# Results of the First Italian External Quality Assurance Scheme for Somatic *EGFR* Mutation Testing in Non–Small-Cell Lung Cancer

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**Introduction:** The Italian Association of Medical Oncology (AIOM) and the Italian Society of Pathology and Cytology organized an external quality assessment (EQA) scheme for *EGFR* mutation testing in non–small-cell lung cancer.

**Methods:** Ten specimens, including three small biopsies with known epidermal growth factor receptor (EGFR) mutation status, were validated in three referral laboratories and provided to 47 participating centers. The participants were requested to perform mutational analysis, using their usual method, and to submit results within a 4-week time frame. According to a predefined scoring system, two points were assigned to correct genotype and zero points to false-negative or false-positive results. The threshold to pass the EQA was set at higher than 18 of 20 points. Two rounds were preplanned.

**Results:** All participating centers submitted the results within the time frame. Polymerase chain reaction (PCR)/sequencing was the main methodology used ($n$ = 37 laboratories), although a few centers did use pyrosequencing ($n$ = 8) or real-time PCR ($n$ = 2). A significant number of analytical errors were observed ($n$ = 20), with a high frequency of false-positive results ($n$ = 16). The lower scores were obtained for the small biopsies. Fourteen of 47 centers (30%) that did not pass the first round, having a score less than or equal to 18 points, used PCR/sequencing, whereas 10 of 10 laboratories, using pyrosequencing or real-time PCR, passed the first round. Eight laboratories passed the second round. Overall, 41of 47 centers (87%) passed the EQA.

**Conclusion:** The results of the EQA for EGFR testing in non–small-cell lung cancer suggest that good quality EGFR mutational analysis is performed in Italian laboratories, although differences between testing methods were observed, especially for small biopsies.

**Key Words:** Non–small-cell lung carcinoma, EGFR mutations, Quality assessment.

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Activating mutations of the epidermal growth factor receptor (EGFR) in non–small-cell lung (NSCLC) cancer have been discovered after analysis of the *EGFR* gene in patients who responded to the EGFR tyrosine kinase inhibitors (TKI) gefitinib or erlotinib in early clinical trials.[1–3] Indeed, almost all patients who respond to EGFR TKIs have been shown to carry activating mutations, usually found in exons 18 through 21 of the tyrosine kinase domain of EGFR, and are either point mutations, or in-frame small deletions or insertions.[4,5] Although more than 250 mutations of the *EGFR* have been described up to now, two mutations—a single point mutation in exon 21, the L858R, and a series of small in-frame deletions in exon 19—account for approximately 90% of all *EGFR* mutations.[4,6] *EGFR* mutations are not frequent in unselected Caucasian NSCLC patients.[7] However, a peculiar feature of these mutations is that they are strongly associated with defined clinical and pathological features. In particular, EGFR mutations are far more frequent in female patients as compared with male; in adenocarcinoma as compared with other histological types; in nonsmokers as compared with current smokers or former smokers; and in East Asian NSCLC patients as compared with non–East Asian patients.[8]

Results of randomized phase III clinical trials have demonstrated that first-line administration of an EGFR TKI results in a prolonged progression-free survival as compared with chemotherapy in patients carrying *EGFR* mutations.[9–14] These studies have also confirmed that *EGFR* mutations are the only reliable markers that predict sensitivity to EGFR TKIs. After these findings, treatment with EGFR TKIs is the

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recommended first-line therapy for *EGFR*-mutant patients. As a consequence, assessment of the mutational status of the EGFR has become mandatory to choose the most appropriate first-line treatment for NSCLC patients.

In May 2010, gefitinib was approved in Italy for treatment of NSCLC patients carrying mutant EGFR. Guidelines for EGFR mutational analysis in NSCLC patients were prepared in 2010 by a steering committee of members of the Italian Association of Medical Oncology (AIOM), and the Italian Society of Anatomic Pathology and Diagnostic Cytopathology–Italian Division of the International Academy of Pathology (SIAPEC-IAP).[15] After the publication of the guidelines, the Italian scientific societies started an educational program with presentation and discussion of the guidelines at several national-level meetings.

External quality assessment (EQA) is a system of checking laboratory results objectively by an external agency. The main objective of an EQA program is to establish interlaboratory consistency. In this respect, the EQA process can identify systematic errors in methodology, which may not be revealed by internal QA processes. AIOM and SIAPEC have formed a permanent scientific board for the molecular characterization of tumors, with particular regard to identification of predictive biomarkers. The board has already released guidelines for *KRAS*, *EGFR*, and *BRAF* testing, and has completed a EQA program for *KRAS* testing in colorectal carcinoma.[16] In this article, we present the results of the first Italian EQA scheme for *EGFR* testing, which was completed in 2011.

## MATERIALS AND METHODS

### Organization of the Scheme

AIOM and SIAPEC identified a board of experts who were assigned to organize the EQA scheme and were the coauthors of this article. Within the group, three surgical pathology departments (Department of Human Pathology and Oncology, University of Florence, Florence, Italy; Department of Pathology, University-Foundation, Chieti, Italy; and Division of Pathology, Ospedale Niguarda Ca' Granda, Milan, Italy), and three laboratories (Department of Pathology, University-Foundation, Chieti, Italy; Department of Pathology and Laboratory Medicine, European Institute of Oncology, Milan, Italy; and Pharmacogenomic Laboratory, Centro di Ricerche Oncologiche di Mercogliano (CROM) Avellino, Italy), were identified as referral centers for the Italian EQA program for *EGFR* testing.

### Validation of Samples

Specimens from resected NSCLCs were collected at referral surgical pathology departments. Because it was decided that the EQA was to be focused exclusively on the analytical phase (i.e., genotyping), samples with adequate content of tumor cells (≥50%) were selected. Dissection was not usually required to perform molecular analysis for these samples.[15] Samples mimicking small biopsies were prepared from resected NSCLCs at the Department of Pathology at the University-Foundation, Chieti, Italy. In each case, tissue cores (1 $mm^2$) were obtained from areas with adequate content of tumor cells (>50%). Considering that three cores approximately corresponded to a standard bronchial biopsy, 15 cores were assembled together in a paraffin block. A single 10-μm section from this block roughly mimicked the amount of tissue obtained from five 10-μm sections of a small NSCLC biopsy.

For each specimen, one slide of 10-μm thickness was sent to the three referral laboratories for mutational analysis in a blind fashion. The referral laboratories analyzed the samples by using three different approaches: direct sequencing of the polymerase chain reaction (PCR) product for exons 18 to 21 mutations; fragment analysis for exon 19 deletions and an allelic discrimination-based real-time PCR assay for the L858R mutation in exon 21; and the Therascreen EGFR RGQ kit (Qiagen, Milan, Italy).

Ten cases (7 resected NSCLC samples, and 3 samples mimicking NSCLC small biopsies) were selected by the referral laboratories. The first, the twentieth, and the last sections of the block were reanalyzed for *EGFR* mutation status to ensure that the mutation was homogeneously represented within the block.

### Registration of the Participants and Shipment of the Samples

Italian laboratories that performed *EGFR* mutational analysis were invited to participate to the EQA. Participating laboratories registered at the www.egfrquality.it Web site, and were requested to perform DNA extraction and analysis, using their usual method. One slide of 10-μm thickness of each sample was sent to the participating laboratories. A random code, different for each center, was automatically assigned to the samples by a program of the Web site, to avoid exchange of information among participants. The laboratories were given 4 weeks to complete the analyses and submit the results of genotyping, through the EGFR quality Web site. The centers were also requested to provide information on the technique used for mutational analysis.

### Evaluation of the Results

The scheme included two rounds: the laboratories that failed the first round had the chance to register for a second round. A board of assessors from AIOM and SIAPEC evaluated the results according to a predefined scoring system, in agreement with recently published European guidelines for external quality assessment in molecular pathology.[17] The scoring system assigned two points to correct genotype and

**TABLE 1.** Scoring System

| Criteria | Marks |
|---|---|
| Correct genotype | 2.00 |
| Error in the nomenclature that might lead to misinterpretation of the results | 1.5[a] |
| Genotype mispositioned or miscalled (e.g., incorrect base/amino acid detected) | 1.0 |
| Test failure giving no result on the sample | 1.0 for the first error, 0 from the second |
| Incorrect genotype (false-positive or false-negative) | 0 |

[a] Deduction applied only once.

**TABLE 2.** Mutational Status of the Samples Used for the EQA Scheme

| Sample No. | Sample Type | PCR/Sequencing | Fragment Analysis/ Real-Time PCR | Therascreen | Round Number[a] |
|---|---|---|---|---|---|
| 1 | Simulated biopsy | Wild-type | Wild-type | Wild-type | A1 and B1 |
| 2 | Simulated biopsy | c.2235_2249del (p.E746_A750del) | 15bp deletion exon 19 | Exon 19 deletion | A2 and B2 |
| 3 | Simulated biopsy | Wild-type | Wild-type | Wild-type | A3 and B3 |
| 4 | Resected sample | Wild-type | Wild-type | Wild-type | |
| 5 | Resected sample | Wild-type | Wild-type | Wild-type | |
| 6 | Resected sample | c.2573T>G (p.L858R) | c.2573T>G (p.L858R) | c.2573T>G (p.L858R) | A4 and B4 |
| 7 | Resected sample | Wild-type | Wild-type | Wild-type | A5 and B6 |
| 8 | Resected sample | c.2573T>G (p.L858R) | c.2573T>G (p.L858R) | c.2573T>G (p.L858R) | B5 |
| 9 | Resected sample | Wild-type | Wild-type | Wild-type | A6 and B7 |
| 10 | Resected sample | c.2236_2250del (p.E746_A750del) | 15bp deletion exon 19 | Exon 19 deletion | A10 and B10 |
| 11 | Resected sample | Wild-type | Wild-type | c.2573T>G (p.L858R) | |
| 12 | Resected sample | Wild-type | Wild-type | Wild-type | A7 and B8 |
| 13 | Resected sample | Wild-type | Wild-type | Wild-type | A8 |
| 14 | Resected sample | Wild-type | Wild-type | Wild-type | A9 and B9 |

[a] Samples selected for the first (A-label) and second (B-label) round of the EQA.
EQA, external quality assessment; PCR, polymerase chain reaction.

zero points to false-positive or -negative results (Table 1). In case of errors in the nomenclature, which might lead to misinterpretation of the results (e.g., *deletion* without specifying the exon in which the deletion occurs), 1.5 points were assigned. This deduction was applied only once for each center, generally to the first sample in which the error was found. One point was awarded for cases in which the genotype was mispositioned or miscalled—this error sometimes occurred with exon 19 deletions, where it could be difficult to define the precise base or amino acid in which the deletion starts or ends. If a test failed and did not give a result on the sample, one point was assigned for the first error, and zero from the second onward. The threshold to pass the EQA was set at higher than 18 of 20 points.

## RESULTS

### Selection of the Samples for the EQA

The first step of the EGFR EQA organized by AIOM and SIAPEC was the selection and the validation of the samples. Three referral pathological centers selected 14 formalin fixed paraffin embedded (FFPE) NSCLC specimens (11 resected NSCLC samples and 3 simulated small biopsies) (Table 2). The mutational status of these samples was assessed in three referral laboratories by using different techniques (Table 2). A good agreement on *EGFR* mutational status of the selected specimens was found among the three referral laboratories. A discordant result was reported only for sample 11, in which PCR/sequencing and the allelic discrimination-based real-time PCR assay for the L858R mutation were negative, whereas the Therascreen kit identified an L858R point mutation (Table 2). The Δ Ct for the L858R mutation was high (4, 82), and this might justify its lack of detection with techniques that have a lower sensitivity as compared with Therascreen. Samples for which a total concordance on the mutational status was found between the three methods, and from which a good yield of genomic DNA was obtained, were selected for the quality assessment scheme. In particular, samples N. 1, 2, 3, 6, 7, 9, 10, 12, 13, and 14 (7 resected NSCLC samples and 3 simulated small biopsies; 3 mutant and 7 wild-type cases) were used for the first round (Table 2; samples A1–A10).

### First Round

Fifty-three laboratories registered, of which six declined and 47 laboratories participated in the first round of the EQA scheme. All the participating centers submitted the results within the time frame. The main methodology used by the participants was PCR/sequencing (37 laboratories; 79%) followed by pyrosequencing (8; 17%) and real-time PCR (2; 4%) (Fig. 1).

A significant number of analytical errors ($n$ = 20) were observed. In particular, a high frequency of false-positive results occurred ($n$ = 16), with two laboratories reporting two false-positive, and one laboratory with five false-positive results. Therefore, 56% of the false-positive results were concentrated in three laboratories. Four false-negative results were reported, with one laboratory having two false-negatives. Test failure occurred in eight cases, with one center reporting test failure for four cases. Finally, in four cases the genotype was mispositioned. This error occurred for exon 19 deletions. Some laboratories were able to detect the presence of a deletion by using PCR/sequencing, but failed to recognize the precise amino acid or base sequence of the deletion. Only one center had a deduction because of errors in the nomenclature. As shown in Table 3, the three samples that mimicked small

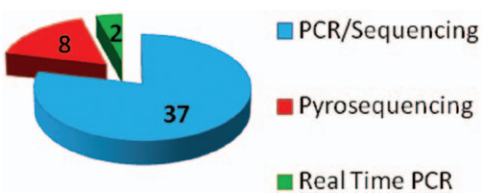


**FIGURE 1.** Methods used for EGFR genotyping by centers participating to the Italian external quality assessment.

**TABLE 3.** Score Per Case of the Samples Analyzed in the First Round

| Sample Number | Sample Type | Average Score |
|---|---|---|
| A1 | Simulated biopsy | 1.79 |
| A2 | Simulated biopsy | 1.78 |
| A3 | Simulated biopsy | 1.64 |
| A4 | Resected sample | 1.91 |
| A5 | Resected sample | 1.89 |
| A6 | Resected sample | 1.87 |
| A7 | Resected sample | 2.00 |
| A8 | Resected sample | 1.96 |
| A9 | Resected sample | 1.96 |
| A10 | Resected sample | 2.00 |

biopsies showed lower scores, suggesting that these samples were particularly challenging for laboratories with low experience in *EGFR* mutational analysis.

Overall, 14 of 47 centers (30%) did not pass the first round, having reached a score of 18 points or lesser; two laboratories had a very poor performance reporting scores of nine and 11, respectively (Table 4 and Fig. 2). Among the centers that did not pass the first round, 13 laboratories reported at least one false-positive or -negative result; in one center, the test for two samples failed and, according to the adopted scoring system, obtained a total score of 17. A difference was observed between the testing methods: all the 10 laboratories that used pyrosequencing or real-time PCR passed the first round, whereas 14 of 37 laboratories (38%) that employed PCR/sequencing made analytical errors and did not pass the first round ($p = 0.0218$; Fisher's exact test). One of these laboratories also used a next-generation sequencing approach in addition to PCR/sequencing.

## Second Round

According to the guidelines of the scheme, the centers that did not pass the first round were given the offer to participate in a second round. The same set of samples used in the first round were sent to the centers, with the exception of sample 13, which was almost completely used up in the first round and was replaced with sample 8 (Table 2; samples B1–B10). A code number, different from the one assigned in the first round, was given to the samples. The centers were informed of the type and number of errors that they made, but

**TABLE 4.** Score Per Center

| Scores | Number of Centers |
|---|---|
| 20 | 28 |
| 19,5 | 1 |
| 19 | 4 |
| 18 | 7 |
| 17 | 2 |
| 16 | 3 |
| 11 | 1 |
| 9 | 1 |

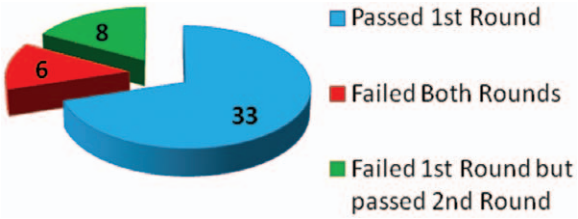


**FIGURE 2.** Results of the first Italian external quality assessment for EGFR testing.

they did not receive information on the results of the specific samples that they analyzed in the first round.

Six centers did not pass the second round: two centers did not submit the results within the 4-week time frame, and four centers once again scored below 18 (Fig. 2). One of the centers with poor performance did not submit the results on time, and the other had a score of 14. The laboratories that did not pass the second round reported false-positive (N. 4) and false-negative results (N. 4) again, as well as test failures (N. 1), mispositioning of the genotype or errors in the nomenclature (N. 1).

Overall, 41 of 47 of the Italian centers (87%) had a score greater than 18 in the first or the second round and passed the EGFR EQA (Fig. 2). The list of the centers that passed the scheme is published on the Web site of AIOM and SIAPEC (www.aiom.it; www.siapec.it).

## DISCUSSION

The approval of drugs based on mutational tests has represented not only a significant innovation for medical oncology but also a major challenge for oncologists and pathologists. This is particularly true for Italy, because at the time when gefitinib was approved by the European Medical Agency for treatment of metastatic NSCLC with *EGFR* mutations, few Italian laboratories were equipped to run molecular pathology analyses. In addition, these laboratories were unevenly distributed in the country, with the majority of the centers concentrated in the northern regions and very few in southern Italy. The Italian health system is organized on a regional basis, with significant differences between regions. However, neither regional or national Departments of Health released guidelines or organized EQA programs for molecular pathology. For this reason, AIOM and SIAPEC decided to start a program to improve *EGFR* testing in Italy. In this regard, the EQA scheme is only a part of this program. In fact, the EQA was preceded by the publication of guidelines and by a number of training courses.[15] All these activities together are indeed required to improve molecular testing.

The EGFR EQA organized by AIOM and SIAPEC was related only to genotyping. For this reason, samples for which dissection was not required were selected for molecular analysis. Other EQA schemes also addressed the preanalytical phase, and laboratories were required to assess the percentage of neoplastic cells and perform dissection, if needed. However, scoring of this phase is not easy because no consensus on the estimation of tumor cell content has been reached, and a huge variability had been reported in previous EQAs.[18,19] Our approach reduces the interlaboratory variability related

to the type of technique used for the dissection, and allows a comparative evaluation of the sensitivity and specificity of the methods used for the mutational analysis.

Different types of samples can be used for EQA. In a pilot phase of a European EQA for *EGFR* mutation testing, FFPE cell lines had been used.[20] The use of cell lines allows the possibility of providing the same sample to all the participating laboratories, even when these are numerous. In addition, it is possible to generate samples with variable content of mutant alleles by mixing wild-type and mutant cells. However, the use of tissue samples ensures a closer relation between the EQA and the routine clinical activity. In this regard, our approach was innovative because we generated three samples mimicking small biopsies, which represent the majority of specimens that are available for mutation analysis in lung cancer patients. Therefore, our EQA differs from other schemes in using material that closely resembles the clinical scenario. We used a total of 10 samples, which has been suggested as the adequate number of cases for a proficiency testing.[19]

Overall, the results of the EQA suggest that *EGFR* testing is performed with good quality in the majority of Italian centers because 41 of 47 laboratories (87%) passed the EQA. Notably, the threshold that was used in this EQA (>18 points) is significantly higher as compared with the majority of EQA schemes that have set the threshold to 18 points or even lower.[17] With our scoring system, a laboratory reporting a single false-positive or -negative result would not pass.

Nevertheless, we noticed a disturbingly high number of analytical errors. In the first round, 13 laboratories reported at least one false-positive or -negative result. Both these results are extremely harmful for NSCLC patients. In fact, a negative result will lead to treatment of an *EGFR*-mutant patient with chemotherapy as first-line of therapy, which is less effective as compared with EGFR TKIs in this subgroup of patients.[9] However, false-positive findings will lead to treatment of *EGFR* wild-type patients with EGFR TKIs that have been shown to have little activity or none as first-line treatment in this subgroup of patients who would benefit more from first-line chemotherapy.[9]

False-negative results might be because of the low sensitivity of the method used for mutational analysis. The three laboratories that had the four false-negative results used PCR/sequencing, and the errors were concentrated in two samples: case 2, a small biopsy with an exon 19 deletion, and case 6, a surgical specimen with an L858R mutation with a relatively low-intensity peak of mutant DNA in the chromatogram. Although the specimens used in this EQA were selected to have a tumor cell content greater than 50%, approximately 60% of *EGFR*-mutant samples have been shown to contain cells with both wild-type and mutated *EGFR*, with the proportion of *EGFR*-mutant cells ranging from 30% to 90%.[21] If the proportion of *EGFR*-mutant alleles is low, this might lead to a false-negative results when low sensitive techniques such as PCR/sequencing are used, even in presence of an adequate level of tumor cells. Nevertheless, these mutations were identified by referral laboratories that used PCR/sequencing as their method. These findings confirm that the sensitivity of PCR/sequencing techniques might significantly differ among laboratories, and should be assessed in every center to define the limits of detection of the technique.

Surprisingly, the rate of false-positive results was much higher as compared with false-negative (16 versus 4), with nine laboratories reporting false-positive results by using PCR/sequencing as main technique of analysis. One center used both standard PCR/sequencing and a next-generation sequencing approach, and reported an *EGFR* mutation represented in less than 5% of the alleles in a sample that was wild-type according to the techniques that are routinely used in clinical diagnostics. The sample was reanalyzed with a Roche 454 sequencer (Roche, Milan, Italy) in one of the reference laboratories (University of Chieti) and *EGFR* mutations were not detected, leading the board of assessors to evaluate this result as a false-positive. Next-generation sequencing techniques are highly sensitive, and they are likely to detect mutant alleles at a very low frequency. However, the clinical significance of these findings, that is, the sensitivity of tumors with very low levels of *EGFR* mutations to TKIs, is not yet known. Therefore, the low prevalence mutations should not be considered *positive*, when using highly sensitive assays. Of the 15 remaining false-positive results, nine were concentrated in three laboratories that were likely to have serious problems of contamination from post-PCR product. This type of contamination could be either intra- or interexperimental, and can be prevented by accurately separating the pre- and post-PCR processing of the samples. In addition, the use of an adequate number of negative controls should allow easy identification of the presence of any contamination. Five of the six errors of laboratories with one false-positive result were concentrated in the two wild-type samples mimicking small biopsies. Previous studies have suggested that analysis with PCR/sequencing of very low amounts of DNA might lead to sequence artifacts and, therefore, false-positive results.[22] However, these artifacts are usually novel or rare transitions,[23] whereas exon 19 deletions or the L858R point mutation were erroneously reported in the EQA, thus suggesting that contamination from post-PCR product was the most likely cause of these false-positive results. Although we did not collect this information, several laboratories likely performed a preamplification or a nested-PCR approach when the amount of DNA was extremely low, and this could have enhanced the possibility of cross-contaminations. In this respect, we are planning to collect more detailed information on the techniques used by the laboratories in the next EGFR EQA that is scheduled to start in 2013. In addition, we will increase the number of simulated biopsies in the next EGFR EQA because these were the most challenging cases that also represented the majority of specimens for lung cancer patients with advanced disease.

Finally, the results of this EQA showed that all laboratories using pyrosequencing or real-time PCR-based commercially available kits did not make analytical errors and passed the first round. The number of centers using these techniques in the EQA were low ($n = 10$), and these findings must be cautiously interpreted. There is no clear evidence that pyrosequencing or real-time PCR-based techniques are superior to PCR/sequencing, at least in samples with an adequate content of tumor cells. However, commercially available kits contain both positive and negative controls, and they are easier to standardize. Therefore, the use of these kits might be advisable, at least in laboratories that have little

 

experience in molecular biology techniques. In addition, the number of times that tubes are opened during PCR/sequencing is significantly higher as compared with pyrosequencing or real-time PCR, and this might account for the higher frequency of false-positive results.

In conclusion, the results of this first quality assessment for *EGFR* testing in Italy, indicates that *EGFR* mutational analysis is performed with good quality in the majority of Italian centers. These findings also underline the importance of EQA to reveal errors in methodology, and to ensure an adequate quality of molecular testing. The Italian EGFR quality assessment scheme might represent a model for other national and international organizations operating in this field.

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