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RESEARCH PAPER

Novel Molecular Insights of the miRNA's Gene and Its Relation to Carcinogenesis EGFR Mutated Gene in Non-Small Cell Lung Cancer (NSCLC) in Kurdistan Region – Iraq

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A B S T R A C T:

Non-small cell lung cancer is the 2nd major cause of cancer-related deaths in Iraq and all over the world due to its delayed diagnosis. Due to its multifactorial nature, studies are underway to identify novel molecular targets which can serve as potential biomarkers for diagnosis and treatment to reduce the disease burden in the Kurdistan region of Iraq. Thus, the research study aimed to evaluate the association of *epidemic growth factor receptor (EGFR) gene* mutation with *microRNA-146a gene* polymorphism in non-small cell lung cancer development. For this purpose, a histochemical study was performed on lung cancer biopsy samples. EGFR, which was recently identified as a critical molecule biomarker in the investigation of lung cancer, was the subject of a novel therapeutic approach based on molecular analysis. *EGFR* and *microRNA-146a genes* were amplified by the amplification refractory mutation system (ARMS)-PCR and allele-specific polymerase chain reaction (AS-PCR), respectively. The EGFR exons with the highest percentage of mutations were EGFR20, which was present in 64 (91.42 %) of the patients, or 70 (50 males and 10 females), and EGFR21, which was present in 59 (84.28 %) patients (47 males and 8 females). While only 35 (50%) patients confirmed positive for mutated EGFR19, 41 (58.57%) patients had mutated EGFR18. Males constituted the majority of the mutant EGFRs. The GC genotype of miRNA-146a was found to be slightly higher in cancer patients with odd ratio=4.8 (confidence interval= 0.75-55.6, *p-value*=0.2532) as compared to healthy controls. More susceptible to get lung cancer between EGFR mutations and miRNA-146a rs2910164 C>G nucleotide polymorphism was observed in this study. Thus, miRNA-146a could act as a potential target for treatment.

KEYWORDS: Lung Cancer; NSCLC; Polymorphism; miRNA-146a gene; EGFR gene. AS-PCR, ARMS-PCR.
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1.INTRODUCTION :

Lung cancer (LC) is considered the second most prevalent cancer worldwide (Pasello et al., 2023). Around 2.21 million annual incidences of lung cancer are reported globally which makes up almost 11.4% of all cancer cases. Globally, the most deaths caused by any cancer reported in GLOBOCAN 2020 are due to lung cancer which is estimated at almost 1.79 million or even more annually. Recent data also showed that the rate of death caused by cancer is highest in Asian countries (Chhikara and Parang, 2023).

According to the most recent Iraqi Cancer Registry (ICR), 31,502 new cases of cancer were reported in 2018, with an incidence rate of 82.6 cases per 100,000 people.

43% of these cases were male, while 57% were female. The top three malignancies in males were leukemia (7.8%), urinary bladder (8.6%), and bronchus and lung (13.4%, with an incidence of 9.5 cases per 100,000 male population). The most frequent causes of malignancy-related mortality in Iraq were leukemia (12.1%), breast (12.3%), and bronchial and pulmonary cancers (18.9%) (Al Alwan, 2022). The prevalence rates of all types of cancer spiked in the Kurdistan region between 2013 to 2019 compared to other areas of Iraq (Hussain and Lafta, 2021). Lung, breast, and blood-related cancers are the most common 3 cancer types found in the Kurdistan region, based on information regarding the prevalence of cancer in the region. In Erbil and Duhok cities, the most prevalent form of cancer in men is lung cancer. According to early reports, there were 50.0 and 61.5 cancer cases per 100,000

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people in Erbil and Duhok, respectively. On the contrary, in the Sulaymaniyah city, the incidence rate was found to increase from 38.5 to 71.7 cases per 100,000 people between 2006-2013 (M-amen et al., 2022).

Depending on the originating cell type, lung cancer is classified as small-cell lung cancer (SCLC) or non-small-cell lung cancer (NSCLC) (Nawaz, et al. 2023). About 80-85% of lung cancer cases are due to NSCLC while almost 15-20% of lung cancer cases are due to SCLC (Petrek and Yu, 2019). NSCLC further constitutes three major subtypes of lung cancer based on histology. These are large cell carcinoma (LCC) (almost 15%), squamous cell carcinoma (SCC) (almost 30%), and adenocarcinoma (ADC) (about 40-50-%) (Shafat et al., 2022).

NSCLC incidence and progression are impacted by both environmental and genetic risk factors. Smoking is one of the most important environmental risk factors for NSCLC (Oliver, 2022, O'Keeffe et al., 2018). Some other environmental risk factors are family history, secondhand smoke, air pollution, human immunodeficiency viral infection, and radiation exposure (Kirk et al., 2007, Akhtar and Bansal, 2017). Several molecular determinants or genetic mutations associated with NSCLC tumors have been identified which serve as the basis for the diagnosis, prediction, and prognosis, and also to use as targets for drugs and treatments. In almost 50% of the NSCLC cases, major target mutations were observed in the *TP53* gene (Consortium et al., 2017). As a member of the ErbB receptor tyrosine kinase (TK) family, the epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein receptor with tyrosine kinase activity. The *EGFR gene*, which spans around 200 kb, contains 28 exons, and encodes a protein with 1210 amino acids, is found at the short arm of chromosome 7 (7p11.2) (Hodoglugil et al., 2013). Cell survival, proliferation, migration, and metastasis are induced by the activation of the EGFR with its particular ligands. This activation also causes receptor dimerization and tyrosine autophosphorylation (Boustany et al., 2022). EGFR activation has frequently been linked to the onset and progression of NSCLC, *EGFR* is a proto-oncogene that makes up one of four tyrosine kinase receptors (Sharma et al., 2007). As EGFR overexpression was associated with a poor prognosis in a variety of cancer forms, such as NSCLC, it has been utilized as a prognostic marker for many years. However, there is conflict over whether or not *EGFR* overexpression can be used as a prognostic marker (Fujino et al., 1996). The activation and overexpression of this mechanism might end up in aberrant gene expression, which may also ultimately cause lung cell malignancy (Pradhan et al., 2019). Around 10-35% of lung cancer cases are due to mutations in a tyrosine kinase receptor known as *EGFR*. While the epidemiological study carried out in Iraq to ascertain the prevalence of EGFR mutations among NSCLC patients reported a 27.53% incidence rate, this contrasts the lower rates in Western countries with the greater prevalence rate among Asian people (Ramadhan et al., 2021). These mutations result in the dysfunctionality of MAPK (Mitogen-activated protein kinases) signaling and AKT (Protein kinase B) because they improve cell survival rate and increase cell proliferation (Massarelli et al., 2013). About 90% of the EGFR mutations are due to single nucleotide polymorphisms of L858R in exons 21, while others are due to of amino acids LREA of exon 19 (Petrek and Yu, 2019).

The binding of the ligands of EGFR stimulates abnormal cell division and propagation due to somatic mutations (Benbrahim et al., 2018). These EGFR mutations are more common in ADC patients as compared to SCC patients due to which these receptors can serve as a potential drug target.

Despite all the recent research and treatment options, the diagnosis of NSCLC still occurs at its late stage which results in very low the survival rate of patients with NSCLC (Inamura and Ishikawa, 2016). The development of potential treatment approaches and novel diagnostic biomarkers is crucial in increasing the survival rate and proper diagnosis at early stages. Frydrychowicz, et al. (2023) stated the role of miRNA in carcinogenesis and identified it as a predictor of the onset and progression of cancer, a potential marker of treatment effectiveness, and ultimately as an achievable therapeutic target.

The microRNAs (miRNAs) are short nucleotide sequences of almost 22 nucleotides which consist of noncoding RNA sequences responsible for the regulation of almost half of the human genes. They are responsible for various biological activities like cell differentiation, proliferation, migration, disease initiation, disease progression, and eventually apoptosis (Barbato et al., 2017). MiRNA works by modulating the gene

function at the posttranscriptional level. Various studies have proposed that miRNAs can act as potential diagnostic biomarkers for NSCLC (Zhan et al., 2016, Berghmans et al., 2013). It has been observed that miRNA-146a plays a crucial role in regulating the activity of various significant genes which are involved in the progression of lung cancer. These genes are *interleukin (IL)-1 receptor-associated kinase (IRAK1), EGFR, tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6),* and *nuclear factor kB (NFkB),* etc. (Qiu et al., 2021, Mohamed et al., 2019).

Mutations in mature miRNAs, such as single nucleotide polymorphisms (SNPs), can alter their functioning by promoting cancer growth. Numerous studies indicate evidence of a link between miRNA polymorphisms and the development of tumors like lung cancer (Iacona et al., 2018). For example, it has been reported that polymorphism in miRNA-146a rs2910164 G>C is linked with numerous malignancies such as cervical, breast, gastric, thyroid, and ovarian cancers (Wilczyński et al., 2017, Khan et al., 2022, Qiu et al., 2011). Various researchers have also reported that in diverse populations, miRNA-146a rs2910164 polymorphism plays a role in lung cancer progression (Wani et al., 2021, Tan et al., 2018, Xiao et al., 2018). Though, the susceptibility of NSCLC by genetic variants of miRNA-146a in the Kurdistan region of Iraq is yet to be discovered. In order to assess if miR-146a is associated with EGFR mutations in NSCLC patients, the current study aims to ascertain and find out the distribution of miR-146a polymorphism and its relationship to the *EGFR gene* for the development of NSCLC in the Kurdistan community.

2. MATERIAL AND METHODOLOGY

2.1 Sample Collection:

In the current study, 70 patients with advanced NSCLC were recruited from PAR Hospital in Erbil, Kurdistan Region, Iraq, between December 2021 and May 2022. They were performed for *EGFR exons gene* mutations and *miRNA146a gene* polymorphism by molecular PCR analysis, and their average age ranged from 65 (25-94) years. Additionally, on 15th January 2023 (Reference number: 45/265), the proposal was approved and authorized by the ethical committee of the Biology Department of the College of Science at Salahaddin University-Erbil (SUE) and included patients with histopathological and immunohistochemistry confirmed NSCLC lung cancer and. The all methods were carried out in accordance with the approved guidelines.

2.2 Instruments and Equipment:

EGFR exons gene mutations and *miRNA146a gene* polymorphism were analyzed and identified with the PCR thermal cycler machine (Applied Biosystems Veriti® Company, USA), Nanodrop spectrophotometer (Thermo Fisher Scientific, USA), and Gel Electrophoresis System apparatus (Cleaver Scientific, UK).

2.3 Genotype Analysis:

Genomic DNA was extracted from tissue of each participant by following manufacturer's protocol of DNA FFPE Tissue Kit (Solar Bio, China) (Yi et al., 2020). The quantity and quality of the extracted DNA was measured by using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, United States) in ng/µl unit and Gel Electrophoresis System apparatus (Cleaver Scientific, UK), respectively. The ratio of absorbance at 260 nm/280 nm was used to determine the purity of DNA (Talukdar et al., 2022)

2.4 Genotyping and Polymerase Chain Reaction (PCR) Amplification:

The target genes, *EGFR and miRNA-146a*, were amplified by the amplification refractory mutation system (ARMS)-PCR and the allele-specific polymerase chain reaction (AS-PCR), respectively. Two primers were used to assess the *EGFR gene* for each exons included Exon18, Exon19, Exon20, and Exon21(Zheng et al., 2020) and four primers were used to determine the rs2910164 C>G polymorphism in the *miRNA-146a gene* (Javid et al., 2019). Tables 1 and 2 indicate the primer sequences for the two genes.

Genotyping was performed in a 25 µl reaction mixture. The reaction mixture for *EGFR gene* amplification contained 1.5 µl (10 pmole) of both reverse and forward primers, 12.5 µl (1X) master mixture, 8 µl nuclease-free water, and 3 µl of genomic DNA. The reaction mixture for the *miRNA-146 gene* comprised 1 µl of each (miRNA-146a FC, miRNA-146a RC, miRNA-146a FG, miRNA-146a RG),13 µl master mix (1 X), 6 µl *nuclease*-free water and 2 µl (25-50 ng) genomic DNA extract. The profile of the thermal cycler for both genes was set as described in Tables 3 and 4 (Zheng et al., 2020, Javid et al.,

2019). The polymerase chain reaction products were analyzed by UV trans-illuminator (UVP, USA) on 2% agarose gel (Green and Sambrook, 2019).

The *EGFR* and *miRNA146-a exons'* amplified PCR products were separated using horizontal gel electrophoresis on a 2% agarose gel as illustrated in Figure 1, 2, 3, and 4. Two grams of agarose and one hundred milliliters of 1X TBE solution were added to the gel. The DNA banding patterns (DNA polymorphisms) were seen using UV Transillumination (UVP) at wave lengths of 240 and 366 nm after staining the gel with DNA dye safe stain. The gel was lit from below by placing it on the transilluminator glass and the 100bp DNA Ladder (Norgen Biotech, Canada) was used as a molecular marker (Russell and Sambrook, 2001, Chong, 2001).

The PCR products for *EGFR gene* mutation were expressed as (Exon 18, Exon 19, Exon 20, Exon 21) and for genotypes of *miRNA146a gene* polymorphism. The genotypes of the *miRNA146a gene* polymorphism were designated as CC (homozygous) as a dominant while depending on the development of bands when primers specific for either allele G (as GG) or allele C (as CC) were used. When bands were seen with both primers, the samples were categorized as heterozygotes (GC).

Table 1. List of primers for *EGFR gene* amplification.

Target Gene	Primer Sequence	Annealing temperature (˚C)	PCR product size (bp)	Reference
EGFR-18F	5'-TGAGGATCGAAGGAAACT-3'	57	159	(Zheng et al., 2020)
EGFR-18R	5'-TCCCACCAGACCATGAGA-3'	57	159	
EGFR-19F	5'-CCCCAGCAATATCAGCCTTA-3'	57	447	
EGFR-19R	5'-TGCCTGTTTCCAGCCTTTTA-3'	57	447	
EGFR-20F	5'-CCTCCTCCAGGAAGCCTAC-3'	57	191	
EGFR-20R	5'-CGATCTGCACACACCAGTTG-3’	57	191	
EGFR-21F	5'-GGATGCAGAGCTTCTTCCCAT-3'	57	187	
EGFR-21R	5'-ATCTTGACAGCTGCGGTGT-3'	57	187	

Table 2. List of primers for *miRNA-146a gene* amplification.

mir146a Gene	Primer Sequence	Annealing temperature (˚C)	PCR product size (bp)	Reference
CC F1 Genotypes	5'-ATGGGTTGTGTCAGTGTCAGACGTC-3'	62	249	(Javid et al., 2019)
CC R1 Genotypes	5'-ATACCTTCAGAGCCTGAGACTCTGCC-3'	62	249	
GG F2 Genotypes	5'-GGCCTGGTCTCCTCCAGATGTTTAT-3'	62	168	
GG R2 Genotypes	5'-GATATCCCAGCTGAAGAACTGAATTTGAC-3'	62	168	

Table 3. The conditions of AS-PCR for *EGFR gene* amplification.

Steps	Temperature (˚C)	Time
Initial denaturation	95	3 minutes
Denaturation	94	30 seconds
Annealing	57	30 minutes
Extension	72	45 seconds
Final extension	72	5 minutes
No. of cycles	40	

2.5 Statistical Data Analysis:

For statistical analysis, GraphPad Prism 9 was used. Age and tumor size were represented in median (maximum-minimum) as they are non-parametric data. Fisher's exact test was used to find the Odds ratio and association of *EGFR exons' gene* and *miRNA146a* genotypes with lung cancer. Kruskal-Wallis's test followed by Dunn's multiple comparisons test and Mann Whitney test was used to compare the numerical variables among the patient groups. In addition, Chi-square was used to compare the categorical variables among the patient groups. Certainly, in statistical analysis, the odds ratio (OR) measures the strength and direction of the association between two variables. An odds ratio greater than 1 indicates a positive association, meaning that the presence of one variable increases the odds of the other variable being present. On the other hand, an odds ratio less than 1 indicates a negative association. The *p-value* is a measure of statistical significance and helps determine if the observed association is statistically significant or could be due to chance. Typically, *a p-value* threshold of 0.05 is commonly used to determine statistical significance.

3. RESULTS

The present study was conducted to evaluate the association of *miRNA-146a* gene with *EGFR gene* mutations and their role in NSCLC progression. A total of 70 NSCLS patients were recruited for this study. In the current study as shown in Figure 5 and Table 5, the male-to-female ratio was 4.8:1. The median age of the patients was 65 (25-94), comprising 58 (82.8%) males and 12 (17.1%) females. The tumor size was 1 (0.02-10.2) centimeters (cm). The most common type of lung cancer was ADC (33 patients) 47% and SCC (33 patients) 47% while only 4 patients (6%) were found to have LCC. The most positive immunomarkers in the lung cancer patients were TTF-1 and Ck7, while only 4 patients were positive for Napsin-A.

The Figure 1, 2, and 3 as well as the Table 6 depicted the frequency of mutated and non-mutated EGFR exons in lung cancer patients. The most mutated EGFR exons were EGFR20 that were present in 64 (91.42 %) of the patients *i.e.*, 70 (50 males and 10 females) followed by EGFR21 with 59 (84.28 %) patients (47 males and 8 females). While 41 (58.57%) patients had mutated EGFR18 and only 35 (50%) patients were positive for mutated EGFR19. The majority of mutated EGFRs were males. The frequency of mutated EGFR18 was least (50%). It was also observed that the frequency of mutated EGFR was higher in male patients as compared to female patients. This study found no significant but slight association of the miRNA-146a genotype with all types of mutated EGFR which indicated that an association was present between miRNA-146a and NSCLC onset in the population of the Kurdistan region of Iraq.

However, the *miRNA-146a* (rs2910164 C>G) polymorphism findings demonstrated (as shown in Figure 4) that more likely to get and effect on susceptibility lung cancer disease but significantly not highly related (Table 7), due to modest scales of number of patients receiving lung cancer. The GC and CC were significant risk factors with an odds ratio (OR) of 4.8 and 1.014, respectively. In the dominant model, 80.43% of the patients were GC+CC genotype and 19.56% were GG. The GC+CC showed a non-significant risk factor. In addition, the CC in the Recessive model was non-significantly increased the probability of lung cancer development. Besides, the frequency of the alleles of miR-146a was 47.82% for the C allele in patients and 64% in

healthy participants. Moreover, the presence of the C allele could contribute a nearly significant association (*p-value*= 0.0789) in lung cancer patients.

Furthermore, the association of miRNA-146a-genotypes with the patient's characteristics is shown in Table 8 in which GC genotype was higher in both males with 23 patients and females with only 7 patients. While CC genotype has a nearly significant association (*p-value*0.658) with increasing tumor size in which median tumor size was 5 (1.3-9) cm. Moreover, GC genotypes for ADC is 19% while for SCC is 8% which is non-significantly associated with these two types of lung cancer.

Not significant but slight association between all EGFR exons and gender was found as shown in Tables 9 and 10. Regarding tumor size, the tumor size in mutated and non-mutated EGFR 18 was nearly equal which were 1 (0.3-10.2) cm and 1 (0.02-9) cm, respectively, this is also the same for EGFR 19 with a median tumor size of 1 (0.3-9) cm and 1 (0.02-102) cm for mutated and non-mutated respectively. In EGFR 20 and EGFR 21 (table 10), all are mutated with the same median tumor size of 1 (0.4-10.2) cm. In addition, a slight association of mutated EGFR exons with histological types was found. High frequency of mutated EGFR 18 and EGFR 19 were observed in patients with ADC type of NSCLC which were 23 and 17 patients, respectively. Furthermore, mutated EGFR 20 and EGFR 21 were found to be more prevalent in patients with ADC and SCC type. It was observed that 34 patients with ADC type have mutated EGFR 20 and 21, while 32 and 31 cases with SCC had mutated EGFR 20 and EGFR 21, respectively.

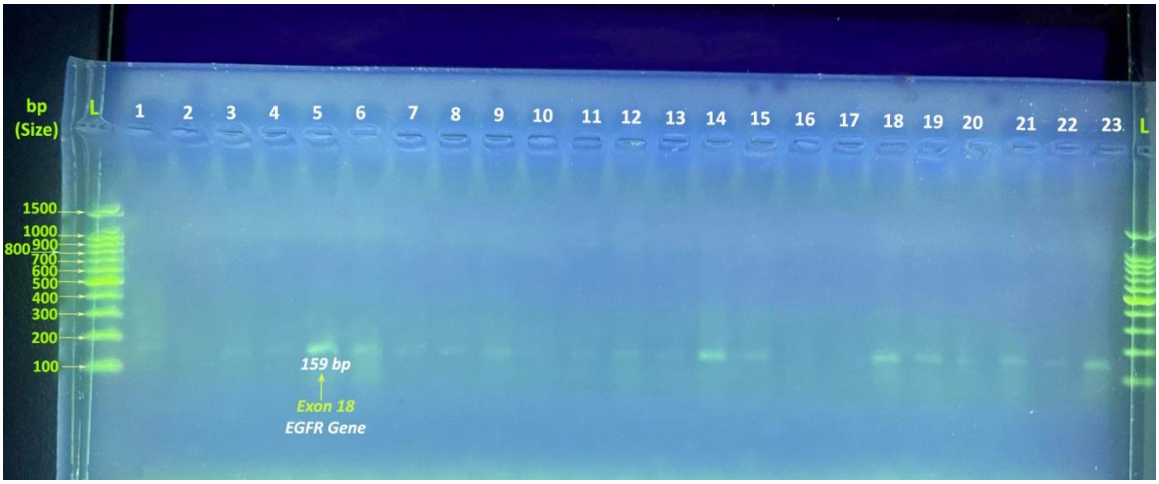

Figure 1: Agarose gel electrophoresis (2%) exhibits 159 bp ARMS-PCR products corresponding to amplification of *EGFR Exon 18 gene* in lung cancer patients. Lane L: 100 bp DNA ladder. Lanes 1, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22 and Lane 23 exhibit positive PCR DNA amplicons sizes (159 bp), which correlate to individuals with lung cancer disease when used with the designated specific primers for *exon 18 of EGFR gene*. While the lanes 2, 10, 16 and lane 17 correspond to lung cancer patients and represent negative PCR results (no amplification).

Table 4. The conditions of AS-PCR for of *miRNA-146a gene* (rs2910164 G>C).

Steps	Temperature (˚C)	Time
Initial denaturation	95	10 minutes
Denaturation	95	45 seconds
Annealing	62	45 minutes
Extension	72	45 seconds
Final extension	72	10 minutes
No. of cycles	40	

Table 5. Characteristics of NSCLC patients.

Variables	Lung Cancer Cases (*n*)
Gender: Male/Female	58/12
Age:	65 (25-94)

Histological type (NSLC):	LC Cases (*n*)	Male	Female
ADC	33	26	7
SCC	33	30	3
LCS	4	2	2
Tumor size (cm):	1 (0.02-10.2)		

Immunohistochemistry:	Positive	M	F	Negative	M	F
Ck5/6	6	5	1	8	6	2
P63	8	6	2	8	6	2
TTF-1	11	8	3	16	13	3
Napsin-A	4	2	2	9	7	2
Ck7	9	7	2	8	6	2
P40	6	5	1	3	2	1
High molecular weight Ck	8	6	2	1	1	0+

Age and tumor size are represented as median (maximum-minimum).

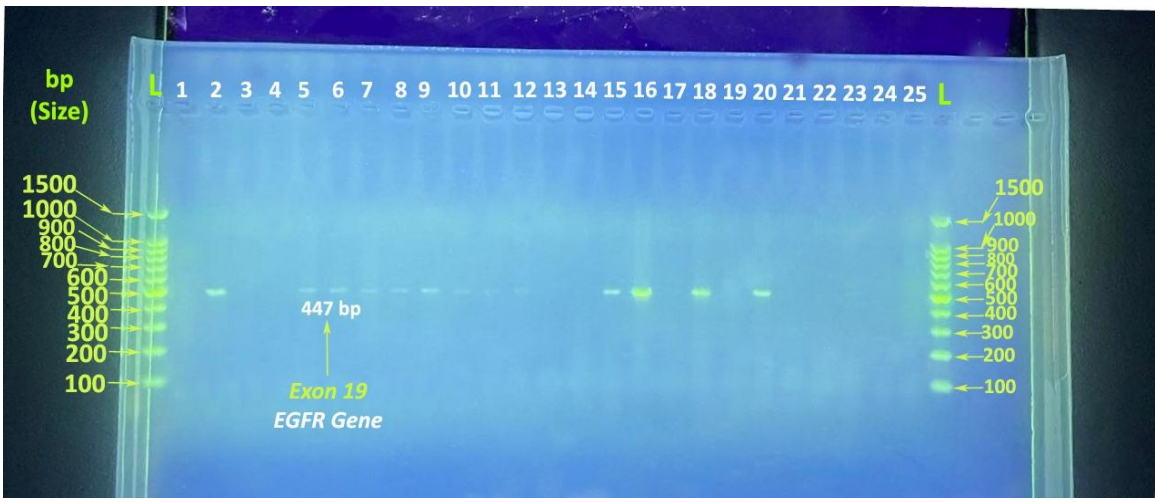

Figure 2: Agarose gel electrophoresis (2%) reveals 447 bp ARMS-PCR products, which are associated with amplification of *EGFR Exon 19 gene* in lung cancer patients. Lane L: 100 bp DNA ladder. Lanes 2, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18 and Lane 23 show positive PCR DNA amplicons sizes (447 bp), which correlate to individuals with lung cancer disease when used with the selected specific primers for *exon 19 of EGFR gene*. While the lanes 1, 3, 4, 13, 14, 19, 21, 22, 24 and lane 25 correspond to lung cancer patients and represent negative PCR results (no amplification).

Table 6. The frequency of the mutated and non-mutated EGFR exons among NSCLC patients**.**

Positive (Mutated Type)		Patients (n=70)	Male	Female
EGFR18	Mutated	41 (58.57 %)	34	7
	Non-Mutated	29 (41.42 %)	23	6
EGFR19	Mutated	35 (50 %)	27	8
	Non-Mutated	35 (50 %)	30	5
EGFR20	Mutated	64 (91.42 %)	50	10
	Non-Mutated	6 (8.57%)	7	3
EGFR21	Mutated	59 (84.28 %)	47	8
	Non-Mutated	11 (15.71 %)	10	5

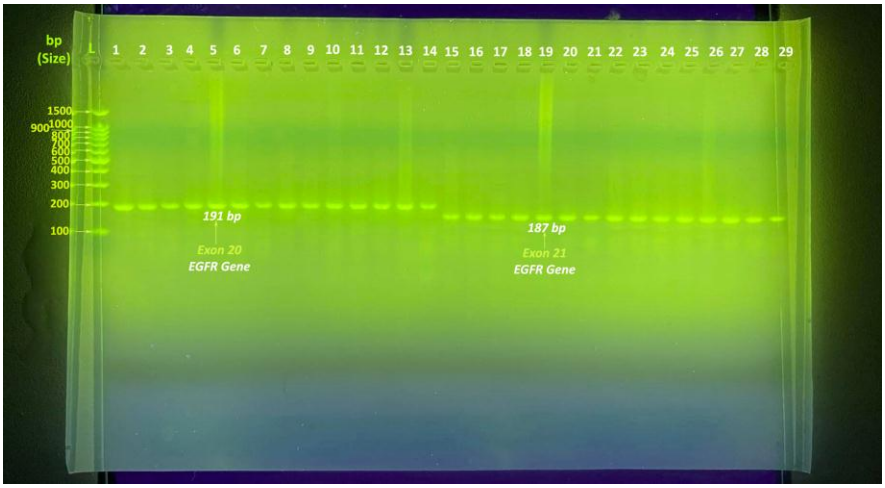

Figure 3: Agarose gel electrophoresis (2%) illustrats 191 bp and 187 ARMS-PCR products, which are associated with amplification of the *EGFR Exon 20 gene* and *EGFR Exon 21 gene* in lung cancer patients, respectively. Lane L: 100 bp DNA ladder. When the designated specific primers for the *EGFR Exon 20 gene* and *EGFR Exon 21 gene*, respectively, were used, the lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and lane 14 display positive PCR DNA amplicons sizes (191 bp) while the lanes 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and lane 29 demonstrate positive PCR DNA amplicons sizes (187 bp), which correlate to individuals with lung cancer disease.

Table. 7. The genotype and allele frequency of *miRNA146a rs2910164G>C gene* polymorphism

miRNA146a-Genotypes	Patients (n=46)	Healthy Controls (n=25)	Odd Ratio (OR) (CI)	*p-value*
Codominant				
GG (Wild Type)	9(19.56%)	1(4%)	Ref.	
GC (Heterozygous)	30(65.22%)	16(64%)	4.8 (0.75-55.6)	0.2532 ns
CC (Homozygous)	7(15.22%)	8(32%)	1.014 (0.144-12.4)	>0.9999 ns
Dominant				
GG	9(19.56%)	1(4%)	-	-
GC+CC	37(80.43%)	24(96%)	5.838 (0.801-66.43)	0.0869 ns
Recessive				
GG+GC	39(84.78%)	17(68%)	-	-
CC	7(15.22%)	8(32%)	2.622 (0.88-8.1)	0.1305 ns
G allele	48(52.17%)	18(36%)	-	-
C allele	44(47.82%)	32(64%)	1.939 (0.968-3.84)	0.0789 ns

Fisher's exact test

Table 8. The association of miRNA146a-Genotypes with tumor characteristics

Variables	MiRNA146a-Genotypes			*p-values*
	GG (%)	GC (%)	CC (%)	
Gender				
Male	8	23	5	0.658 ns
Female	1	7	2	
Tumor size (cm)	0.6 (0.04-8)	1 (0.02-10.2)	5 (1.3-9)	0.0293 * [a]
Histological Type (NSLC)				
ADC	3	19	4	5.472 ns
SCC	6	8	2	
LCS	0	3	1	

a Kruskal-Wallis's test followed by Dunn's multiple comparisons test
Chi-square Tumor size are represented as median (maximum-minimum).

Table 9: The association of EGFR 18 and 19 with tumor characteristics

Variables	EGFR 18		OR	*p-values*	EGFR 19		OR	*p-values*
	Mutated Type	Non-Mutated Type			Mutated Type	Non-Mutated Type		
Gender								
Male	34	23	1.26	0.76	27	30	0.56	0.54
Female	7	6			8	5		
Tumor size	1 (0.3-10.2)	1 (0.02-9)		0.64 [a]	1 (0.3-9)	1 (0.02-102)		0.35 [a]
Histological Type (NSCLC)								
ADC	23	10		0.203	17	16		0.521
SCC	16	17			15	18		
LCS	2	2			3	1		

a Mann Whitney test, Chi-square test, **OR** Odd Ratio

Table 10: The association of EGFR 20 and 21 with tumor characteristics

Variables	EGFR 20		OR	*p-values*	EGFR 21		OR	*p-values*
	Mutated Type	Non-Mutated Type			Mutated Type	Non-Mutated Type		
Gender								
Male	57	0	4.25	1	56	1	1.39	1
Female	13	0			13	0		
Tumor size	1 (0.4-10.2)	-		-	1 (0.04-10.2)	-		-
Histological Type (NSCLC)								
ADC	34	0			34	0		0.547
SCC	32	0			31	1		
LCS	4	0			4	0		

Table.11: The association of *miRNA 146a* genotypes and *EGFR exons* together with lung cancer

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miRNA 146a	***Exons of EGFR***			
	Mutated	Non-Mutated	OR	*p-values*
	EGFR18			
GC and CC (Mutated Type)	6	2	1.75	0.6942 ns
GG (Wild Type)	24	14		
	EGFR19		OR	*p-values*
GC and CC (Mutated Type)	18	6	1.400	0.6079 ns
GG (Wild Type)	15	7		
	EGFR20		OR	*p-values*
GC and CC (Mutated Type)	30	7	2.143	0.3471 ns
GG (Wild Type)	6	3		
	EGFR21		OR	*p-Values*
GC and CC (Mutated Type)	33	6	4.125	0.0916 ns
GG (Wild Type)	4	3		

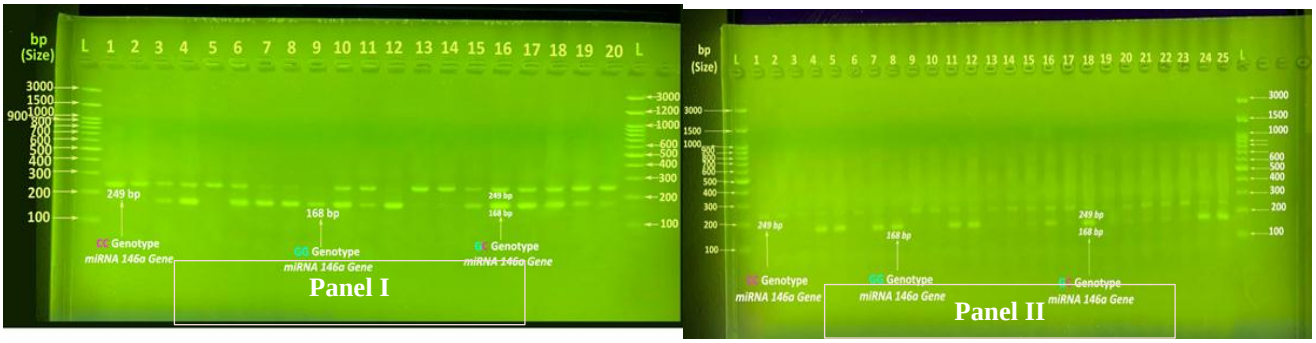

Figure 4: Illustrates 2% agarose gel electrophoresis for PCR products of *miRNA146a rs2910164G>C gene polymorphism* by the allele-specific polymerase chain reaction (AS-PCR) method:
Panel I for C allele and for G allele of lung cancer (NSCLC) patients, while **Panel II** for C allele and for G allele of normal person (healthy). Genotypes were typed as CC and GG depending on the development of **249 bp** and **168 bp** bands, respectively, when allele-specific primers for or *allele C* (as CC) or *allele G* (as GG) were used. Samples were categorized as heterozygotes (GA) when bands were detected with both the primers. Lane L represent DNA Ladder. The lane **1** is ***CC genotype***, lane ***9*** is ***GG genotype***, lane **16** is ***GC genotype*** were shown in the **Panel I** figure 4.

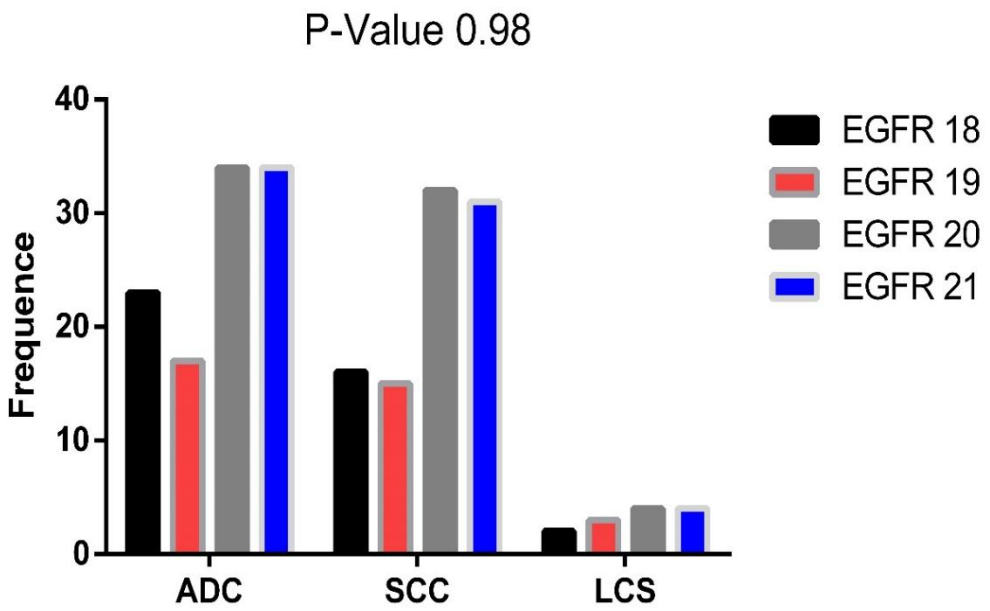

Figure 5. The comparison of the *EGFR gene exons* according to the histological type.

4. DISCUSSION

In the Kurdistan region of Iraq, the lung cancer continues to be a major cause of morbidity and mortality of all other cancers, with an increasing incidence in recent years. In Iraq, lung cancer account for 11.8% overall mortality rate across all malignant cancers, and 80% of lung cancer cases have NSCLC (Aldujaily et al., 2020) and lung cancer is the top most common type of cancer diagnosed in Kurdistan region as compared to other regions of Iraq (M-amen et al., 2022). The most commonly used treatment regime for NSCLC is chemotherapy, but it does not greatly increase the survival rates. EGFR-TKIs are currently the most successful molecularly targeted therapy for NSCLC that may enhance patients' overall health. NSCLC Patients with *EGFR gene* mutations can benefit significantly from EGFR-TKIs, but patients without mutations have not shown effective outcomes. Thus, prior to using an EGFR-TKI in lung cancer patients, the genetic profiling of the *EGFR gene* of patients should be done (Petrek and Yu, 2019). miRNAs are emerging as novel biological markers for several types of cancer detection. Studies have reported that miRNA can alter or suppress the functions of several coding and cancer-related genes including *EGFR*. miRNA has been found to play an important role in the EGFR signaling pathway. Dysregulation of EGFR signaling can lead to the development of lung cancer (Javid et al., 2019). Thus, miRNAs can act as oncomiRs *i.e.*, involved in oncogenesis or tumor suppressors by regulation of the signaling pathway of EGFR. However, NSCLC showed both a reduced expression of tumor-suppressive miRNAs and increased production of oncogenic miRNAs (Inamura and Ishikawa, 2016).

In the current study, these *EGFR gene exons* and *miRNA genes* were found to be positively correlated in lung cancer patients based on the correlation of miRNA-146a genotypes with all EGFR exons. The findings of the correlation between all EGFR exons and miRNA-146a genotypes in lung cancer patients indicated a positive relationship between these *EGFR gene exons and miRNA genes*. Thus, the occurrence of the mutated EGFR exons in lung cancer patients with any of the genotypes has affected but only a slight association with lung cancer was found.

In the present study, the odds ratio for the association between the *EGFR gene* exons and *miRNA gene* in lung cancer patients is greater than 1. It means that there was a potential positive relationship between these genes and lung cancer. In other words, it exhibited that there might be a positive relationship between *EGFR* and *miRNA genes*, suggesting that increased expression or activity of *EGFR gene* is associated with altered expression or regulation of *miRNA genes* in lung cancer. This suggests that these genes might play

a role in the development or progression of lung cancer.

However, the *p-value* being more than 0.05 indicated that the observed association between the *EGFR gene* and *miRNA gene* might not be statistically strong significant at the conventional p-value threshold of 0.05 and could potentially be due to chance (random variation in the data) or might not represent a true association between the *EGFR* and *miRNA146 genes* and lung cancer patients.

However, previous studies have reported a significant association of miRNA-146a with lung cancer. Javid et al. (2019) in their study reported that the risk of lung cancer onset is correlated with a mutation in the *miRNA-146a gene* (Javid et al., 2019). According to the results of this study, in comparison to healthy individuals, lung cancer patients had a considerably greater percentage of the miRNA-146a CC homozygous genotype. The miRNA-146a CC genotype was found to be associated with a 4.3 times higher chance of lung cancer development. The frequency of the miRNA-146a C allele was higher in all lung cancer stages *i.e*., I-IV and histological grades. Also, the frequency of the C allele was much higher in SCC patients as compared to ADC patients. In another study, pre-miRNA-146a C to G polymorphism (rs2910164) was observed to be associated with the risk of developing lung cancer in the Korean population (Jeon et al., 2014). Additionally, the expression level of miRNA-146a has been demonstrated to be downregulated in lung cancers. Also, in lung cancer cell lines including H358, H1650, H1975, HCC827, and H292 miR-146a were found to be overexpressed that causing the suppression of the EGFR downstream signaling pathways and decreasing cell proliferation and migration. Notably, induced expression of miRNA-146a may improve the potential of monoclonal antibody therapies such as cetuximab and EGFR-TKIs including gefitinib, erlotinib, and afatinib to suppress the proliferation of cells by targeting the EGFR and NF-kB signaling pathways (Chen et al., 2013).

Moreover, conflicting results were found in investigations of NSCLC. The function of miRNA-146a appears to vary depending on the cancer type. The risk of breast cancer was shown to be indirectly linked with the miR146 mutant allele (Alshatwi et al., 2012). In the case of anaplastic papillary thyroid cancer and tissues from cervical, miR-146a was found to be overexpressed (He et al., 2005; Wang et al., 2008). According to one study, NSCLC tissues have decreased miRNA-146a-5p expression (Chen et al., 2013) whereas a different investigation found that the higher miRNA-146a-5p serum level in NSCLC patients (Chen et al., 2013). It was also reported that NSCLC cells with transfected miRNA-146a mimic showed the downregulation of EGFR mRNA, however, transfection of miRNA-146a inhibitor showed opposite results EGFR mRNA. Moreover, an increased level of miR-146a was found to be linked with longer progression-free survival in tissues of NSCLC patients.

Thus, miRNA-146a acts as a potential biomarker for NSCLC diagnosis and treatment (Chen et al., 2013). However, both *in vivo* and *in vitro* studies have reported the usefulness of miRNA therapy to reduce the drug resistance against EGFR-TKIs of tumor cells. In NSCLC, the miRNA-146a expression has been reported to boost the curative efficacy of EGFR-TKIs such as cetuximab, gefitinib, erlotinib, and afatinib (Zhong et al., 2010). The reactivation of miR-146a in NSCLC cell lines improved the resistance to cisplatin, cell cycle halt, cell proliferation inhibition, and apoptosis of cells. Further research revealed that miR-146a directly regulated cyclin J, resulting in these outcomes (Shi et al., 2017). DDP-resistant (cisplatin) NSCLC samples and cell lines (A549, Calu-1) were found to have continuously reduced the levels of miR-146a expression as well as higher levels of NF-kB activity and TNF signaling adapter proteins like TRAF6 and IRAK1. miRNA-146a was found to modulate DDP sensitivity by preventing activation of the NF-kB inflammation pathway (Jiang et al., 2017). Consequently, when combined with conventional chemotherapeutic drugs, miRNA-146a acts as a new approach to decrease resistance against cisplatin in NSCLC patients.

5. CONCLUSION

In conclusion, our study found more likely to get and effect on susceptibility lung cancer disease with a nearly significant association of *miRNA-146a rs2910164 C>G* polymorphism with EGFR mutation in NSCLC patients in the Kurdistan region of Iraq. Thus, *miRNA-146a* could serve as a biomarker for the diagnosis and treatment of NSCLC. It's important to note that the understanding of presented results should consider other factors such as the sample size, the specific study design and potential confounding

variables and other factors. Additionally, replication of findings in independent studies is crucial to establish the validity of the association and might evolve over time as new evidence emerges. Further recent researches are needed to confirm and understand the relationship between these genes in the situation of lung cancer (NSCLC). However, further investigations comprising large-scale patient specimens are necessary to understand *miRNA-146a*-dependent *EGFR gene* regulation in a cancer microenvironment influenced by the immune system and other microenvironmental components to validate its therapeutic use.

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