IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 22, Issue 11 Ser.2 (November. 2023), PP 01-05
www.iosrjournals.org

EGFR Mutations In Tissue And Plasma Detected By Real Time PCR Technique In Iraqi Patients With Advanced Non-Small Cell Lung Cancer

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Abstract
Background
*Detection mutations of Epidermal Growth Factor Receptor (*EGFR) *in plasma is essential for guiding clinical decision regarding prediction of patient's treatment. The appropriate use of* EGFR *mutation testing in plasma has been shown to deliver both clinical and economic benefits, especially in clinical situations where biopsy material is inadequate or unavailable. The study aims to highlight the importance of using plasma samples in identifying EGFR mutations compared to Formalin fixed paraffin imbedded tissue samples (FFPET), specially that the detection of EGFR in plasma have not been previously evaluated among Iraqi lung cancer patients.*
Methods:
Plasma and FFPET samples collected from non-small cell lung cancer (NSCLC) patients. Real time PCR technique is used to detect EGFR Mutations in both plasma and FFPET samples. Two hundred and fourteen samples were tested for EGFR mutations, plasma samples were used in addition to the FFPET sample.
Results:
EGFR Mutations in plasma was higher than that of FFPET samples, no significant difference were found when compared the results of EGFR mutations that obtained from FFPET and Plasma samples. The mutations results in females were higher than that in male and the predominant types of mutations were found in exon-19 (EX19Del). Combination of mutations from Exon-20 & Exone-21 (S7681 & L858R) found in (3%) of positive cases.
Conclusions
This study has shown the importance of substituting a plasma sample instead of FFPET sample for EGFR mutation testing, especially when biopsy material is insufficient or unavailable and this leads to fewer tissue biopsies. The type of mutations in Iraq was analyzed, and the dominant mutation in Iraq was EX19 Del.

Date of Submission: 29-10-2023 Date of Acceptance: 09-11-2023

I. Introduction

The clinical management of patients with advance non-small cell lung cancer (NSCLC) has shifted from a histology based to a molecularly approach because of the identification of actionable genetic alterations and subsequent development of effective targeted therapies (Perdigones&Murtaza, 2017) &(Michael Oellerich et al, 2019). Many studies have confirmed that activating EGFR gene mutations are effective markers for EGFR-TKIs sensitivity (Al Dayel, 2012) & (Castro et al, 2013). Tyrosine kinase inhibitor (TKIs), that targets epidermal growth factor receptor (EGFR) have superior efficacy in conjunction with chemotherapy in NSCLC patients with positive results of EGFR mutations (Douillard et al,2014) & (Zhou et al, 2011).

Although tumor tissue is still recognized to be the preferred standard sample for EGFR mutation detection, it is not readily available from every patient (Kawamura et al, 2016) & (Errico, 2014). EGFR mutations detected in plasma might be predictive of response to EGFR TKIs (Jian, et al, 2010). However, recently commercial real-time PCR kits developed and have successfully increased sensitivity, reducing the amount of tumor DNA required to detect the mutation in a patient sample (E.A. Collisson, 2014). EGFR mutation detection in the plasma cell aims to develop a novel method, with the potential advantage for evaluation of patient's response and resistance to treatment (Helman et al, 2018).

The purpose of the study is to identify EGFR mutations in the FFPET samples of lung cancer patients as well as in the plasma sample and to demonstrate the importance of using plasma instead of a tissue sample to

show the possibility of benefiting from the blood sample in detecting these mutations in the event that it is not possible to obtain a tissue sample for any reason.

II. Materials and Methods:

Sampling:

Specimens of patients diagnosed with NSCLC (metastatic cancer and non-metastatic cancer) received from different Hospitals and Oncological Centers in Iraq for testing EGFR Mutation test in Molecular Biology Unit at Central Public Health Laboratory of Baghdad. Samples collected from August/2020 to July/2021. Patient's information of the study was obtained from all participants.

251 Formalin-Fixed Paraffin-Embedded Tissue (FFPET) and 20 plasma samples from NSCLC patients were enrolled. All FFPET specimens were histologically diagnosed and pathologically evaluated to confirm the diagnosis of NSCLC.

Five to seven sections (5 µm thick each) from qualified tissue blocks were putting in sterile Eppendorf tubes. Blood samples were collected in EDTA tubes, Centrifugation was performed at 4000 g for 5 min, and then the plasma was separated and either directly begins with extraction process or stored at -20°C.

Methods:

DNA extraction from plasma:

Cobas Plasma Cell-free DNA sample preparation Kit (Roche, Germany) was used for DNA extraction. Two milliliters (2 ml) of Plasma required for testing each sample, then the extraction procedures performed according to the manufacturer's manual.

DNA extraction from FFPE tissue sections:

FFPET specimens are processed and genomic DNA isolated using the cobas® DNA Sample Preparation Kit (Roche, Germany). Deparaffinized5µ section of an FFPET specimen is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNAase. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. The amount of genomic DNA is determined by spectrophotometer and adjusted to a fixed concentration (2 ng/µ) to be added to the amplification/detection mixture.

Amplification method

The target DNA is then amplified and detected on the cobas z 480 analyzer using the amplification and detection reagents provided in the cobas® EGFR Test. The cobas® EGFR Test is based on two major processes:

(1) Manual specimen preparation to obtain DNA from FFPET or plasma; and

(2) PCR amplification and detection of target DNA using complementary primer pairs and oligonucleotide probes labeled with fluorescent dyes. The cobas® EGFR Test is designed to detect the following mutations:

• Exon 18: G719X (G719A, G719C, and G719S)
• Exon 19: deletions and complex mutations
• Exon 20: S768I, T790M, and insertions
• Exon 21: L858R and L861Q

A mutant control and negative control are included in each run to confirm the validity of the run.

III. Results:

In this study, (214) samples of FFPET were tested for EGFR mutations and thirty seven (37) samples found to be invalid although that the test repeated more than two times. The percentage of mutations in FFPET and plasma were evaluated according to the sex, the female appeared high percentage for EGFR mutations (26.7%) when compared to males (10.2%) and the statistical analysis showed significant differences ($p < .01$) between them, while no significant differences were found between male and female when comparing the results obtained from the plasma samples (Table1).

Table (1): Frequency of EGFR mutations according to gender for FFPET samples and plasma samples in NSCLC patients

Sex	FFPET samples			Plasma samples		
	Mutations of EGFR (%)	No Mutations of EGFR (%)	Total	Mutations of EGFR (%)	No Mutations of EGFR (%)	Total
Male	13 (10.2%)	115 (89.8%)	128	1 (0.8%)	11 (92.2%)	12

Female	23 (26.7%)	63 (73.3%)	86	4 (50%)	4 (50%)	8
Total	36 (17%)	178 (83%)	214	5 (25%)	15 (75%)	20
Z value= -3.18 p value=0.00148, p < .01, significant.				Z value= -1.5936, P value=0.11184. p ›0.05, not significant		

A comparison was made between plasma and FFPET samples (Table-2). Although that the EGFR results of plasma samples were higher (25%) than the results obtained from FFPET samples (16.8%) samples, no significant differences (p>0.5) were found between them.

Table (2): comparison of EGFR mutations between FFPET and Plasma samples taken from NSCLC patients

Type of samples	Mutations of EGFR (%)	No Mutations of EGFR (%)	Invalid	Total
FFPET	36 (16.8%)	178 (83.2%)	37	251
Plasma	5 (25%)	15 (75%)	0	20
	Z test = 1.7 . The value of p is 0.1488, Not significant p › 0.05.			

EGFR Mutations results of plasma samples and FFPET samples for metastatic and non-metastatic patients were evaluated. The statistical analysis shows that there was no significant difference (p > .05) between the plasma/metastatic samples and FFPET/metastatic samples for the positive results of EGFR mutations (Table 3). There were (37) invalid FFPET samples and no results were obtained from those samples and all were excluded from the statistical analysis of this study.

Table (3): comparison of EGFR mutations results in Metastatic and non-metastatic NSCLC patients according to samples type.

Results	FFPET		Plasma	
	Metastatic	Non metastatic	Metastatic	Non metastatic
Mutations	26	10	5	0
No Mutations	105	73	14	1
Total	131	83	19	1
Invalid	21	16	0	0
The value of z is -0.6507. The value of p is 0.5157. The result is not significant at p › 0.05.				

The predominant mutations of EGFR gene in Iraqi patients were found in exon-19 which was (86%) of FFPET samples and (80%) of plasma samples, It is followed by exon 20, then exon 21 as shown in (Table 4).

Table (4): Frequency of Genotypes of EGFR Mutations among NSCLC patient for both FFPET and Plasma samples

Mutations Types	Exon	FFPET		Plasma	
		Number	(%)	Number	(%)
EX19 Del	**EX-19**	31	86%	4	80%
L858R	**EX-20**	4	11%	1	20%
S7681 & L858R	**EX-20 & 21**	1	3%	0	0
TOTAL		36	100%	5	100%

IV. Discussion:

In clinical practice, tumor tissues are insufficient for EGFR genotyping in at least 20% of advanced NSCLC patients for various reasons including insufficient availability of neoplastic tissue or lack of appropriate tumor tissue for biopsy, or that a biopsy is not technically feasible (Hiley CT et al, 2016)&(Bernabe R et al, 2017).

The results of this study shows that there were (37) FFPET sample not tested due to infeasible sample which gave invalid results. For this reason, a single biopsy could not enough to give the decision and repeat biopsy for treatment monitoring is challenging. For this reason plasma sample evaluated to used instead of the wax tissue due to the difficulty of obtaining a second tissue sample from some patients due to their poor health. The second sample is requested when the first sample was not possible to obtain results due to that it was too tiny or it was bad and could not give the desired results. In addition to that, the DNA extract from paraffin-embedded tissues varies widely in quality. Unfortunately, the process of FFPE sample causes fragmentation and chemical modification in DNA. Such modifications result lead to loss of quality and number of amplifiable DNA templates and pose significant challenges to PCR sensitivity and specificity. DNA quantitation may also impact PCR efficiency (Dushyant Kumar et al, 2016).

Detecting EGFR in plasma has been studied in several areas for molecular analysis in cancer patient most studies are consist with the results of this study (MichaelOellerich et al, 2019), (Diaz LA et al, 2014) & (Murtaza M et al 2013).

Although that the percentages of positive results of EGFR mutations in plasma samples (25%) were higher than that found in FFPET (17%), the statistical analysis showed no significant differences when compared the results obtained from FFPET with the results of plasma. It's may be due to that the sample size of plasma cases was too low and these finding mean that the plasma samples may be used instead of FFPET for monitoring the progress of the disease among NSCLC patients. Furthermore, the plasma samples is needed because the tumor genome often changes over time in response to therapy, so the repetitive Real Time PCR testing of the tumor is required for drug resistance mutations in patients with advanced NSCLC (K.S. Thress et al, 2015). For this reason, detecting mutations in plasma is very necessary because it is not possible to follow up developments unless we get a sample of the patient and it is illogical to use a tissue sample constantly so it is necessary to replace this sample with another easy sample such as plasma to follow up.

The relationship between metastases and EGFR mutation status was investigated in this study. The above results indicated that plasma samples in metastatic cases can be used instead of FFPET. It's because that the percentages of positive results from Metastatic/plasma were higher than that obtained from Metastatic/FFPET and these finding is compatible with Qiao-meiGuo et al, 2019. The number of plasma samples was very small compared to the number of FFPET samples, and the size of the samples may be the reason for the negative results of the statistical analysis when comparing the above two groups.

Additionally, when EGFR independent resistance mechanisms occur alternative sequencing methods of circulating tumor DNA (ctDNA) are required, especially when use immunotherapy (S.M. Lim et al, 2015). It has been shown that EGFR mutation testing in plasma is essential for clinical decisions, or adjustment of treatment and tracking resistance. In addition to that using plasma samples is very important where biopsy material is inadequate or unavailable or when it is difficult to take a biopsy from the patient due to his poor health condition. (Michael Oellerich et al, 2019).

The frequency of EGFR mutation was higher in females and this result is compatible with (Tomoaki Tanaka et al, 2010 and Xiuzhi Zhou et al , 2018). Another studies showed different results, which appeared high percentage of mutations in males compared females (Ebru Derici Eker et al, 2019), (Hayfa H et al, 2014) & (Hanan et al, 2021). This difference in these results may be due to the difference in geographical distribution of the individuals under study, or it may be due to different races and origins, in addition to their influence by the external environment.

Regarding to EGFR genotypes, the predominant types of EGFR mutations in Iraq were EX19Del followed by the L858R mutation and these results are consistent with several previous research conducted in Iraq (Sanaa Alizi et al, 2022) & (Hanan et al , 2021). About (3%) of multiple mutations were founded in (Exon 20 and 21 combined together) and it is differ from that founded by Hanan et al research in 2021 which were found the combination in (Exon 20 and 19).

As above, we find differences in results in the few studies conducted on EGFR gene, and there were clear differences in the frequency of mutations and genetic types, as well as in the percentage of their presence in females and males. Conducting a large study with large numbers of samples is required to confirm the importance of using plasma sample for monitoring lung cancer patients, as well as to determine predominant genotype of EGFR mutations in Iraq.

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