each sample. All centers extracted DNA using similar principles, i.e., affinity-column-based protocols excepted for two centers using protocols based on magnetic particles, with extraction controls (water) in each series (see supplementary data, http://links.lww.com/JTO/A89; Table Si, http://links.lww.com/JTO/A90). DNAs from the 16 extractions were pooled. The median quantity of pooled DNA by sample was 13 μg (range: 0.7–127 μg) by Nano Drop (Wilmington, DE) (see supplementary data, Table Sii, http://links.lww.com/JTO/A91). Quality of pooled DNA samples was evaluated by a ladder amplification technique analysis[15] (see supplementary data, Table Sii, http://links.lww.com/JTO/A91). The 74 pooled DNA sam-

Recherche Translationnelle, Institut Gustave Roussy, Villejuif; ¶¶¶Laboratoire de Biochimie et Biologie moléculaire, CHU Angers; ###Laboratoire de Génétique moléculaire, CHU de Caen, Caen; ****Service d'Anatomie pathologique, Hôpital Européen Georges Pompidou, Paris; ††††Département de Pathologie, INSERM U823/Université Joseph Fourier, CHU de Grenoble, Grenoble; ‡‡‡‡Service de Pneumologie, Hôpital Saint-Antoine, APHP, Faculté de Médecine Pierre et Marie Curie, Université Paris VI, Paris; §§§§Service de Pneumologie, CHU de Caen, Caen; ||||||Service de Pneumologie, Assistance Publique Hôpitaux de Paris, Hôpital Tenon, Faculté de Médecine Pierre et Marie Curie, Université Paris VI, Paris, France.

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Address for correspondence: Jacques Cadranel, MD, PhD, Service de Pneumologie, Hôpital Tenon, 4 rue de la Chine 75970, Paris Cedex 20, France. E-mail: jacques.cadranel@tnn.aphp.fr

Armelle Degeorges contributed equally for this study.

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ples were shipped to the coordination center to be aliquoted, blinded, and redistributed to each of the 15 ERMETIC centers, which received the same quantity (1/16 of pooled DNA samples) of the extracted DNA for each sample.

Pooled DNA extracts from paraffin samples and snap-frozen paired specimens were also sent to the external molecular laboratory (P. Hainaut, IARC, Lyon, France).

Sequencing Procedure

Sequencing was performed in the external molecular laboratory as described previously[16] and in the 15 different ERMETIC centers by using their own procedures and internal quality controls (see supplementary data, Table Siii, http://links.lww.com/JTO/A92). A common decision algorithm was adopted (M.B.-F., A.D., V.B., F.C., and J.-L.P.), including a minimum of three polymerase chain reaction (PCR) amplification attempts for each exon, before a sample was considered nonamplificable (NA). All mutations had to be detected in both strands and confirmed by sequencing analysis of a second, independent PCR product. Each ERMETIC center sequenced *EGFR* (exon 18–21) and *KRAS* (exon 2) genes in all blinded paraffin DNA extracts, and the external molecular laboratory sequenced *EGFR* and *KRAS* genes in all paraffin and paired snap-frozen DNA samples, to provide a gold standard reference value. Centers used direct or nested sequencing, but each used their own standard procedure to reflect the different center molecular procedures. Therefore, testing was done as if the different laboratories had to analyze the samples in routine diagnosis.

Frozen samples were used as the gold standard because unfixed samples have a lower risk of DNA artifacts.[17] For six samples, frozen tissue was not proper (absence or $<30\%$ of tumor cells) to be used as the gold standard. For those six samples, more sensitive molecular techniques were performed on fixed paraffin-embedded samples: for *KRAS*, *KRAS* Therascreen DxS kit (DxS, Manchester, UK) (A.D.), peptide-nucleic-acid (PNA)-mediated PCR clamping[18] (Eurogentec, Liège, Belgium) (M.B.-F.), allele-specific oligonucleotide PCR (N.R.), Taqman allelic discrimination[19] (H.B.) and for *EGFR*, fragment size separation for insertions/deletions of exon 19 (M.B.-F., A.D., and H.B.) and PCR-restriction fragment length polymorphism (RFLP) for L858R (A.D. and H.B.).[20] These assays were also used to document discrepancies between the external laboratory and ERMETIC centers for a few samples that were found mutated by ERMETIC centers and wild type (WT) by the external laboratory.

Data Collection/Nomenclature

Mutations were described using nomenclature of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). Unknown variants (UVs) were classified as mutations not described in the database at the time of analysis. All results were reviewed by two molecular biologists to identify ambiguities in nomenclature (M.B.-F. and A.D.). Each centers completed a questionnaire to inventory their "Materials and Methods."

Statistical Analysis

The sample size was determined to have a specific precision on the estimated discordance rate between DNA sequencing from paraffin-embedded samples and snap-frozen samples. For a confidence interval of a width of 10% around an expected discordance rate of 15%, at least 49 samples were requested. This number was increased to 74 samples to anticipate frozen specimens with too few tumor cells to perform DNA sequencing and the addition of samples with SCC histology. Discordances between paraffin and frozen samples were addressed. A false-positive result was defined as presence of a mutation in paraffin and WT in paired frozen sample/gold standard (see "Results" section). A false negative was defined as WT in paraffin and presence of a mutation in paired frozen sample/gold standard (see "Results" section). Paraffin-embedded and frozen sample DNA analyses were compared by discordance rates, i.e., proportion of false-positive and false-negative results. Discordance was not defined if one of the samples was NA. Each center was compared with the external molecular center using a standard kappa statistic. Reproducibility of paraffin-embedded sample analysis among the 15 centers was assessed using a global kappa test.[21] According to Landis and Koch classification,[22] concordance was considered bad if kappa was less than 0.20, poor if kappa was 0.20–0.40, moderate if kappa was 0.40–0.60, and good if kappa was greater than 0.60. An exploratory sensitivity analysis was performed, excluding samples with high NA rates, i.e., not amplified by half of centers. Statistical tests were two sided, and p values less than 0.05 were considered significant.

RESULTS

Clinical and Pathological Characteristics

Clinical and pathological characteristics of the tumor bank (74 samples) are listed in Table 1. Of the 63 ADC samples in the tumor bank, 39 (62%) were from nonsmoking patients of whom 33 (85%) were female. The majority ($n = 60$; 83%) of paraffin-embedded blocks were less than 5 years old, and all except three were formalin fixed. In most cases, paraffin samples had a greater percentage of tumor cells than paired frozen samples (frozen versus paraffin, Fisher's exact test $p = 0.04$).

Phase A: Sequencing of Cryopreserved and Fixed Paraffin-Embedded Samples by External Molecular Center

Direct sequencing of *EGFR* exons 18 to 21 and *KRAS* exon 2 was performed by the external molecular laboratory on blinded tumor DNA extracts from 74 paraffin and 68 paired frozen specimens. Six frozen samples were excluded as they contained less than 30% tumor cells. Results are summarized in Table 2 and Figure 1.

Cryopreserved Samples (n = 68)

All mutations except four detected in DNA from frozen samples were previously described (Hope database http://www.egfr.org and http://www.sanger.ac.uk/genetics/CGP/cosmic/) (Table 2). *EGFR* mutations were described in 22 (32%) frozen samples, including three samples with two *EGFR* mutations. All 22 *EGFR* mutated samples were from females with ADC of whom 19 were never smokers and three

TABLE 1. Clinical and Pathological Characteristics of ERMETIC Tumor Bank

	All (n = 74), N (%)	ADC (n = 63), N (%)	SCC (n = 11), N (%)
Age (yr), median (range)	65 (39–84)	65 (42–84)	62 (39–80)
Gender			
Female	56 (76)	53 (84)	3 (27)
Male	18 (24)	10 (16)	8 (73)
Smoking status			
Never smoker	39 (53)	39 (62)	0 (0)
Current/former smoker	35 (47)	24 (38)	11 (100)
Paraffin tumor cells percentage, median (range)	80 (30–90)	80 (30–90)	80 (50–90)
Frozen tumor cells percentage, median (range)	70 (0–100)	70 (0–90)	70 (0–100)
Type of fixation			
Formol	71 (96)	60 (95)	11 (100)
Other or unknown	3 (4)	3 (5)	0 (0)
Age of paraffin embedded blocks[a] (2 MD)			
≤5 yr	60 (83)	52 (85)	8 (73)
>5 yr	12 (17)	9 (15)	3 (27)

[a] Age was calculated from the time of tumor sample fixation until May 2006 (date of ERMETIC bank collection).

ADC, adenocarcinoma; SCC, squamous cell carcinoma; MD, missing data.

were current/former smokers. One mutation was found in exon 18 (G719A), associated with an exon 21 mutation (L861Q). One UV insertion was found in exon 20. Thirteen samples had a deletion in exon 19 and one also contained an UV single mutation. Eight samples had a mutation in exon 21 (seven L858R and one L861Q mutations), one also had a single UV mutation. Two DNA samples were nonamplificable (NA) for exon 19. *KRAS* mutations were found in 14 (21%) ADC samples including 10 females and 4 males of which nine were current/former smokers and five were never smokers. All DNA samples were amplifiable for *KRAS*. *EGFR* and *KRAS* mutations were mutually exclusive.

Fixed Paraffin-Embedded Samples (n = 74)

All *EGFR* and *KRAS* mutations found in paraffin samples were also found in the 68 paired frozen samples. No discordance (see "Patients and Methods" section) was found in 46 amplified samples for exon 19. A single discordance (2%) was found in 54 samples amplified for *EGFR* exon 21 (L858R), and two discordances (4%) were found in 64 samples amplified for *KRAS* (G12C and G12V) (Table 2). All discordances were mutations found in frozen but not in paraffin samples and were classified as three false-negative results.

DNA was NA from 75 of 370 exons (20%) analyzed from paraffin samples (Figure. 1). Nine samples accounted for 39 NA exons (52%), these samples were NA for all four

TABLE 2. *EGFR* and *KRAS* Mutations Identified by the External Molecular Center in Frozen and Paired Paraffin Samples

Frozen (n = 68)	Type of Mutation	Paraffin (n = 68)
25	*EGFR*	18
1	*EGFR* exon 18 mutations	0
1	c.2156G > C, p.Gly719Ala (G719A)[a]	NA
14	*EGFR* exon 19 mutations	12
5	c.2235_2249del; p.Glu746_Ala750del[b]	5
2	c.2236_2250del; p.Glu746_Ala750del	2
1	c.2239_2248delinsC; p.Leu747_Glu749del,p.Ala750Pro	NA
1	c.2239_2251delinsC; p.Leu747_Thr751delinsPro	1
1	c.2260A > G; p.Lys754Glu (UV)[b]	1
1	c.2237_2251del; p.Glu746_Thr751delinsAla	1
1	c.2239_2240TT > CC; p.Leu747Pro (UV)	NA
1	c.2239_2256del; p.Leu747_Ser752del	1
1	c.2240_2257del; p.Leu747_Pro753delinsSer	1
1	*EGFR* exon 20 mutations	0
1	c.2311_2312insACCGGC; p.Asp771_772insArg-His (UV)	NA
9	*EGFR* exon 21 mutations	6
7	c.2573T > G; p.Leu858Arg (L858R)[c]	5 + 1WT + 1NA
1	c.2543T > C; p.Pro848Leu (UV)[c]	NA
1	c.2582T > A; p.Leu861Gln (L861Q)[a]	1
14	*KRAS*	11
6	c.35G > A; p.Gly012Asp (G12D)	5 + 1NA
6	c.34G > T; p.Gly012Cys (G12C)	5 + 1WT
2	c.35G > T; p.Gly012Val (G12V)	1 + 1WT

[a,b,c] Presence of a double mutant.

NA, nonamplificable; WT, wild type; UV, unknown variant; EGFR, epidermal growth factor receptor.

EGFR exons and three were also NA for *KRAS*. Of these nine paraffin samples, five were more than 5 years old, and one was Bouin fixed.

Analysis of paraffin DNA samples produced very few false-negative and no false-positive mutations compared with frozen specimens, so the project progressed to phase B.

Phase B: Sequencing of Blinded Paraffin Samples by the 15 ERMETIC Centers

Gold Standard

A gold standard was built with results from (1) sequencing 68 frozen amplifiable DNA samples and (2) for six paraffin samples for which the paired frozen sample had less than 30% tumor cells, at least two more sensitive molecular techniques (described earlier) were performed, adding three supplementary *KRAS* mutations. Sequencing results for the 74 blinded paraffin samples, obtained by each of the 15 ERMETIC centers, were compared with the gold standard.

Descriptive Analysis of Mutations

Figure 2 summarizes the assessment of *EGFR* and *KRAS* mutations in the 15 participating centers. None of the ERMETIC centers detected the exon 20 *EGFR* mutation included in the gold standard (data not shown). Two

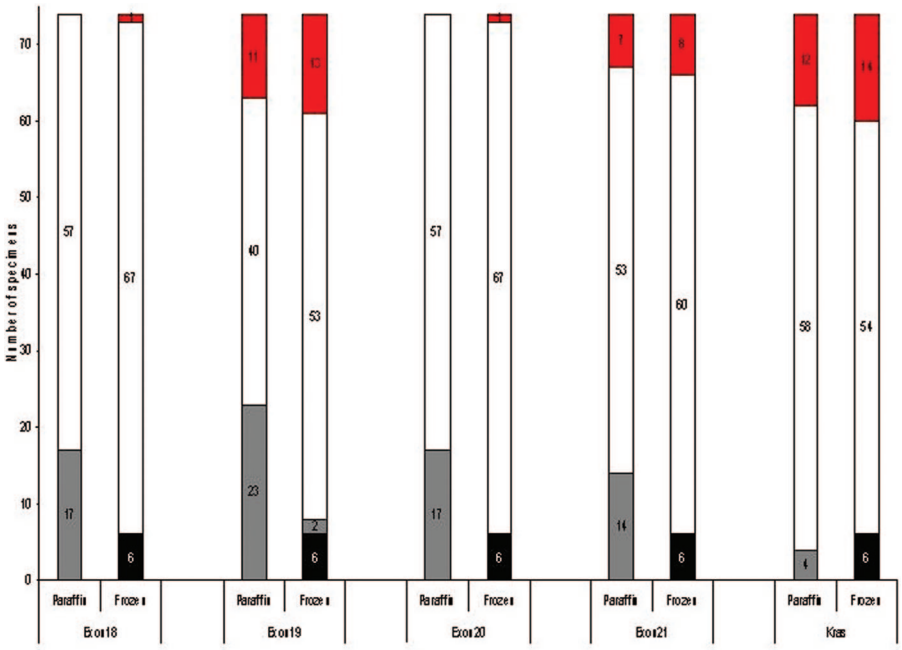

FIGURE 1. Phase A, external molecular center sequencing of frozen and paraffin samples. Six frozen tumors were not available for analysis (black bars). Proportion of mutated, wild-type and nonamplifiable DNA specimens are represented by red, white, and gray bars, respectively.

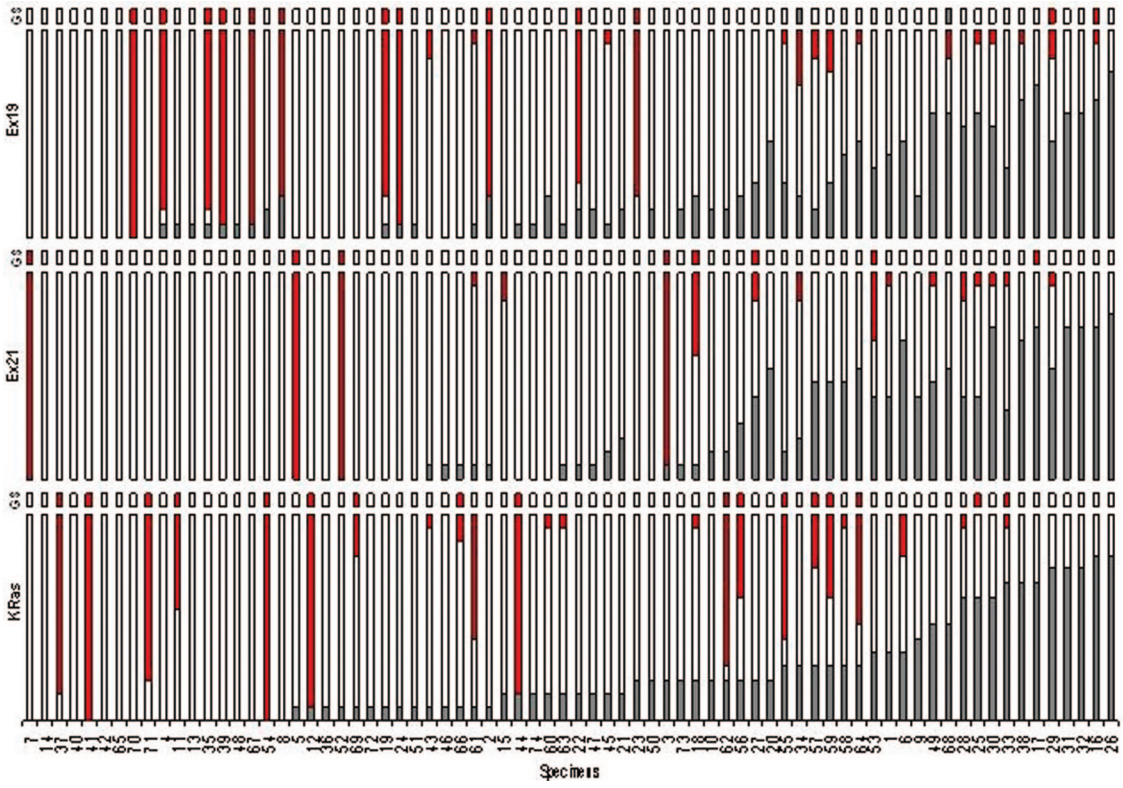

FIGURE 2. Phase B, sequencing of 74 blinded paraffin samples by the 15 ERMETIC centers. In abscissa are the 74 samples and in ordinate the 15 ERMETIC centers. Proportion of mutated, wild type, and nonamplicable DNA specimens are represented by red, white, and gray bars, respectively. GS, gold standard; ERMETIC, evaluation of the epidermal growth factor receptor mutation status for the administration of EGFR-tyrosine kinase inhibitor in non-small cell lung carcinoma.

ERMETIC centers detected the G719A mutation in exon 18 that was included in the gold standard (data not shown). For exon 19 *EGFR* analysis, 11 of 13 (85%) mutations in the gold standard were found by 11 of 15 ERMETIC centers (Figure. 2). For exon 21 *EGFR* analysis, only four of eight (50%) mutations (three L858R and one L861Q), in

TABLE 3. Results by Sample (n= 74): False Positive and False Negative Results of Sequencing, Compared with the Gold Standard

	EGFR Exon 19	*EGFR* Exon 21	*KRAS*
False-positive/negative results by sample (n = 74)			
Number of samples with at least one center with a false-positive result	10/74	10/74	7/74
Number of samples with at least one center with false-negative results	7/74	4/74	12/74

EGFR, epidermal growth factor receptor.

TABLE 4. Results by Center (n=15): False Positive and False Negative Results of Sequencing, Compared with the Gold Standard

	EGFR Exon 19	*EGFR* Exon 21	*KRAS*
False-positive/negative results by center (n = 15)			
False-positive results			
Median rate of false-positive results by center, (range)	0% (0–27%)	17% (0–37%)	8% (0–25%)
Number of centers with a false-positive rate of			
0%	9/15	7/15	5/15
>0%, <10%	1/15	0/15	4/15
≥10%	5/15	8/15	6/15
False-negative results			
Median rate of false-negative results by center, (range)	2% (0–9%)	3% (0–6%)	9% (2–17%)
Number of centers with a false-negative rate of			
0%	6/15	6/15	0/15
>0%, <10%	9/15	9/15	8/15
≥10%	0/15	0/15	7/15

EGFR, epidermal growth factor receptor.

TABLE 5. Kappa Concordance Values: Concordance of Each ERMETIC Center Sequencing with the Gold Standard

Kappa Score Rating	Number of Centers with Kappa Score Rating		
	EGFR Exon 19	*EGFR* Exon 21	*KRAS*
Good	9/15	0/15	3/15
Moderate	1/15	6/15	3/15
Poor	4/15	9/15	8/15
Bad	0/15	1/15	2/15
Median Kappa score (range)	0.52 (0.23–0.73)	0.37 (0.20–0.57)	0.39 (0.15–0.66)

EGFR, epidermal growth factor receptor.

the gold standard were found by 14 of 15 ERMETIC centers (Figure. 2). For *KRAS* analysis, 7 of 17 (41%) of mutations in the gold standard were found by 11 of 15 ERMETIC centers (Figure. 2).

False-positive and false-negative mutation results (see "Patients and Methods" section for definition) by sample, compared with the gold standard, are listed in Table 3. More samples harbor false-positive results for *EGFR* mutations than false-negative results, and vice versa for *KRAS*. False-positive and false-negative mutation results by center are listed in Table 4. The highest median rate of false-positive results by center was for *EGFR* exon 21, whereas *KRAS* exon 2 showed the highest median rate of false-negative results by center. In contrast, *EGFR* exon 19 showed a low median rate of either false-positive or false-negative results by centers.

Nonamplificable Paraffin Samples

The proportion of NA samples was highly variable (Figure. 2). Nine samples (12%) were amplified at exon 19, 21 *EGFR* and *KRAS* by all ERMETIC centers. The other 65 samples were categorized as slightly (<5 centers with nonamplified samples, n = 42, 57%), moderately (5–7 centers with nonamplified samples, n = 7, 9%), and highly (>7 centers with nonamplified samples, n = 16, 22%) NA for these three exons. Six centers were found to have the highest rates of amplification for all three exons. For exon 19, 21 *EGFR* and *KRAS* analysis, the percentage of NA samples for each center was comparable. The median rate of NA samples was 26% (0–34%) for *EGFR* exon 18, 19% (0–49%) for *EGFR* exon 19, 28% (1–61%) for *EGFR* exon 20, 20% (1–43%) for *EGFR* exon 21, and 15% (4–53%) for *KRAS*.

Kappa Concordance Statistics

Kappa concordance values were not calculated for *EGFR* exons 18 and 20 because they had a very low frequency of *EGFR* mutations. Kappa concordance statistics between each of the 15 ERMETIC centers and the gold standard was performed for *EGFR* exons 19 and 21, and *KRAS* mutation analyses (Table 5). Exon 19 had the highest concordance value, whereas exon 21 and *KRAS* had mostly moderate or poor ratings. Results of global kappa tests for concordance among the 15 ERMETIC centers are presented in Table 6. The low level of concordance between centers may reflect the number of NA DNA samples. A sensitivity analysis excluding the 16 samples qualified as highly NA resulted in increased global kappa test scores (Table 6).

DISCUSSION

ERMETIC part 1 is the first nationwide study assessing in a blinded tumor DNA bank the accuracy of detection of *EGFR* and *KRAS* mutations as molecular biomarkers for NSCLC EGFR-targeted therapy. At the beginning of the ERMETIC project (2005), limited information was available on the frequency and type of *EGFR* sensitizing or resistance mutations. DNA sequencing was considered the most effec-

TABLE 6. Kappa Concordance Values: Concordance between the 15 ERMETIC Centers

Samples Compared	Global Kappa Score (95% CI)		
	***EGFR* Exon 19**	***EGFR* Exon 21**	***KRAS***
All samples ($n = 74$)	0.47 (0.45–0.49)	0.47 (0.45–0.49)	0.42 (0.40–0.43)
Sensitivity analysis[a] ($n = 58$)	0.60 (0.58–0.61)	0.56 (0.55–0.58)	0.47 (0.45–0.49)

[a] Sensitivity analysis was performed, excluding 16 samples with high nonamplifiable rates, i.e., not amplified by half of centers.
EGFR, epidermal growth factor receptor.

tive approach to acquire detailed, specific, and comparable information. Therefore, international experts designated by the French National Cancer Institute to evaluate the ERMETIC project, recommended that external molecular laboratory, as well as ERMETIC centers, performed direct sequencing.

The external molecular laboratory analyzed 74 paraffin embedded and 68 paired frozen samples for *EGFR* and *KRAS* mutations (phase A). Our study verifies the current database of known *EGFR* single-base mutations (G719A in exon 18, and L858R and L861Q in exon 21) and known insertion/deletions in exons 19 and 20.[6] No false-positive results and only three false-negative results were identified when paraffin samples were compared with paired frozen samples. Detection of *EGFR* and *KRAS* mutations can, therefore, be accurately performed using paraffin-embedded tissues prepared for routine diagnosis. In addition, the use of these materials makes it possible to verify the presence of tumor cells and to select areas containing a high proportion of tumor cells in cases with low tumor cells (macrodissection).

Progressing into a nationwide study, the project was to compare *EGFR* and *KRAS* mutation sequencing results from 15 ERMETIC centers with the gold standard (phase B). In phases A and B of the ERMETIC project, NA DNA was the main difficulty resulting from the use of paraffin-embedded samples and also the major source of variation among results observed between ERMETIC centers. Interestingly, the NA samples often originated from the same centers, and the DNA extracted from these samples was often difficult to amplify for all exons analyzed (Table Sii). Four factors seem to be critical. The first one is the fixation procedure. The presence of trace of additives in fixatives such as Bouin's is known to inhibit the PCR amplification reactions, as found for three samples from one center (Table 7). Over fixation with formalin-based fixative may also induce DNA fragmentation and cross-linking, preventing DNA amplification and might explain some difficulty of PCR reactions in some samples from other different centers. Nevertheless, duration of fixation did not affect the rate of amplification failure in our study. Furthermore, length of amplificated DNA fragments, which are related to DNA quality, appeared smaller in the highly nonamplicable samples (Table 7). The second factor is the age and conditions of storage of the tissue block, which may result in DNA oxidation and fragmentation. Indeed, five of our nine (55%) higher nonamplificable samples were more than 5 years old (Table 7). As all tissue blocks were stored at room temperature, in all the ERMETIC centers, amplification failure was not related to conditions of storage. The third factor is the DNA extraction procedure, which may not yield sufficient amounts of DNA and/or introduce traces of reagents that impair PCR reactions. The use of xylene for DNA extraction seems to induce more difficulties for DNA amplification for several ERMETIC centers (Table 7). Although DNA concentrations seemed higher in successfully amplificated cases, there was no statistically significant differences in the levels of DNA concentration between the different categories of amplification failure (see "Results" section for definition) (Table 7). Although our data did not show any significant relationship between poor DNA quality extraction and amplification failure, this question may be addressed using newer generation mutation analysis techniques in an ongoing second ERMETIC project. Finally, ease of DNA amplification also depends on the amplicon size. Among the five exons analyzed, exon 20 amplicon had the highest size in 13 of 15 centers, may be explaining that this exon was responsible for the highest rate (311/1110, 28%) of amplification failure (data not shown). Furthermore, rate of nonamplificable cases seemed related to higher length of amplicons, for *EGFR* exons 19 and 21, as well as for exon 2 *KRAS* (Table 8).

False-negative results were observed in nine centers for *EGFR* exons 19 and 21 and in all the centers for *KRAS*. The mutations were identified by sequencing analysis sensitivity of which is known to be approximately 25% of mutated cells,[13] and therefore, all evaluated paraffin samples should contain more than 50% of tumor cells. This low sensitivity of sequencing makes this technique probably inadequate for analysis of low proportion of tumor cell samples and may have resulted in some false-negative results.[13] In our study, only 1 of 12 false-negative *KRAS* mutated samples (found falsely negative by eight centers) contained less than 40% of tumor cells. Other reasons should be hypothesized. DNA extraction procedure could be involved as extraction with xylene presented the highest percentage of false-negative results (Table 7). The mutation type may also have affected detection rates, as *EGFR* exon 19 deletions are easier to detect than point mutations in exon 21 or in *KRAS* exon 2. Interestingly, in DNA from paraffin-embedded samples, deleted fragments could be selected in the amplification procedure, whereas single-base mutations (*EGFR* exon 21 or *KRAS*) were not, an observation that would point out a different rate of mutant allele amplification efficacy on DNA altered by the fixative. This might also explain why *KRAS* analysis was so difficult for many centers. This problem may be addressed by implementing more sensitive, mutant allele-specific detection techniques.

The clinical consequence of inaccurate detection of *EGFR* or *KRAS* mutations is an important consideration because patients would potentially receive inappropriate treatments. False-positive detection of *EGFR*-activating mutations could lead to counter-productive patient treatment with EGFR-TKIs, as recently demonstrated in the Asian IPASS phase III trial.[10] Critically, patients whose tumors

TABLE 7. Paraffin Blocks Characteristics and DNA Quantity/Quality Findings in Relationship with the Rate of Amplification Failure and Number of False-Positive and False-Negative Cases

	H-NA ($n = 16$)	M-NA ($n = 7$)	S-NA ($n = 42$)	A ($n = 9$)	False-Positive Cases ($n = 22$)	False-Negative Cases ($n = 22$)
Blocks characteristics						
Fixation procedure (*n*)						
Formalin	13	7	42	9	21	21
Bouin	3	0	0	0	1	1
Cell count (%), median (range)	70 (30–90)	80 (40–90)	80 (40–90)	80 (50–90)	70 (30–90)	70 (40–90)
Age of block (yr), median (range)	5 (0.8–15.6)	4 (1.2–5)	2.8 (0.4–6.7)	1 (0.1–4.5)	4 (0.8–15.6)	2.7 (0.1–15.6)
Time of fixation						
≤48 h, *n* (%)	10 (62)	3 (43)	29 (69)	4 (44)	14 (64)	13 (59)
>48 h, *n* (%)	6 (38)	4 (57)	13 (31)	5 (56)	8 (36)	9 (41)
Extraction, *n* (%)						
Heating	5 (31)	2 (29)	21 (50)	6 (67)	7 (32)	5 (23)
Scrapping	2 (12.5)	0 (0)	3 (7)	3 (33)	3 (14)	4 (18)
Toluene	1 (6)	2 (28)	6 (14)	0 (0)	7 (32)	4 (18)
Xylene	8 (50)	3 (43)	12 (28)	0 (0)	5 (22)	9 (40)
DNA quantity/quality						
Concentration (ng/μl), median (range)	93.5 (18–270)	88 (8–260)	64 (3–1274)	101 (65–573)	53 (3–223)	64 (3–430)
260/280 ratio, median (range)	1.79 (1.65–2.22)	1.84 (1.4–1.92)	1.99 (1.78–2.08)	1.95 (1.92–2.06)	1.79 (1.55–2.22)	1.95 (1.63–2.22)
Ladder (bp), median (range)	80 (80–200)	100 (100–200)	200 (200–300)	300 (300–300)	100 (80–200)	200 (80–300)

H-NA, highly nonamplificable; M-NA, moderately nonamplificable; S-NA, slightly nonamplificable; A, amplificable (as defined in "Results" section, in "NA paraffin samples" section).

TABLE 8. Amplicon Size in Relationship with the Amplification Failure, False-Positive and False-Negative Results

	NA (%)	False-Positive Results (%)	False-Negative Results (%)
Amplicon size <160 bp			
EGFR exon 19	20.5	0	0
EGFR exon 21	16	22.2	0
KRAS	16	7.8	8
Amplicon size ≥160 pb, <300 bp			
EGFR exon 19	19.6	8	3
EGFR exon 21	19.5	11.25	2.65
KRAS	21	11.7	10.2
Amplicon size ≥300 pb			
EGFR exon 19	31.8	9.78	3.86
EGFR exon 21	21.5	8.75	2.35
KRAS[a]	—	—	—

[a] *KRAS* analysis, no amplicon was higher than 300 bp.
NA, nonamplificable; bp, base pairs; EGFR, epidermal growth factor receptor.

were *EGFR* wild type had a marked decline in PFS compared with those who received chemotherapy (hazard ratio: 2.85; 95% confidence interval: 2.05–3.98; $p < 0.001$).[10] In contrast, false-positive detection of *KRAS* mutations may prevent patients from receiving effective EGFR-TKIs. Nevertheless, the prognostic and predictive significance of *KRAS* mutations in the context or EGFR-TKI therapy are still a matter of debate. The frequency of false-positive results varied between centers, samples, and also exons (Table 3). One ERMETIC center using toluene reagent for its DNA extraction procedure was responsible for at least one false-positive result of *EGFR* exon 19, *EGFR* exon 21, and *KRAS* mutation by four, one, and two centers, respectively. All centers used more than 20 ng of DNA for sequencing analysis, suggesting that false-positive results did not come from rate-limiting amount of DNA template. By contrast, the rate of false-positive results was highest in highly and moderately nonamplificable samples (14/23 [61%] versus 8/51 [15%] for others; $p < 0.01$). Technical explanation could be proposed for the three centers having the highest false-positive rate for *EGFR* exon 19 deletions with two centers using more than 40 PCR cycles and the other one using a nested PCR (Table Siii). Two other centers were responsible from all five L858R *EGFR* exon 21 false-positive mutations suggesting DNA contamination. Finally, among 13 false-positive *EGFR* exon 21 mutations, five are transition type of whom three related to highly nonamplificable samples.[17]

As mutation analysis was based on low-sensitive sequencing technique in the ERMETIC project—part 1, two samples with "false-positive" results found by ERMETIC centers were finally confirmed mutations, by alternative more sensitive techniques. These results concerned one sample with L858R *EGFR* mutation found by two centers and wild type by the 13 other centers, and one sample with G12S *KRAS* mutation found by nine centers, wild type by five centers, and nonamplificable by one center. These low-signal mutants could be subclones in the tumor, similar to the T790M *EGFR* resistance mutation or *KRAS* mutation previously described in tumoral subclones.[23–25]

Finally, despite a relative heterogeneity in sequencing procedures, there was moderate agreement in mutation detection

between the centers and the gold standard, and among the centers, as indicated by global kappa values. Nevertheless, if poor-quality samples/DNA were excluded from statistical analyses (i.e., Bouin fixed, >5 years old and toluene extracted), global kappa values were clearly improved, reflecting the true level of agreement between centers.

In conclusion, our results indicate that paraffin-embedded specimens may represent a suitable source of DNA for sequencing analyses in ERMETIC centers. Nevertheless, ease and accuracy of results obtained depended more on the quality of samples and DNA extraction, as well as on the type of exon than the difference in molecular sequencing procedures between centers. This emphasizes the need for rigorous stepwise quality control programs including preanalytic recommendations, to ensure accurate detection of biomarkers for EGFR-targeted therapies. It will be true not only for direct sequencing but also for screening mutations by newer generation analysis techniques for which the role of DNA extractions might be also important. Finally, sequencing is probably not enough sensitive to be applied in a routine diagnostic strategy of *EGFR* mutations performed on poor-quality DNA samples, as in particular for small biopsy samples that often contains less than 50% of tumor cells. This question will be addressed in the second step of the ERMETIC project, which will test for biomarkers in response to EGFR-TKIs in a prospective cohort of more than 500 patients including 70% with the small paraffin-embedded NSCLC biopsy samples.

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