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The Comparison of Real-Time PCR and Mutation-Specific Immunohistochemistry in EGFR Mutation Analysis of Non-Small Cell Lung Carcinomas

Küçük Hücreli Dışı Akciğer Karsinomlarında EGFR Mutasyon Analizinde Real-Time PCR ve Mutasyon Özgü İmmünohistokimya Karşılaştırması

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The Comparison of Real-Time PCR and Mutation-Specific Immunohistochemistry in EGFR Mutation Analysis of Non-Small Cell Lung Carcinomas

ABSTRACT

Objective: This study aims to identify activating mutations in the epidermal growth factor receptor (EGFR) gene in patients with non-small cell lung cancer (NSCLC) and to evaluate their correlation with responses to EGFR-tyrosine kinase inhibitors (TKI) treatment. This study aims to identify activating mutations in the epidermal growth factor receptor (EGFR) gene in patients with non-small cell lung cancer (NSCLC) and to evaluate their correlation with responses to EGFR-tyrosine kinase inhibitors (TKI) treatment. We conducted a comparative analysis of Real-Time PCR and immunohistochemistry to detect EGFR mutation status in non-small cell lung cancer patients, focusing on the sensitivity, specificity, and predictive values of immunohistochemistry.

Material and Method: We evaluated 788 non-small cell lung cancer samples which were analyzed for EGFR mutation status by RT-PCR. We detected 126 EGFR mutated cases among these patients. We evaluated mutation-specific EGFR immunohistochemistry directed towards the exon 19 deletions (15 bp E746-A750) and exon 21point mutation (L858R) to the 47 EGFR mutated patients histologic material and cell blocks of cytologic specimens.

Results: 32 of the 47 cases (68%) had exon 19 deletion, 14 of them (30%) had point mutation in exon 21, and one of them (2%) showed exon 18 mutation. EGFR exon 19 (15 bp E746-A750 deletion) antibody showed a sensitivity of 100%, specificity of 40%, negative predictive value of 100%, and positive predictive value of 78%. The sensitivity of the exon 21 (L858R point mutation) antibody was 93%, specificity was 91%, negative predictive value was 97% and positive predictive value was 82%.

Conclusion: Our investigation indicates that mutation-specific EGFR immunohistochemistry has demonstrated a notable sensitivity and specificity for exon 21. However, while sensitive, the exon 19 (15 bp E746-A750 deletion) antibody lacked specificity. While positive immunohistochemical staining may suggest the presence of an EGFR mutation, making the patient potentially eligible for TKI treatment, it should not be the sole determinant. If immunohistochemistry results are negative, it is essential to resort to molecular tests to ensure accurate diagnosis and appropriate therapeutic guidance. With evolving diagnostic landscapes, it is crucial to harness both IHC and molecular techniques judiciously for optimal patient care.

Keywords: EGFR, Immunohistochemistry, Non-small cell lung cancer.

ÖZET

Amaç: Bu çalışmanın amacı, Küçük Hücreli Dışı Akciğer Kanseri (KHDAK) hastalarında EGFR genindeki aktivasyon mutasyonlarını tespit etmek ve EGFR-tirozin kinaz inhibitörlerinin (TKI) tedavi yanıtlarıyla olan ilişkisini değerlendirmektir. Küçük hücreli dışı akciğer kanseri (KHDAK) hastalarında epidermal büyüme faktörü reseptörü (EGFR) genindeki aktive edici mutasyonların belirlenmesi, EGFR-tirozin kinaz inhibitörleri (TKI) tedavi yanıtlarıyla ilişkilidir. Mutasyon spesifik antikorlar kullanılarak yapılan immünohistokimya, belirli mutant EGFR proteinlerini tespit edebilmektedir. KHDAK hastalarında EGFR mutasyon durumunu tespit etmek için Real-Time PCR ve immünohistokimyayı karşılaştırdık ve immünohistokimyanın duyarlılık, özgüllük, pozitif ve negatif öngörü değerlerini analiz ettik.

Gereç ve Yöntem: RT- PCR ile EGFR mutasyon durumu için analiz edilen 788 küçük hücreli dışı akciğer kanseri örneğini değerlendirildi. Bu hastalar arasında 126 EGFR mutasyonlu vakayı tespit edildi. 47 EGFR mutasyonlu hastanın histolojik materyali ve sitolojik örneklerin hücre bloklarına yönelik ekzon 19 delesyonları (15 bp E746-A750) ve ekzon 21 nokta mutasyonu (L858R) için mutasyon spesifik EGFR immünohistokimyası (IHK) çalışıldı ve sonuçlar boyama kuvveti ve yaygınlığına göre değerlendirildi.

Bulgular: 47 vakadan 32'si (%68) ekzon 19 delesyonuna sahipti, bunların 14'ünde (%30) ekzon 21'de nokta mutasyonu vardı ve birinde (%2) ekzon 18 mutasyonu gözlendi. EGFR ekzon 19 (15 bp E746-A750 delesyon) antikoru %100 duyarlılık, %40 özgüllük, %100 negatif öngörü değeri ve %78 pozitif öngörü değeri gösterdi. Ekzon 21 (L858R nokta mutasyonu) antikorunun duyarlılığı %93, özgüllüğü %91, negatif öngörü değeri %97 ve pozitif öngörü değeri %82 idi.

Sonuç: Araştırmamız, EGFR immünohistokimyasının, ekzon 21 mutasyonu için belirgin bir duyarlılık ve özgüllük sergilediğini göstermektedir. Ancak, duyarlı olan ekzon 19 (15 bp E746-A750 delesyon) antikoru özgüllükten yoksundur. Pozitif immünohistokimya sonucu, hastada EGFR mutasyonu olabileceğini ön görebilir ve hasta potansiyel olarak TKI tedavisine uygun olabilir; ancak bu durum tek başına belirleyici olmamalıdır. İmmünohistokimya sonuçları negatifse, doğru tanı ve uygun tedavi rehberliği için moleküler testlere başvurulması esastır.

Anahtar Sözcükler: EGFR, İmmünohistokimya, Küçük hücreli dışı akciğer karsinomu.

Introduction

Lung cancer is the most frequent cause of cancer-related mortality worldwide. Moreover, lung carcinoma is the 2nd most common cancer type by gender after prostate cancer for men and breast cancer for women. As most patients are diagnosed at advanced stages, the 5-year survival rate of people diagnosed with advanced stage non-small cell lung cancer (NSCLC) is around 6%, and of small cell lung cancer is around %3(1, 2).

Several driver mutations have been described in the pathogenesis of non-small cell lung cancers. While FGFR1 amplification and TP53 mutations are common driver events in SCC, mutations in receptor tyrosine kinases (most commonly in EGFR, ALK, ROS1, and MET) are mainly responsible for the initiation of adenocarcinoma. Today, even the detection of only a set of these mutations, such as activating EGFR mutations in lung adenocarcinomas, harbor clinical importance as targeted therapeutic agents are available (3-5).

EGFR is a transmembrane receptor protein that constitutes 486 amino acids and contains 4 extracellular and 3 intracellular domains. Approximately 25% of lung adenocarcinomas harbor an EGFR mutation where most commonly exons 18, 19, 20, and 21 are affected (6). More specifically, about 90% of EGFR mutations encountered in adenocarcinoma of the lung are found in exons 19 and 21. These activating mutations are associated with sensitivity to tyrosine kinase inhibitors (TKI). Thus, the detection of such mutations is of utmost importance in NSCLCs.

There is no approved gold-standard method to detect EGFR mutations in non-small cell lung carcinoma patients, yet real-time PCR, Sanger sequencing, Pyrosequencing, and next-generation sequencing techniques are widely used. Real-time PCR and NGS are the most commonly used methods for their specificity, sensitivity, and speed. On the other hand, monoclonal antibodies directed at the mutant protein have also been developed and considered as an alternative method.

Mutation-specific antibodies are designed to detect the two most frequent mutations; the 15-base pair deletion at exon 19 (p.Glu746_Ala750del) and the L858R point mutations at exon 21 (p.Leu858Arg). Currently, four different clones are commercially available. Antibodies directed to detect the p.Glu746_Ala750del mutation have been reported to have a range sensitivity of 47-100% in the literature (7) (8, 9). Similarly, sensitivity ranges between 36% and 100% for antibodies that detect L858R (7-10). In contrast, specificity rates are around 90-100% for exon 19 and 80-100% for exon 21 (8, 11, 12).

This study aims to compare the sensitivity and specificity of these mutation-specific antibodies with that of real-time PCR. Besides, we also aim to evaluate the utility of the detection of activating EGFR mutations by immunohistochemistry in routine practice.

Material and Methods

The principles of the Helsinki Declaration conducted this study, and after obtaining ethical approval from the Hacettepe University Non-Interventional Clinical Research Ethics Committee (decision no: Go13/519-24, at 08.11.2013), it was supported as scientific research project number 1146 by the Hacettepe University Scientific Research Projects Coordination Unit.

Patient Selection

A retrospective search was conducted to select archival cases of primary lung carcinoma whose EGFR mutation analyses were carried out using real-time PCR at the Hacettepe University Department of Pathology. The search yielded 788 such cases. The digital hospital database was used to collect data for patient age, gender, biopsy/aspiration localization, the procedure used to obtain the sample, additional techniques to aid diagnosis (histochemistry and immunohistochemistry), and survival. One hundred and twenty-six cases with EGFR mutations were selected and the remaining tissue in these blocks was reviewed for sufficiency. Cases that had insufficient tissue in blocks or cases that consisted only of aspiration cytology smears without cell blocks were excluded. The biopsy and cytology samples of the remaining 47 cases were included in the study. All H&E tumor slides were reviewed, and the block previously used for molecular analysis was preferably chosen for immunohistochemical staining.

EGFR Mutation Analysis with RT-PCR

Mutation analyses were carried out as follows: 5x8-micron thick slides were prepared from the block with the tumor. QIAamp® DNA FFPE Tissue

Kit's (QIAGEN, Hilden, Germany) protocol was followed after the deparaffinization step for DNA isolation. DNA quality was assessed by running the products in an agarose gel. The Real-time PCR EntroGen EGFR Mutation analysis kit was used according to the manufacturer's instructions using the ABI StepOnePlus Real-Time PCR platform. Every assay included appropriate controls.

Immunohistochemical Detection of Mutant EGFR

Out of 126 cases chosen for the study, 47 had sufficient tissue for immunohistochemistry. Five-micron thick slides prepared from formalin-fixed paraffin-embedded samples were stained with the primary antibody directed to the protein with p.Glu746_Ala750del mutation (clone: 6B6, dilution: 1/100, Cell Signaling Technology, Boston, MA, USA) and the primary antibody directed to the protein with L858R mutation (clone: 43B2, dilution: 1/100, Cell Signaling Technology, Boston, MA, USA). The standard streptavidin-biotin procedure was followed for the staining.

Slides were reviewed at x20 for membranous and/or cytoplasmic staining, depending on the suitable staining pattern of the antibody. Strength (intensity) and the extent (percentage) of staining were recorded for each case. Staining in >10% of tumor cells was scored as 1, and <10% was scored as 0. Cases with >10% staining were re-assessed for the intensity of staining as follows: Score 1; staining as strong as the positive control (Figure IA-B), score 2; intermediate strength of staining between scores 1 and 3 (Figure IC-D), and score 3; no staining or barely discernable staining (Figure IE-F).

Statistical Analysis

Categorical variables were described with numbers and percentages, and continuous variables were noted with medians, standard deviations, and minimum and maximum values. ROC (receiver operating characteristic) analysis was implemented for inter-variable cutoff. Scores obtained by immunohistochemical staining were compared with the RT-PCR results; sensitivity, specificity, and positive and negative predictive values were calculated. The cutoff of the p-values for statistical significance was 0.05. IBM SPSS v20 statistical analysis software package was used for these analyses.

Results

The RT-PCR results of the 47 cases that underwent immunohistochemical staining were as follows: 32 (68%) had exon 19 deletion, 14 (30%) had exon 21 point mutation and 1 (2%) had exon 18 mutation.

Thirty-seven (79%) of the samples were from the lungs while 10 (21%) were from extrapulmonary sites. Twenty-two of the pulmonary samples were small biopsies, 3 were cytology material, and 12 were resections (wedge biopsy, lobectomy, or pneumectomy). Extrapulmonary sites included 3 (7%) liver biopsies, 2 (4%) pleural biopsies, 2 (4%) lymph node biopsies, 2 (2%) soft tissue and one (2%) brain excision. Twenty-seven (57%) of the immune-stained cases were received and processed in our lab. Three of them were cell blocks prepared during the adequacy assessment in the sampling process. The remaining 20 cases (43%) were received and processed in other labs and were sent to our lab for consultation.

Table I. Staining intensity of cases with exon 19 mutation-specific immunohistochemistry

	Staining intensities in immunohistochemical exon 19 deletion specific antibody			
EGFR mutation status	Negative	Score 1	Score 2	Score 3
Exon 21 L858R mutation (n=14)	6 (%43)	1 (%7)	4 (%29)	3 (%21)
Exon 19 deletion (n=32)	-	6 (%19)	14 (%44)	12 (%37)
Exon 18 mutation (n=1)	-	-	-	1 (%100)

Immunostaining for the E746-A750 deletion at exon 19 identified diverse staining scores: 16 cases scored 3, 18 scored 2, and 7 scored 1; six were negative. Notably, a few cases with high staining scores also showed mutations in exons 21 and 18. Detailed distribution of mutations across different staining scores is presented in Table I.

When score 1 was set as a cut-off point for immunostaining intensity, the sensitivity of the immunohistochemistry assay for exon 19 mutations

was 100%, its specificity was 40%, its negative predictive value was 100% and its positive predictive value was 78%. Higher scores demonstrated varied sensitivity and specificity, indicating a trade-off between the two metrics (Table II)

Table II. Comparison of sensitivity, specificity, negative and positive predictive values when changing the threshold value in the study with mutation-specific antibodies for EGFR exon 19 and exon 21

Mutation specific antibody	IHC threshold value (positive)	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)
EGFR exon 19 E746-A750 deletion	≥ score 1	100	40	100	78
	≥ score 2	81	47	54	76
	score 3	38	73	35	75
EGFR exon 21 L858R point mutation	≥ score 1	93	64	95	52
	≥ score 2	93	91	97	82
	score 3	71	100	89	100

In the exon 21 L858R mutation-specific immunohistochemistry assay, staining intensity scores varied: 10 cases scored 3, six scored 2, and nine scored 1, with 22 cases testing negative. All cases scoring 3 harbored the exon 21 mutation. Among cases scoring 2, three had exon 21 mutations and three had exon 19 mutations. A case scoring 1 displayed an exon 18 mutation, while the rest had exon 19 mutations. Of the negative cases, 21 had exon 19 mutations and one had an exon 21 mutation, with one such case showing nuclear positivity and originating from an external institution (Table III).

Table III. Staining intensity of cases with exon 21 mutation-specific immunohistochemistry

	Staining intensities in immunohistochemical exon 21 mutation specific antibody			
EGFR mutation status	Negative	Score 1	Score 2	Score 3
Exon 21 L858R mutation (n=14)	1 (%7)	-	3 (%21)	10 (%72)
Exon 19 deletion (n=32)	21 (%66)	7 (%22)	3 (%12)	-
Exon 18 mutation (n=1)	-	1 (%100)	-	-

Comprehensive sensitivity, specificity, and predictive values for exon 21 immunostaining were derived for each score, highlighting the assay's diagnostic accuracy across different thresholds (Table III). A cut-off point of score 2 revealed the highest sensitivity, specificity, and negative and positive predictive values of 93, 91, 97, and 82%, respectively. (Table II).

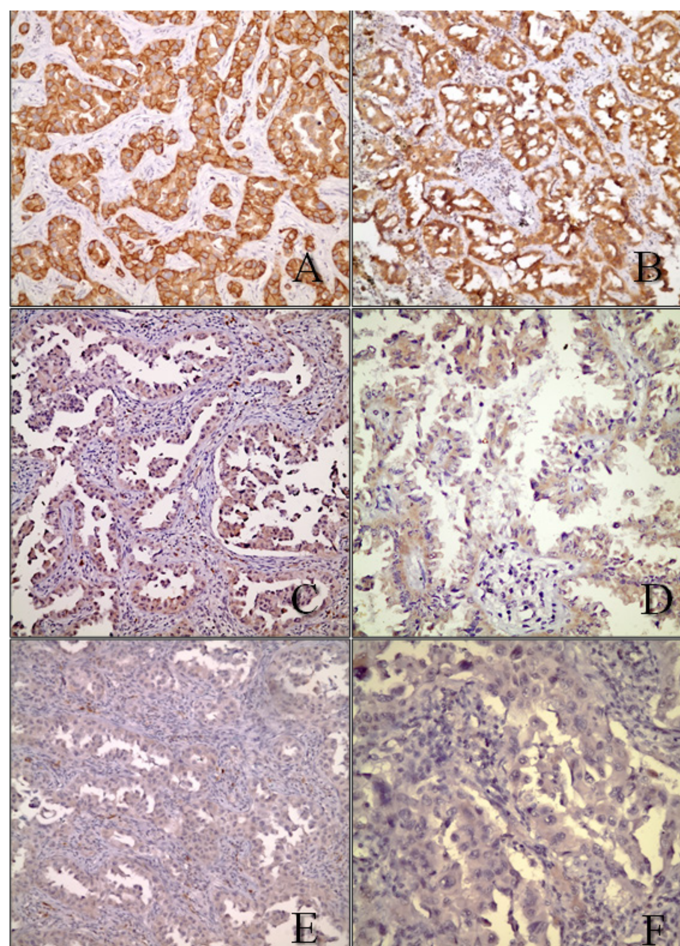

Figure I: Score 3 staining intensity in an immunohistochemical study with exon 21 mutation-specific antibody (A), Score 3 staining intensity in an immunohistochemical study with exon 19 mutation-specific antibody (B), Score 2 staining intensity in an immunohistochemical study with exon 21 mutation-specific antibody (C), Score 2 staining intensity in an immunohistochemical study with exon 19 mutation-specific antibody (D), Score 1 staining intensity in an immunohistochemical study with exon 21 mutation-specific antibody (E), Score 1 staining intensity in an immunohistochemical study with exon 19 mutation-specific antibody.

Several different staining scores were set as cut-off values in the literature. Separate ROC analyses were carried out per score to determine the ideal cut-off points. For exon 19 immunostaining, the best cut-off point was attained when scores 1 and higher were considered positive; the p-value at this point was

0.065. When scores 2 and above were considered positive in the exon 21 immunostaining, the p-value was 0.0001.

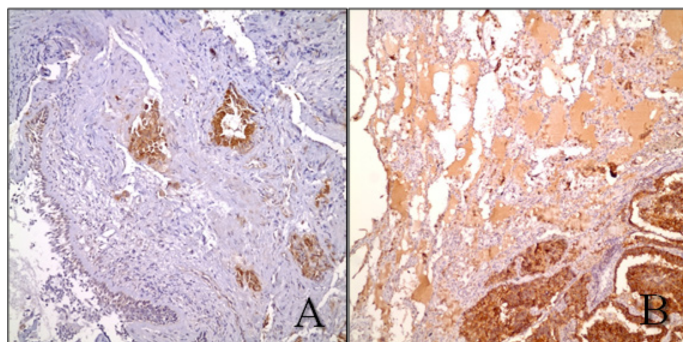

Figure II. Weak (score 1) staining in non-neoplastic bronchial epithelial cells (A), False staining in inflammatory cells and alveolar spaces (B).

A single case that harbored an exon 18 mutation revealed a score of 3 staining intensity with the exon 19 antibody and a score of 1 staining intensity with the exon 21 antibody.

In non-neoplastic tissues, both antibodies displayed weak (score 1) staining in bronchial epithelial cells, while alveolar pneumocystis were negative (Figure IIA). Weak cytoplasmic staining was occasionally encountered in alveolar macrophages, inflammatory cells, and necrotic areas (Figure IIB).

Discussion

EGFR is a transmembrane receptor tyrosine kinase involved in cell survival and development under normal conditions (13) Activating EGFR mutations are detected in 10-35% of lung adenocarcinomas (14-16). Because of the activation of the cascade, in EGFR-mutant cases, EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib attain much better success in disease-free survival than carboplatin or paclitaxel (17). According to the National Comprehensive Cancer Network (NCCN) guidelines, EGFR-tyrosine kinase inhibitors are the first-line therapy in advanced, recurrent, or metastatic lung adenocarcinomas with mutant EGFR (17, 18). Therefore, EFGR mutation analysis in advanced-stage non-small cell lung carcinomas has become mandatory.

PCR-based molecular modalities for mutation detection are the most commonly implemented techniques after the discovery of the clinical significance of EGFR mutations. Most molecular techniques rely on the amplification of the mutant DNA among the wild-type DNA. In many studies, the method at hand is compared to the sequencing technique that is already known. Direct sequencing is the most common screening method; its main limitation is its low sensitivity. This method requires at least 20% of mutant DNA (19).

Macro or microdissection before DNA extraction may yield a higher tumor/non-tumor tissue ratio. These processes of sample preparation are time-consuming, labor-intensive, and require experienced/well-trained personnel. However, with its high sensitivity and easy application, RT-PCR is presently the method of choice.

As mentioned above, because EGFR mutation status depicts possible targeted therapy sensitivity, it should be determined routinely by a standard method. Yet, as most of the patients are inoperable at the time of diagnosis, small biopsies or cytology specimens are frequently the sole samples for mutation detection. Instead of techniques that require DNA isolation using a substantial amount of tissue, immunohistochemistry could be a useful method to detect mutant protein on just a single 4-um thick section.

Therefore, mutation-specific immunohistochemistry is a method that can be used to detect EFGR mutations in small samples with few tumor cells; it can be performed on intraoperative consultation slides, paraffin blocks, and even on cytology samples (cell blocks or smears). It is much less costly when compared to the other methods; results are revealed relatively faster and can be carried out in many laboratories without additional equipment. To this end, antibodies are developed to detect two of the most frequent EFGR mutations; exon 19 (15 bp, E746-A750) and exon 21 (L858R point mutation). So far, there are four commercially available mutant EGFR-specific antibody clones. These are directed at the two most frequent mutations: 6B6 and SP111 clones for exon 19 deletion at E746-A750 and 43B2 and SP125 clones for exon 21 point mutation at Leu858Arg.

In our study, the most significant ROC value for exon 19 mutation-specific immunohistochemistry was attained when the cutoff value was set at score 1, with the assay's sensitivity, specificity, and positive predictive value being 100%, 40%, and

78%, respectively. The relatively lower specificity, compared to that reported in the literature, can be attributed to the use of exon 21 or exon 18 mutant cases as negative controls, potentially leading to cross-reactivity with the exon 19 mutation-specific antibody. Despite the high specificity (98.8%) and sensitivity (100%) for detecting the 15-bp deletion in exon 19 (8, 11, 20, 21), the sensitivity for detecting various 3-8 amino acid deletions distinct from the frequent 15bp/5AA E746-A750 alteration drops to 20-67% (22, 23). According to the Catalog of Somatic Mutations in Cancer (COSMIC), these less common deletions constitute 35% of all deletions in exon 19, emphasizing the need for comprehensive molecular testing for samples that test negative with E746-A750 deletion-specific IHC to ensure no other mutations are missed. This approach aligns with the latest CAP guidelines which recommend extending molecular screenings beyond the two most frequent alterations to ensure accurate diagnosis and appropriate therapy initiation based on reliable IHC results (24).

In exon 21 L858R mutation-specific immunohistochemistry, the sensitivity and specificity values are found to be high when scores 2 and 3 are considered positive. In the present study, the sensitivity, specificity, and positive predictive values for a score 2 cutoff are 92%, 91%, and 91% respectively, showing a strong similarity to the 'perfect test' in ROC analyses with a p-value of 0.0001. Despite this high accuracy, there was a case where RT-PCR detected an exon 21 mutation that the immunohistochemistry failed to reveal, displaying only nuclear positivity and considered negative due to the criteria for positivity being membranous and/or cytoplasmic staining. This discrepancy could result from a fixation or processing artifact since the sample was processed outside our institution. Immunohistochemistry directed at the exon 21 L858R point mutation is noted to be more sensitive and specific than that for exon 19 E746-A750(8, 12, 20, 22, 25). The monoclonal antibody for exon 21 only detects the L858R mutation and misses L861Q(11), another alteration, but as per the COSMIC database, over 90% of exon 21 mutations are L858R, ensuring the antibody's effectiveness for most clinical scenarios. Therefore, cases that test positive with this antibody can typically commence tyrosine kinase inhibitor therapy without further molecular confirmation; however, negative results necessitate additional molecular diagnostics to rule out rare mutations. Moreover, the challenge of detecting less common mutations, as seen in exon 21 cases, highlights broader issues in mutation-specific testing that extend to challenges in intratumoral heterogeneity.

Intratumoral heterogeneity is defined as the presence of variable morphological and phenotypical features in different tumor cells of the same tumor. Intratumoral heterogeneity concerning EGFR mutations is a controversial issue. Some studies report up to 13% intratumoral heterogeneity of EGFR status (26). Other studies maintain that such data results from methodological disparities (27). In the present study, we had 14 cases with an extent of staining less than 90%. The areas that are devoid of EGFR expression and the positive areas do not display any morphological difference. As immunohistochemistry allows for wider areas of tissue for assessment, theoretically, mutant protein detection can be carried out with higher sensitivity and thus be less susceptible to intratumoral heterogeneity. However, at least in some cases, the possibility of staining heterogeneity being due to fixation and processing artifacts can never be fully ruled out.

Three cases included in the study had samples of cell blocks prepared in specimen adequacy assessment. Two of these cases had exon 19 and one had exon 21 mutations. All three displayed immunohistochemical positivity. Specimen adequacy assessment during sampling increases the diagnostic value of the biopsy procedure while attaining samples for further use in research or diagnostics (28). As mentioned in the literature and the CAP molecular guideline, EGFR mutation assessment cell blocks are preferred over smears (29). Immunohistochemical assays similarly yield better results with cell blocks (30). The present study does not include smear preparations so such a comparison was not attempted. Understanding all of these variations is crucial, especially when considering the overall reliability of diagnostic methods and the current guidelines that influence clinical decision-making.

If we overlook disadvantages such as the small number of patients and the absence of a true

negative group in our study, we observed that mutation-specific antibodies yield varying levels of sensitivity and specificity at different staining intensities. Furthermore, the reproducibility of such scoring assessments in routine pathological practice can be low due to variations in laboratory conditions, such as changes in the technician performing the immunohistochemistry. Current guidelines generally do not recommend the use of EGFR IHC for testing the presence of EGFR mutations due to these types of variability and the differences discussed above, like base pair differences. Despite its utility for certain molecular targets like ALK, ROS, BRAF, and PD-L1 (31-33), the role of EGFR-specific IHC is diminishing. This shift is due to the superior accuracy of newer sequencing technologies that can analyze even single cells. Given the variations in test results and reproducibility issues under different laboratory conditions, current guidelines advise against using EGFR IHC for detecting EGFR mutations. As such, we advocate for a transition to advanced genomic testing methods that provide greater precision and reliability, ensuring that our diagnostic strategies evolve to deliver the best patient outcomes.

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