Performance Assessment of Epidermal Growth Factor Receptor Gene Sequencing According to Sample Size in Daily Practice Conditions

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Abstract: Lung carcinoma is the main cause of cancer death worldwide. Adenocarcinoma molecular biomarkers have been discovered, and targeted therapies have been developed with encouraging results. The epidermal growth factor receptor gene is one of these biomarkers. Exons 18 to 21 should be studied in patients with advanced adenocarcinoma, who are candidates for treatment with tyrosine kinase inhibitors. The objective was to compare the performance of the determination in large and small samples in daily practice conditions, trying to adjust to published consensus guidelines. A retrospective observational study of 141 cases was carried out, with exons 19 and 21 sequencing. Sample size (small vs. large), including number of satisfactory polymerase chain reaction (PCR), sequencing, deletions, and mutations, were evaluated. In small biopsies, sample type, fragment number, and percentage of tumor per sample were analyzed. The results shown 114/141 (80.8) cases that met selection criteria; 60/114 (53%) were large (surgical) and 54/114 (47%) were small samples (19/54 endoscopic, 17/54 fine needle aspiration clots, 4/54 lymph nodes, 14/54 core and other). All large samples were satisfactory PCR, 56/60 (93%) satisfactory sequencing, and 12/56 (21%) had deletions in exon 19. Small samples were satisfactory PCRs in 50/54 (93%) cases, and satisfactory sequencing in 35/50 (65%), 8/35 (23%) showed alterations in exon 19, and 1/35 (3%) in exon 21. In conclusion, the proportion of samples unfit for the study of the epidermal growth factor receptor gene mutational status increased from 7% in large samples to 35% in small ones. Nineteen small samples were inconclusive, with cell blocks predominating, 10/19 (53%).

Key Words: EGFR, small samples, adenocarcinoma, lung

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Lung cancer is the leading cause of cancer death in both sexes in the world, with a poor prognosis because at diagnosis only <15% of patients have a surgical tumor. The overall 5-year survival is 16%.[1] In Argentina, lung cancer causes about 9000 deaths per year in both sexes, which accounts for 16% of all cancer deaths. Significant sex differences were observed, with 72% of deaths in men.[2] Most cancers are carcinomas (99%), and 75% to 80% comprise non–small cell carcinomas (NSCLC). NSCLC are a heterogeneous group including 3 major types: adenocarcinoma (ADC), squamous cell, and large cell carcinomas; the first being the most common type (50% of cases).[3,4] Conventional chemotherapy and radiotherapy were the only alternatives for patients at advanced stages, until ADC molecular biomarkers were discovered in 2004 to 2005. This resulted in the development of targeted therapies with encouraging results.[5]

Kris and colleagues identified 10 driver mutations in 64% of 1007 lung ADC samples. The most frequent were *KRAS* (25%), epidermal growth factor receptor gene (*EGFR*) (17%), anaplastic lymphoma kinase (*ALK*) rearrangements (8%), *ERBB2* (3%), *BRAF* (2%), *PIK3CA* (< 1%), *MET* amplification (< 1%), etc. Of these mutations, 97% were mutually exclusive. These findings are similar to those reported by other researchers.[6–8]

EGFR is a member of the membrane receptors family, with tyrosine kinase activity known as ErbB. The most frequent alterations (about 90%) are deletion in exon 19, and L858R mutation in exon 21. Nowadays, certain drugs (Erlotinib, Gefitinib) produce responses in 70% to 80% of patients with *EGFR* deletions/mutations, which are longer and have less toxicity than conventional chemotherapy.[9–11] The most common method to detect complete *EGFR* deletions/mutations status is direct sequencing.[10] In 2007, a fusion oncogene involving the *ALK* gene and *EML-4* was described in NSCLC. This alteration result is present in 3% to 13% of patients. The only technique currently validated for detection is fluorescence in situ hybridization. Crizotinib is an agent that proved efficient in the inhibition of the tyrosine kinase activity of *ALK* in these groups. The determination of these 2 biomarkers (*EGFR* and *ALK*) is currently recommended and other determinations will be probably made with different methodologies. Subclone development has resulted in an early resistance to therapeutic agents.[9,12,13] Therefore,

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drug combination treatments associated with the genetic profile of each tumor are postulated to attack those subclones.[14]

As most patients are not surgically treated, such determinations will be made in small biopsies. These small samples are usually obtained from transbronchial needle aspiration and biopsies, transthoracic core biopsies, cell blocks (clots) prepared from fine needle aspiration (FNA) material and by mediastinoscopies.[10,15] Several studies using cytologic smears extracted material have been also reported.[16]

Most studies evaluate technique sensitivity and specificity based on the proportion (%) of tumor cells versus nontumor cells, but generally fail to consider the total amount of tumor DNA in each sample. In contrast, preanalytical variables ideal conditions cannot sometimes be reached in everyday practice, let alone in the developing world. Therefore, the purpose of this work was to evaluate the impact of sample size on the rate of effectiveness of direct sequencing of *EGFR* gene exons 19 and 21 in patients with ADC. Minimum selection criteria and some uncontrolled factors were considered as in daily practice, where not all the recommendations are met.

MATERIALS AND METHODS

A retrospective observational study including 141 cases was reviewed. Polymerase chain reaction (PCR) amplification and subsequent direct sequencing (Sanger method) of the product were carried out to evaluate exons 19 and 21 of the *EGFR* gene, since 85% to 90% of mutations in the tyrosine kinase domain of the *EGFR* gene occur in these exons.[10] Our study only included these 2 exons, since the use of additional analytical variables could complicate outcome analysis.

Subjects with primary lung ADC diagnosis, complete data and hematoxylin and eosin-stained histologic preparation were selected. All tissue samples were formalin-fixed (10% buffered) and paraffin embedded (FFPE). It has been generally agreed that a qualified pathologist should evaluate the proportion of tumor cells and tissue quality in each specimen (fixation, autolysis, necrosis, etc.).[10,12,13]

The preanalytical variable studied was sample size, which was divided into 2 groups: large (blocks from surgical specimens) and small (obtained by transbronchial needle aspiration and biopsies from bronchoscopy, transthoracic core biopsy and FNA clots, nodal biopsies obtained by mediastinoscopy, etc.). In addition, small biopsy sample type, fragment number, and initial tumor rate (proportion of tumor cells) were considered. Material from cytologic smears was not used. Microdissection was performed in most large samples to increase the proportion of neoplastic cells. In small samples, however, this methodology could only be applied in a few cases, given the size of the samples.

Mutational status detection was performed by PCR and amplification product automated sequencing for mutations in exons 19 and 21 of the *EGFR* gene.[10] DNA was purified from FFEP samples. Microdissection of the region with the highest percentage of tumor was performed, reaching a minimum of 70% of tumor cells compared with the presence of normal cells. Slides were deparaffinized with xylene-100% ethanol (Carlo Erba, Italy), and the DNA was purified by column using QIAamp DNA FFPE Tissue (Qiagen; # 55404). DNA purity and yield were measured by spectroscopy. DNA was amplified using primers 5′ and 3′ flanking sequence specific to each exon. Primers sequences are *EGFR*19—forward: 5′-GCACCATCTCACAATTGCCAGTTA-3′; *EGFR*19—reverse: 5′-AAAAGGTGGGCCTGAGGTTCA-3′; *EGFR*21—forward: 5′-CCTCACAGCAGGGTCTTCTCTGT-3′; and *EGFR*21—reverse: 5′-TCAGGAAAATGCTGGCTGACCTA-3′.

The amplicons obtained are 207 and 222 bp, respectively. All PCRs were assessed by 9% polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (Tris-Boric-EDTA), visualized with ethidium bromide and photographed under ultraviolet light (PAGE). Sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) commercial kit. The sequence was separated by capillary electrophoresis (ABI PRISM 310 Genetic analyzer; Applied Biosystems). The sequence obtained was analyzed with Sequencing Analysis Software v5.2 (Applied Biosystems).

The presence of a visible band in PAGE was defined as positive or satisfactory PCR (PCRok), according to the amplicon expected. Thus, optimal sequence (Sok) was defined as chromatogram with background distinguishable from the specific signal. DNA concentration (mg/mL) and number of mutations/deletions in all samples were also studied.

Medians and percentiles were calculated. Both Fisher exact test for dichotomizing variables and Wilcoxon test were used to compare medians.

RESULTS

Although trying to adjust to published consensus guidelines in daily practice, we obtained the following results.

Of the 141 cases studied, 114 (81%) met the selection criteria. There were 60/114 (53%) large (surgical) samples and 54/114 (47%) small biopsies. Small biopsies were distributed in 19/54 (35%) endoscopic biopsies, 17/54 (31%) FNA clots, 4/54 (7%) lymph node biopsies, and 14/54 (26%) core biopsies and other procedures. All large samples had PCRok, 56/60 (93%) Sok, and 12/60 (20%) had exon 19 deletion (Table 1).

Small biopsies were PCRok in 50/54 (93%) cases, and Sok in 35/50 (65%) cases. Exon 19 deletions were observed in 8/35 (23%) cases, and exon 21 mutation in 1/35 (3%) (Table 1).

Table 2 shows a difference in DNA concentration between large and small samples. Regarding small samples, Table 3 discriminates between negative PCR number, inconclusive sequences, fragment number, percentage of

TABLE 1. PCR Results, Sequence, and Genetic Alterations in Large and Small Samples

		n (%)			
Sample Type	**N**	**Positive PCR**	**Optimal Sequencing**	***EGFR* 19 (Deletion)**	***EGFR* 21 (Mutation)**
Large	60	60 (100)	56 (93)	12 (20)	0
Small	54	50 (93)	35 (65)	8 (23)	1 (3)
Total	114	110	91	20	1

EGFR indicates epidermal growth factor receptor; PCR, polymerase chain reaction.

initial tumor (ranging from 5% to 90%, average 40%), and DNA concentration. Table 4 presents the results of the entire study (PCR/sequence) in different types of small samples. Table 5 shows differences in DNA concentration and percentage of initial tumor in positive and negative PCR from small samples. Table 6 shows the same findings, although in optimal and inconclusive sequence.

The sample proportion required to determine *EGFR* gene mutational status increases from 7% in large samples to 35% in small ones. Of the 19 inconclusive small biopsies, 10/19 (53%) were FNA clots.

The proportion of tumors with *EGFR* gene alterations is similar to that reported in the literature, with no significant difference observed in the detection of genetic alterations between large and small samples (Fisher exact $P = 0.411$).

DISCUSSION

Identifying patients with therapeutic molecular targets in their tumors is now a standard of care. However, the initial morphologic diagnosis and the eventual tumor classification by immunohistochemistry, as well as the acquisition, handling and processing of tumor tissue remain to be essential.

In advanced stages patients, a single procedure will provide a relatively small amount of tissue that must be used in the most efficient way for all studies.[17] In this sense, there is a consensus about performing histopathologic diagnosis using as little material as possible, which should be kept for molecular studies.[10,12,13] The combination of less invasive techniques that provide very small samples to carry out an increasing number of determinations is controversial, given the impossibility to increase the amount of tumor cells. Therefore, higher sensitivity and specificity of molecular determinations are required.[15] In fact, several methods are being developed, including free tumor DNA detection in peripheral blood.[14,18] However, most of these determinations are currently experimental and few are validated for clinical use. Thus, until more sophisticated techniques for these and other molecular markers are validated, the amount/size of the samples should be considered. Therefore, the purpose of this manuscript is to compare performance by determining EGFR gene deletions/mutations in large and small samples in daily practice conditions.

TABLE 2. DNA Concentration in Large and Small Samples

Sample Type	**DNA Concentration (p50)**
Large	46.9
Small	3.25
	$P = 0.000$

In our study, the proportion of samples suitable for *EGFR* gene mutation determination by direct sequencing decreases from 93% in large surgical samples to 65% in small samples. In molecular biology, several variables should be considered for optimal results, and preanalytical conditions are herein evaluated. neutral buffered formalin fixation is recommended. Prefixation in alcohol-based fixative, decalcifying acidic solutions, acidic fixatives (such as Bouin) or containing metallic salts may alter DNA antigenicity or integrity. Setting a period of >6 and <48 hours is recommended.[19] Short or excessive fixation time may have deleterious effects on DNA and protein antigenic epitopes.[20,21]

The use of PCR techniques in FFPE tissues is associated with a higher incidence of sequence artifacts and risk of misinterpretation in PCR results, compared with the use of fresh samples.[22,23] As FFPE tissue is currently used for genetic analysis, results should always be carefully interpreted. Mutations detected from FFPE samples by sequencing must be confirmed by independent PCR.

Dideoxynucleotide sequencing is the "gold standard" for identifying genetic changes. However, at least 10% to 20% of allelic presence is required for this detection. Mutations below said threshold due to normal cells high contamination or tumor heterogeneity could not be detected by this method.[24] The percentage of tumor cells must be estimated, since low percentages of neoplastic cells are sometimes associated with unreliable results.[12,25] This estimation can be made through microdissection technique and selection of block interest region. To increase the sensitivity of Sanger sequencing, and to discriminate from technical background, at least 70% of tumor cells are required.[12,13]

PCR was successful in all large samples (60/60), whereas sequencing was inconclusive in 4 cases. The chromatogram obtained failed to discriminate specific signal from background. Such chromatogram type may be determined by preanalytical conditions (prefixing, fixative type, or fixation time). As mentioned above, large surgical specimens were formalin fixed. However, fixation time was variable and difficult to control. Nevertheless, our results support consensus recommendations about formalin being the best fixative, whereas fixation time is often difficult to control, since our laboratory receives samples from other centers.[10,12,13]

TABLE 3. Small Sample Type, Number of Negative PCRs, Inconclusive Sequence, Fragment Number, Initial Tumor Percentage, and DNA Concentration

Small Sample Type	N (%)	Negative PCR [n (%)]	Inconclusive Sequence [n (%)]	Fragment Number (p50)	Initial Percentage of Tumor (p50)	DNA Concentration (p50)
Endoscopic	19 (35)	0	4 (21)	4	30	3
Clots	17 (31)	3 (18)	7 (41)	—	50	3.3
Lymph nodes biopsies	4 (7)	0	0	4	50	3.05
Core biopsies and other	14 (26)	1	4 (29)	3	45	1.8
		$P = 0.209$	$P = 0.195$	$P = 0.244$	$P = 0.564$	$P = 0.211$

PCR indicates polymerase chain reaction.

TABLE 4. Complete Study Results in Different Types of Small Samples

Small Samples	Positive PCR/Sequencing [n (%)]	Negative PCR/Sequencing [n (%)]	Total
Endoscopic	15 (43)	4 (21)	19
Clots	7 (20)	10 (53)	17
Lymph nodes biopsies	4 (11)	—	4
Core biopsies and other	9 (26)	5 (26)	14
Total	35	19	54

Fisher exact test = 0.056.
PCR indicates polymerase chain reaction.

TABLE 5. Initial DNA Concentration and Percentage of Tumor in PCR Positive and Negative Small Samples

Small Samples PCR	N	DNA Concentration (p50)	Percentage of Tumor (p50)
Positive	50	3.25	50
Negative	4	2.9	10
	54	$P = 0.4470$	$P = 0.0376$

PCR indicates polymerase chain reaction.

TABLE 6. DNA Concentration and Percentage of Initial Tumor in Optimal and Inconclusive Sequence

Small Samples Sequence	N	DNA Concentration (p50)	Initial Percentage of Tumor (p50)
Optimal	35	3.7	50
Inconclusive	15	2.5	10
	50	$P = 0.0253$	$P = 0.0108$

As expected, there was a statistically significant difference between large and small samples DNA concentration. However, no significant differences were observed in concentration, fragments number, or tumor initial percentage among different small sample types. The proportion of tumors with *EGFR* gene alterations is similar to literature reports, with no significant differences in the detection of genetic alterations between large and small samples (Fisher exact $P = 0.411$). We can infer that all these types of tissue samples are similarly useful and depend on interdisciplinary medical team (surgeons, radiologists, clinicians, pathologists, and oncologists).[15]

Nearly half unfit samples were FNA cell blocks (clots). As the DNA concentration of the latter showed no significant differences compared with other small samples, this deficit in performance could be due to Bouin fixation, which is commonly used in some laboratories.[10,12,13,17] Regarding the use of clots, a DNA purification method is required to extract hemoglobin. Hemoglobin is one of the main polymerase inhibitors in PCR.[26] In this sense, specific columns for FFPE tissues are efficient (data not shown).

In many patients, FNA is the first (and often the sole) technique used for diagnosis, given its low invasiveness. Therefore, the clot is all the material available for molecular studies. In these cases, formalin fixation is recommended, and although some reports suggest 70% ethanol as an alternative, as mentioned above, alcohol-based fixatives may alter DNA antigenicity or integrity.[12,15,17]

Determining the nature and duration of fixation is a great challenge to our laboratory, which receives samples from other centers. Therefore, we suggest that the type and time of tissue fixation should be registered in the pathology report.

Over the last decade, genomic research of various solid tumors has suddenly progressed through the discovery of several molecular biomarkers that eventually impact on the prognosis and treatment of most common cancers. The treatment of ADC patients with *EGFR* and *ALK* fusion has encouraging results. However, this translates into multiple activities and a heavy burden for surgeons and pathologists, who must obtain and process samples, prepare a pathology report, select the material for molecular biology. Moreover, those molecular biosciences technicians who perform studies must draw up guidelines to standardize these practices, and algorithms to cover both cytopathological and histopathologic diagnoses, immunohistochemistry and molecular studies.[10–13,27,28]

Moreover, recent technical innovations, such as "next or second generation" sequencing or "massively parallel" sequencing, have the potential to detect many abnormalities in a single assay, and are probably the solution to tissue shortage.[27,29] Despite the very promising technologies, there are still insufficient published data on this accuracy, precision, sensitivity, specificity, and validation in clinical practice.[12]

In conclusion, the proportion of samples unfit for the study of *EGFR* gene mutational status increased from 7% in large samples to 35% in small ones. Nineteen small samples were inconclusive, with cell blocks predominating, 10/19 (53%).

We agree with Brega and Brandao, who have postulated that surgical pathologists are witnessing a continuous and inexorable shift in their practice. Their job goes beyond histologic diagnosis. The molecular profile has become an integral part of anatomic pathology practice, and pathologists' adaptation to and training for this new cancer pathology practice is crucial.[30]

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