Mutation Analysis of Epidermal Growth Factor Receptor Gene in Non-small Cell Lung Cancer for Selection of Patients Eligible for Tyrosine Kinase Inhibitor Therapy

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Abstract

Introduction: Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) therapy is effective as a first-line treatment of advanced non-small-cell lung cancer (NSCLC). This research study investigated the distribution of EGFR mutations in patients diagnosed with NSCLC to assist in selecting patients who could benefit from TKI therapy. **Materials and Methods:** This cross-sectional study was conducted between July 2017 and November 2022. A real-time multiplex polymerase chain reaction (PCR) assay supplied by Roche Diagnostics was used to examine DNA obtained from 682 tumor biopsies collected from NSCLC patients. DNA amplification was performed in a Cobas z 480 instrument for mutation analysis. The PCR assay was designed using specific primers and probes to detect 43 different mutations targeting exons 18–21. **Results:** Among the 682 samples, 466 (68.3%) were males, and 216 were females. The male-to-female ratio was 2.1. About 20% of the male and 37% of the female samples were positive for EGFR mutations. The most common mutations were the in-frame deletion of exon 19, followed by L858R in exon 21, exon 20 insertions, and S769I, exon 18 G719X. In addition, three mutations, namely, del exon 19, T790M, and exon 20 insertions were also detected in a patient, suggesting an actively progressive disease. **Conclusions:** This study showed that EGFR mutations are more common in Pakistani female patients than males. Second, in-frame deletion of exon 19 and exon 21 mutation L858R is prevalent in most of the NSCLC patients. The prevalence of common and rare EGFR mutations in Pakistani patients provides an opportunity for a subset of patients' chance of therapy.

Keywords: Epidermal growth factor receptor, mutation analysis, non-small-cell lung cancer, tyrosine kinase inhibitors

Introduction

Lung cancer ranks as one of the leading causes of death in both men and women worldwide.[1] Lung cancer is often diagnosed at advanced stages when treatment options are limited. Pakistan is a highly populous country where lung cancer morbidity and mortality have been underreported.[2] Many lung cancer patients, especially those of Southeast Asian origin, are reported to carry sensitizing mutations in the epidermal growth factor receptor (EGFR) gene. In patients of different ethnicities, EGFR

mutations have been estimated to occur between 15% and 45%.[3,4] This subgroup of non-small-cell lung cancer (NSCLC) responds to tyrosine kinase inhibitor (TKI) therapy targeting mutated EGFR molecules. After discovering an oncogenic role of EGFR mutations in lung cancer, several TKIs were approved as the first line of treatment with proven efficacy, including afatinib, dacomitinib, and erlotinib. EGFR-TKI therapy is considered a better choice for patients with an elevated risk of relapse.

Several studies have reported that in EGFR-positive cases, the partial or complete response rate and disease-free survival are between 30% and 70%, respectively.[5] However, TKI therapy will not be effective for all EGFR mutations in NSCLC cases. In the future, the repertoire of molecular markers will expand to have more possibilities for using targeted agents for NSCLC patients in advanced stages.[6] The molecular mechanisms behind lung cancer are currently the subject of more intense research. Identifying targetable genetic changes has fundamentally altered the pathological strategy for treating lung cancer, particularly NSCLC.

Pathologists today hold a dominant position in lung cancer treatment thanks to their relentless expertise. The therapy and prognosis of NSCLC have changed due to the identification of genetic alterations responsive to targeted therapies. Lung cancer can now be further subdivided into molecular subgroups, and there is a renewed focus on using targeted therapies for its management.[7,8] The discovery of new molecular targets, resistance mechanisms, and uncommon NSCLC genotypes will likely advance drug development and expand therapy options. As lung cancer treatment has become increasingly biomarker-driven and tailored medicines have emerged, clinical organizations must develop best practice guidelines for the type of molecular tests required according to target populations.

Although, in multiple clinical trials, following the discovery of EGFR-sensitizing mutations in 2004, EGFR-TKI has better performed in NSCLC patients than chemotherapy, it was in 2011 that the American Society for Clinical Oncology (ASCO) first proposed clinical testing in lung cancer patients confirmed to have mutations causing uncontrolled activation of EGFR protein.[9,10]

In the absence of a population-based cancer registry, reliable statistics on the incidence, prevalence, and mortality of lung cancer in Pakistan are not known. Previously, we have reported the distribution of EGFR mutations commonly observed in primary lung adenocarcinomas in Pakistan.[11] In this study, we explore the spectrum of EGFR variants in a large cohort of patients diagnosed with adenocarcinoma of the lung. We investigated the distribution of EGFR mutations by age and gender. The patients in this study represented all significant regions and ethnicities of Pakistan.

Material and Methods

This study received approval from the Ethical Review Committee, The Aga Khan University, Pakistan. Participants provided informed verbal consent.

Specimen collection

A retrospective study was performed on NSCLC biopsy specimens received for histopathological workup at the Aga Khan University Hospital. The specimens were obtained between July 2017 and December 2022. The patient's age, gender information, and tumor features were obtained from histopathology reports, including tumor size, lymph node status, presence of recurrence or metastasis, and biopsy site. The histopathological diagnosis of lung adenocarcinoma was confirmed based on morphology and histochemical features. Immunomarker profile of the tumors consisted of makers CK7, TTF1, CK5/6, CK20, and p63. The algorithm used for histopathological diagnosis is shown in Figure 1. Appropriate controls comprising previously tested specimens positive for the immunomarker were run with each assay. In addition, a negative control was also included in every batch.
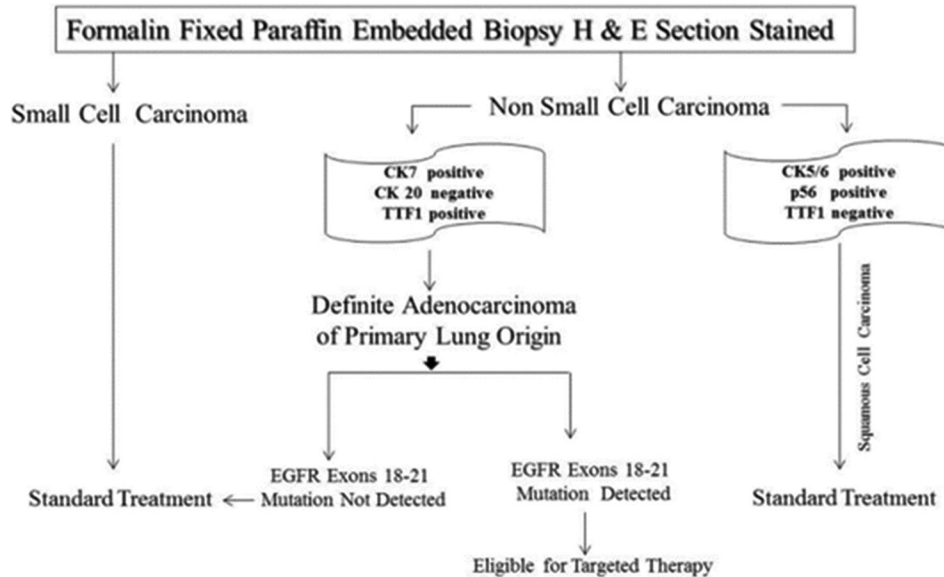
Figure 1: Algorithm for histopathological diagnosis. It has been adapted from Ahmed *et al.*[11]

DNA extraction and real-time polymerase chain reaction (PCR) assay

The tumor area was marked on a hematoxylin-eosin slide by an experienced histopathologist, and more than 20% of the tumor content was confirmed before DNA extraction. Macro dissection was only done if tumor content was less than the desired amount. DNA extraction was accomplished by Cobas DNA sample preparation kit as per the manufacturer's package insert (Roche Diagnostics, NJ, USA). Briefly, a section of 10µm thickness was deparaffinized with xylene from the tumor block. The tissue section was resuspended in lysis buffer with proteinase K solution, and DNA was purified by spin columns. All DNA samples were stored at −20°C for further processing. Cobas EGFR mutation test v2 was used to screen for EGFR mutations in patients' DNA samples as recommended in the kit instructions. To screen EGFR mutations, 150 ng DNA from each patient was amplified in a Cobas z480 analyzer (Roche Diagnostics, NJ, USA).

The EGFR allele-specific multiplex PCR is intended to distinguish 43 rare and common mutations in exons 18–21 of the EGFR gene. It included point mutations, insertions, deletions, and complex mutations such as G719A/C/S, exon 19 deletion, S768I, T790M, and L858R in exons 18–21, respectively. The lower detection limit of the assay has been verified to be 5% mutant allele frequency. The results were analyzed, and mutations were documented following the latest HGVS guidelines. Table 1 lists the EGFR mutations detected by the Cobas EGFR Test according to their respective HGVS nomenclature.

Statistical analysis

Data were summarized and presented using appropriate descriptive statistics. Patients' demographics, histopathological, and mutation data were recorded in a Microsoft Excel sheet. A Chi-square test was performed to assess differences in the proportion of EGFR mutations across age and gender as categories and where applicable, and $P < 0.05$ was considered significant.

Results

In this study, we analyzed tumor specimens for mutations in the EGFR gene by Cobas multiplex PCR assay. The mutations detected by the assay are shown in Table 1. Among the total samples, 466 (68.3%) were males, and 216 (31.7%) were

Table 1: Lists the epidermal growth factor receptor mutations detected by the cobas EGFR test. The list has been adapted from the cobas EGFR mutation test v2 kit insert available on https://diagnostics.roche.com/us/en/products/params/cobas-egfr-mutation-test-v2.html

Exon	EGFR mutation group	EGFR nucleic acid sequence
Exon 18	G719X	2156G>C
		2155G>A
		2155G>T
Exon 19	E×19Del	2240_2251del12
		2239_2247del9
		2238_2255del18
		2235_2249del15
		2236_2250del15
		2239_2253del15
		2239_2256del18
		2237_2254del18
		2240_2254del15
		2240_2257del18
		2239_2248TTAAGAGAAG>C
		2239_2251>C
		2237_2255>T
		2235_2255>AAT
		2237_2252>T
		2239_2258>CA
		2239_2256>CAA
		2237_2253>TTGCT
		2238_2252>GCA
		2238_2248>GC
		2237_2251del15
		2236_2253del18
		2235_2248>AATTC
		2235_2252>AAT
		2235_2251>AATTC
		2253_2276del24
		2237_2257>TCT
		2238_2252del15
		2233_2247del15
Exon 20	S768I	2303G>T
	T790M	2369C>T
	E×20Ins	2307_2308ins9GCCAGCGTG
		2319_2320insCAC
		2310_2311insGGT

(Contd…)

Table 1: (Continued)

Exon	EGFR mutation group	EGFR nucleic acid sequence
		2311_2312ins9GCGTGGACA
		2309_2310AC>CCAGCGTGGAT
Exon 21	L858R	2573T>G
		2573_2574TG>GT
	L861Q	2582T>A

EGFR: Epidermal growth factor receptor

females. In our cohort, male samples comprised two-thirds of all samples Table 2. In males, the mean age was 60.7 (range 5 years to 90 years), whereas in females, the mean age was 55.9 (range 6 years to 91 years). Adenocarcinoma of the lung was most frequently diagnosed (98.7%), as shown in Table 3; several of its histopathological subtypes included poorly differentiated, moderately differentiated, and well-differentiated adenocarcinoma. In addition, metastatic adenocarcinoma constituted 29% of the total specimens, whereas squamous cell carcinoma was positive in 1.7% of the cases. Furthermore, the source of 85.5% of the samples was lung biopsy, and 14.3% belonged to extra pulmonary sites and were metastatic in origin. The main extra pulmonary areas included the liver, brain, endometrium, rectal polyp, adrenal gland, liver, and lymph nodes.

As shown in Table 4, EGFR mutations were positive in 20% of the male samples and 37% of the female patients. Analysis by the Chi-square test showed a statistical difference in the distribution of EGFR mutations between the two patient groups. However, the distribution in different subtypes of lung adenocarcinoma specimens was insignificant.

Table 4 shows EGFR mutation positivity in male and female patients by age group. Moreover, there was no statistical difference between males in age groups ≤50 and above 50 years, but the females in age group above 50 years accounted for a higher number of mutation-positive patients and were statistically significant. Of 682 samples,

Table 2: Patients demographic and histopathological features

Variable	Characteristics	No. (%)
Gender	Male	466 (68.3%)
	Female	216 (31.7%)
Age (years)	Male	Range 5–90 Mean age (60.7)
	Female	Range 6–91 Mean age (55.9)
EGFR mutation status	Positive	171 (25%)
	Negative	511 (75%)
Histopathology	Adenocarcinoma	649 (95%)
	Squamous cell carcinoma	11 (1.6%)
	Other (metastatic malignancy of unknown/undefined primary origin)	22 (3.4%)

EGFR: Epidermal growth factor receptor

171 (25%) were positive for EGFR mutations. A single mutation was found in 668 (98%) patients, whereas compound heterozygous mutations were found in 14 patients. The three common mutations in these cases were del exon 19, S768I, and exon 20 ins. Furthermore, multiple mutations, including del exon 19, T790M, and exon 20 insertions, were detected in one patient with advanced adenocarcinoma. T790M was found in only two cases associated with other mutations, and the tumor was lung adenocarcinoma with metastasis.

Figure 2 illustrates the deletion of exon 19 (61%) as the most common mutation. It was followed by L858R (24.5%), exon 20 ins (7%), and S769I (3.5%), exon 18 G719X (3.5%), whereas L861Q was detected in a single sample. Statistically, there was no difference in the distribution of the above mutations between male and female

Table 3: EGFR mutation status by histopathological characteristics

| Histopathology	EGFR mutation		Total	P value
	Positive	Negative		
Adenocarcinoma	82 (27%)	226 (73%)	308	NS
Poorly differentiated adenocarcinoma	14 (20%)	53 (80%)	67	
Moderately differentiated adenocarcinoma	13 (23%)	43 (77%)	56	
Well-differentiated adenocarcinoma	8 (42%)	11 (58%)	19	
Metastatic adenocarcinoma	55 (27%)	144 (73%)	199	
Squamous cell carcinoma	3 (27%)	8 (73%)	11	

EGFR: Epidermal growth factor receptor

Table 4: EGFR mutation status by gender and age groups

Variable	EGFR mutation		Total	P value
	Positive	Negative		
Gender				
Male	92 (20%)	374 (80%)	466	P<0.05
Female	79 (37%)	137 (63%)	216	
Age				
Male≤50 years	25 (25%)	76 (75%)	101	NS
Male>50 years	67 (18%)	298 (82%)	365	
Female≤50 years	15 (22%)	54 (78%)	69	P<0.05
Female>50 years	64 (44%)	83 (56%)	147	

EGFR: Epidermal growth factor receptor
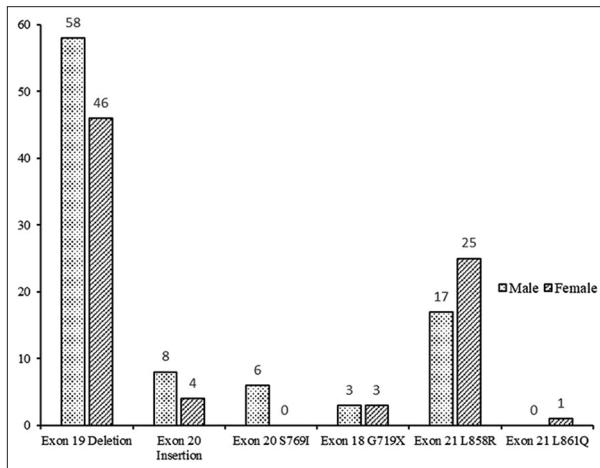
Figure 2: Grouping of epidermal growth factor receptor mutations in male and female lung cancer patients

groups. Besides adenocarcinoma, 11 cases diagnosed as squamous cell carcinoma underwent EGFR mutation analysis; none tested positive for mutations.

Discussion

Globally, more than 2.2 million lung cancer cases were reported in 2021, making it the most frequently diagnosed cancer and the leading cause of death in cancer patients. Approximately 65% of cases were reported in males, with a male-to-female ratio of 1.8 (wcrf.org/cancer-trends/lung-cancer-statistics). In 2004, Pao *et al.* first reported in NSCLC adenocarcinoma oncogenic mutations in the EGFR gene, opening up a new line of therapy choices with TKIs.[12,13] Since then, many clinical trials have demonstrated the efficacy of TKIs in EGFR mutation-positive advanced NSCLC. Worldwide, it is becoming a standard of care to test for EGFR-activating mutations for identifying a subset of NSCLC patients who can benefit from tyrosine kinase-targeted therapies. Although most patients in our study were males (68.3%), the EGFR mutations were found at a statistically higher percentage in the female group compared to males (37% vs. 20%, $P < 0.05$). These results agree with most published studies and clinical trials, confirming that activating somatic mutations in the EGFR gene in female lung cancer patients is more common than in male patients.[14] Overall, in our study, EGFR mutations were found in 25% of the total patients.

Several studies have concluded that sensitizing EGFR mutations is prevalent in lung cancer patients at varying frequencies in different populations; for example, it is estimated to be around 10–15% in the North American and European populations, 19% in Africans, and about 30% in East Asians.[15,16] The frequency of EGFR mutations in our study was close to that reported in the East Asian population. Zeeshan *et al.* reported a 29% EGFR mutation rate in their 94 Pakistani NSCLC patients.[9] On the contrary, in another study based out of India, lung cancer patients reported that 35% of their patients harboring an EGFR mutation, which appears to be considerably higher than our study population. Likewise, in another Indian study, the estimated frequency of EGFR mutations was 51.7%, a likely overestimation due to the small sample size of the study population.[17] Likewise, in Bangladesh, a country in the Indian subcontinent region, the overall frequency of EGFR mutations in male patients was 23% and in females 14%, reporting a lower frequency in females.[18] Our data show that the distribution of EGFR mutations in age groups below and above 50 years of male patients was nearly equal. Noticeably, more females in the age group above 50 than under carried EGFR mutations 43.4% versus 22%. Likewise, to our findings, Wu *et al.* reported that EGFR mutation prevalence in younger Asian patients under 50 was less frequent than in older lung cancer patients.[19]

Most patients who tested positive for EGFR mutations (98.3%) had lung adenocarcinoma, whereas squamous cell carcinoma (SSC) was reported in 1.7% of the patients. Of the 11 squamous carcinoma patients analyzed, 3/11 (27%) were positive for EGFR mutations, contrary to Esteban *et al.* findings, who reported EGFR mutations in 4% of their SSC patients.[20] Similarly, Han *et al.* concluded, based on the IGNITE study, that there was a 4.3% positivity of EGFR mutations in SSC samples of Asia Pacific origin.[15] The small sample number or analytical sensitivity of the

techniques used could be the reason for the failure to detect EGFR mutations in the SSC samples. Occasionally, EGFR mutations have been found in the lung adenocarcinoma compound, presenting a combination of uncommon and common mutations. Some of the following mutations, G719X, T790M, and exon 20 insertions, have been found in conjunction with common mutations.

All patients in our study were tested for EGFR mutations, which include exon 19 in-frame deletions and a point mutation in exon 21 L858R These classical mutations are linked with increased susceptibility to TKI therapy. The prevalence of exon 19 deletion in our patients was 60.8%, and it was 24.5% for L861Q. Both mutations accounted for more than 85% of the total mutations. Even though the frequency of exon 19 deletion was almost equal in both male and female patients, the prevalence of L858R was considerably higher in female patients compared to males (31.6% vs. 18.4%). Similarly, to our study, in the literature, exon 19 deletion and L858R mutations in the EGFR gene have been reported in several studies to account for approximately 90% of mutations in lung adenocarcinoma.[21] The leading rare mutations detected in our study were exon 20 insertions (7%), followed by a point mutation in exon 20 (3.4%), and the least common was L861Q, with a frequency of only 0.5%. In contrast, the overall frequency of rare mutations was 14.5%. According to Hsu, the outcome of TKI therapy in the case of relatively rare mutations has not been completely understood; it is a matter of concern from the point that between 10% and 15% of the patients reported harboring uncommon mutations.[22] For instance, in response to TKI treatment, patients with the L861Q mutation illustrated a shorter progression-free survival than patients with common mutations.[23] Notably, most lung cancer patients who acquire resistance to TKI therapy have also been shown to carry T790M, a mutation that confers resistance.[24] However, no case of T790M as a single mutation was noted in our study. It was found as a double (T790M/del exon 19) and triple mutation (T790M/del exon 19/exon 20 insertions) in two cases. Mulloy *et al*. showed that

T790M/L858R double mutations cooperate to enhance the phosphorylation activity of the kinase domain significantly, thus making the kinase more potent and less sensitive to TKIs.[25]

Our study also reports double mutants, 2.1% (15/682) of the specimens. Double mutations were more common in males compared to females (12 versus 3). There is disagreement in the studies reporting the incidence of double mutations due to differences in the sensitivity of the assays used. Like our results, Wei *et al*. showed an overall prevalence of EGFR double mutations in 2.1% of their study patients.[26] However, unlike our finding, dual modifications were accounted for mainly in the female population. Patients with double mutations had a lower response rate when treated with EGFR TKIs than single EGFR exon 19 or exon 21 mutations. In our study, T790M was present as comutation in two patients; in one patient, it was present with del exon 19; in the second patient, del exon 19 and exon 20 ins. At the time of diagnosis, tumors occasionally have an EGFR-activating mutation in conjunction with the exon 20 T790M resistance mutation.[21] Our study detected T790M as a double mutation in only 0.3% of lung tumors, which could be due to technological limitations. For instance, Su *et al*. described that some technologies like DNA mass array have the potential to capture the simultaneous occurrence of EGFR resistance mutation T790M and activating mutations in patients at a higher rate (25%) when compared with Sanger sequencing at 2.8%.[27]

In our study, almost 25% of the NSCLC specimens received for EGFR mutation screening exhibited sensitizing mutations relevant to TKI therapy. The prevalence of common and rare EGFR mutations in Pakistani patients was like global statistics and provided an opportunity for a subset of patients' chance of therapy. This study has several limitations, such as a small sample size, results from a single tertiary care center that is not a representation of the general Pakistani population target mutation analysis (hot spot mutations) and unavailability of data for any relationship between EGFR mutation

analysis and TKI therapy. Future research should find more EGFR-sensitizing mutations that can be addressed by both existing and new EGFR TKIs, enhancing the prognosis for patients with EGFR-mutated NSCLC. In addition, in the future, the need for complete gene sequencing for the identification of novel pathogenic variants in our population (customized targeted panel) aiding an improved prediction of therapies has been acknowledged in the discussion.

In the present study, we identify the spectrum of EGFR mutations in Pakistani lung cancer patients and associate them with clinical and histopathological parameters. The NSCLC specimens received for EGFR mutation screening exhibited sensitizing mutations relevant to TKI therapy. The prevalence of common and rare EGFR mutations in Pakistani patients provides an opportunity for a subset of patients' chance of therapy.

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Author Contributions

Conceived and designed the analysis: ZA and TM; Collected the data: ZA and TM; Contributed data or analysis tools: ZA, AN, and TM; Performed the analysis: ZA, AN, and TM; Wrote the paper: ZA, AN, and TM.