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TGFβ1, SMAD2, CTNNβ1, and Wnt3a gene mutational status and serum concentrations in individuals with non-small cell lung cancer

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ABSTRACT

The objective of the current investigation was to investigate the diagnostic utility of the serum concentrations and mutational status of TGFβ1, SMAD2, CTNNβ1, and Wnt3a. and the expression levels of human-related genes in patients with non-small cell lung cancer (NSCLC). The serum concentrations were determined using the ELISA technique, and PCR for genotype variations of TGFβ1, SMAD2, CTNNβ1, and Wnt3a were examined using Sanger sequencing in tissue samples obtained from 93 patients with NSCLC and 84 healthy individuals for blood, and 20 Formalin Fixed Paraffin Embedded (FFPE) from normal samples dissected adjacent to the tumour. The findings of the current investigation indicate that individuals diagnosed with NSCLC exhibited significant elevation in the serum levels of CEA and CYFRA21-1, as well as TGFβ1, SMAD2, CTNNβ1, and Wnt3a. In total, 325 mutations in four trialled genes (243 mutations in TGFβ1, 24 mutations in SMAD2,47 mutation Wnt3a and 11 mutations in CTNNβ1) were identified in patients with NSCLC. Furthermore, all mutations were recorded in adenocarcinoma, not squamous and normal adjacent tumour cells. CYFRA21-1 and CEA are more significant between NSCLC and HC, gender, and NSCLC types ($p<0.001$). In detail, TGFβ1 exhibited the highest rate of mutations among other genes and three types of genomic mutations. Elevated levels and genetic polymorphisms of TGFβ1, SMAD2, CTNNβ1, and Wnt3a may play crucial functions in the pathogenesis and angiogenesis of non-small cell lung cancer (NSCLC). These biomarkers might play a role in future immunologic response and pharmacologically targeted NSCLC therapy.

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Introduction

Cancer is a disease that can affect almost all biological human tissues and is currently one of the leading causes of death worldwide (1). GLOBOCAN 2020 indicates lung cancer is the second most commonly diagnosed cancer after breast cancer while still being the leading cause of cancer-related deaths (2). NSCLC (non-small cell lung cancer) is a frequently diagnosed form of cancer on a global scale (3).

Lung cancer, also known as bronchogenic carcinoma, is a major global health problem (4, 5). When normal lung cells' DNA undergoes a succession of driver mutations, the result is aberrant and uncontrolled cell development in the lung that may later metastasise to other organs (6). The molecular alterations under consideration pertain to oncogenes and tumour suppressor genes. Changes in DNA sequence can cause them to occur at the level of upstream or downstream gene regulation (point mutations), loss of heterozygosity (LOH), gene instability, and changes in microsatellite DNA as a result of amplification of a DNA segment or the loss or gain of a whole chromosome (7).

TGFβ1 is a versatile cytokine that is vital for promoting local angiogenesis, extracellular matrix production, immunological evasion, cell heterogeneity adhesion, cell proliferation, invasion, metastasis, and other processes (8). Transforming growth factor β-induced protein is differentially expressed in transformed tissues. Loss of TGFβ1 expression has been described in several cancers, such as lung cancer (9). The cytokine-encoding TGFβ1 gene is situated on the lengthy arm of chromosome 19 and comprises seven exons and six introns that encode TGFβ1 cytokine, and spanning a total length of 52.3 kb (10). Cell differentiation, adult tissue homeostasis, wound healing, and immune modulation are just some of the many biological processes that TGFβ1 plays a role in as a signal molecule released by diverse cell types (10, 11).

Smad2 is a small intracellular effector protein that belongs to the SMADs family and is proposed to be a tumour suppressor protein encoded by the gene present at chromosome 18q21, It is triggered by TGF-β receptors to mediate intracellular TGF-β signaling (12). Furthermore, Smad2 has a critical role in TGF-β induced apoptosis of prostate epithelial cells activated by TGF-β1 (13). Smad2 lack of DNA binding activity can be attributed to a structural change caused by the substitution of the β-hairpin loop to a 30 amino-acid insertion which is expressed by exon 3 (14).

β-Catenin, encoded by the CTNNβ1 gene, is the central component of the Wnt pathway. However, this protein was originally described by its interaction with the cytoplasmic domain of cadherin and α-catenin and its crucial role in cell-cell adhesion (15). The CTNNβ1/β-catenin gene harbors 16 exons (16). Canonical Wnt/β-catenin signalling is

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involved in many developmental and physiological processes, and its deregulation leads to numerous diseases, including cancer development (17). B-catenin is essential for the formation and maintenance of the epithelial layer and is a key component of the canonical Wnt signalling pathway (18). The wnt/β-catenin system has been identified as one of the important oncogenic pathways signaling related to immune evasion (19). Furthermore, β-catenin plays key roles in the canonical Wnt pathway during tumorigenesis by transmitting Wnt signals to the nucleus and regulating oncogenes (20).

Wnt3a is a classical Wnt family member comprising 19 homologous ligands (21). β-catenin-dependent pathway is activated by Wnt3a, which it's called canonical Wnt signaling (22). Wnt3a, one of the Wnt family members regarded as an activator of the canonical Wnt signaling pathway, is expressed in the dorsal midline region responsible for developing the spinal cord, forming a dorsal-to-ventral concentration gradient. Wnt3a is involved in various cellular processes, such as self-renewal, proliferation, differentiation, and motility, during vertebrate embryonic development (23). Lung adenocarcinoma and squamous cell carcinoma are the two main types of NSCLC. These results suggest that Wnt3a could be a potential therapeutic target in the suppression of metastasis and tumorigenesis of lung cancer(1). Recently, Wnt3a has been identified as a cancer-promoting factor in various types of cancer, including colon, breast, lung, and esophageal squamous cell carcinoma (24). However, advanced gene-sequencing technology discovered that ethnicity influences the distribution of genetic variations in populations and has a major impact on the connection between alleged genetic markers and cancer risk (11). The application of molecular and ELISA testing has been found to improve the effectiveness of identifying and evaluating lung cancer patients with NSCLC (25).

Insufficient research has been conducted on the immunological variances among individuals diagnosed with NSCLC. This study aimed to determine and measure the changes in TGFβ1, SMAD2, CTNNβ1, and Wnt3a serum concentrations in patients with NSCLC as well as the genetic polymorphisms of the TGFβ1, SMAD2, CTNNβ1 and Wnt3a genes in the patients.

Materials and Methods

Patient samples

The current investigation utilized a case-control design. The specimens under investigation were collected from the Oncology Department of the Hiwa Hospital in Sulaimaniyah, Iraq. The study was approved by the Human Ethics Committee of the College of Medicine, Suleimani University (N0:230; date, 1/9/2020; Sulaymaniyah) and was conducted according to the principles of the Declaration of Helsinki. All subjects provided written informed consent for the use of their blood and tissues. Any participant with chemotherapy or radiotherapy was excluded from the study. The venous blood samples are taken from 93 patients, 24 female and 69 male patients with NSCLC, with mean ages (of 62.69±10.43) years, and 84 healthy individuals, 25 female and 59 male controls, with mean ages (61.86±9.638) years, with no significant difference (p=0.522). Formalin-fixed paraffin-embedded (FFPE) specimen blocks and blood samples were collected from both patients and controls between July 2020 and November 2021.

Tumors samples

We analyzed samples of NSCLC tissues from 93 patients who had undergone bronchoscopy and surgical operation at the Department of Surgery, Sulaimani Education and Research Hospital. In addition, after resection of the tumours or by bronchoscopy, tissue processing Formalin-fixed and paraffin-embedded (FFPE) samples of all tumours were examined at the Pathology Department of the Shorsh Teaching Hospital.

The tissue samples were procured subsequent to bronchoscopy or resection surgery. Patients with NSCLC, preferentially diagnosed via screening and bronchoscopy, were included in the study. All cases with NSCLC were pathologically diagnosed. These substantial phenotypic and genetic changes are apparent up to 1 cm from the tumour's margins. Consequently, in cancer studies, it is common to designate histologically normal samples that are dissected adjacent to the tumour but beyond the observed aberrations (referred to as NAT or normally adjacent to the tumour) as healthy control (HC) samples. This is based on the assumption that histological normalcy indicates biological normalcy. The aforementioned methodology presents numerous benefits, including the ability to conduct a comparative analysis of samples derived from a single, larger tissue specimen obtained from the same individual. This approach effectively minimizes the impact of individual-specific and anatomical site-specific factors (26). Through written informed consent, subjects were recruited for surgical excision of the NSCLC samples and paired adjacent normal tissue (27, 28). Patients diagnosed with lung cancer received no treatment, drug, and/or chemotherapy before a blood sample was obtained. Pathological diagnosis was classified according to the WHO classification of lung tumours, and staging (TNM classification groups) was performed by an expert pathologist in lung diseases (29).

Genom extraction

Genomic DNA was extracted and quantification. According to the manufacturer's protocol, genomic DNA was purified from NSCLC tissues using DNA extraction from paraffin tissue (QIAamp® DSP DNA FFPE Tissue, RF 60404) DNA isolation KIT. A nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.) was used to determine the concentration (A260) and purity (A260/A280 ratio) of extracted DNA. DNA purity was calculated using an ideal A260/A280 ratio of 1.8 and stored at -20° for later use after the Nanodrop instrument was blanked with the elution buffer.

Blood samples

A blood sample (5 ml) was obtained from the patients and healthy individuals under an aseptic technique. Subsequently, the blood was placed into a clot activator tube for serum preparation. Samples were centrifuged at 5,000 x g for 10 minutes after coagulation of blood samples and then preserved in an Eppendorf tube, which was stored at -80˚C until required for further analysis.

Estimation of CEA and CYFRA21-1 concentration

The concentration of CEA and CYFRA21-1 was deter-

mined using a Cobas e411 analyzer (Roche Diagnostics). This detection method uses electrochemiluminescence to measure immunoreactivity (30, 31).

Determination of TGFβ1, SMAD2, CTNNβ1 and Wnt3a concentrations

To analyze serum biomarkers (Wnt3a/CTNNβ1, TGFβ1/SMAD2) production, we conducted an enzyme-linked immunosorbent assay (ELISA) using the sandwich method on the serum sample using a commercially available KIT (Catalogue numbers: E-EL-H5681, E-EL-H5623, E-EL-0162, and E-EL-H5623) respectively, (Elabscience Biotechnology Inc., Texas, USA) based on the manufacturer's protocol. The concentration of TGFβ1, SMAD2, CTNNβ1, and Wnt3a was measured spectrophotometrically with an ELISA reader (Chromate ELISA Reader, Instruments, Inc.USA) at a wavelength of 450 nm. Subsequently, the concentrations present in the samples were determined through a comparative analysis of their absorbance values against the standard curve.

Genotype determination

The current investigation examined four frequently studied variations of SMAD2, TGFβ1, CTNN β1, and Wnt3a. Initially, the purified DNA was separately amplified using ready to use master mix AddStart Taq DNA Polymerase kit (Addbio,18101M, Korea) according to the manufacturer's protocol, 2.0 µl 10Xbuffer, 1.0 µl Magnesium Chloride (MgCl2), 2.0 µl deoxynucleotide Triphosphate (dNTP), 0.5 µl of each primer, 0.4 µl Taq DNA polymerase, 11.6 µl PCR grade water, and 1.5 µl template DNA were mixed in PCR tube and amplified. For each genetic polymorphism using PCR on an Applied Biosystems thermal cycler (GeneAmp PCR system 9700) using the following primers: **Wnt3a**-Forword 5´-AGCCCTGTTAACCCTGCATC-´3 and Wnt3a-Reverce 5´-CTTTTTCCCAAGCACCCT-TGC-´3, **CTNNβ1**- Forward 5'- CCA ATC TAC TAA TGC TAA TAC TG -' 3 CTNNβ1- Reverse 5'- CTG CAT TCT GAC TTT CAG TAA -' 3, **SMAD2-** Forword 5'-ACTTCCTGAGCTTTTGCCAG -'3, SMAD2-Reverce5´-CTGCATTCTGACTTTCAGTAA-´3, **TGFβ1**-Forword 5' GCTATCGCCTGCACACAGCT -' 3 TGFβ1- Reverse 5'- CCAGGCGGAGAAGGCTTAAT -' 3. The following thermocycling conditions were used for PCR: Initial denaturation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, annealing stepped at (59.0, 55.0, 58.3 and 60.2) ˚C, for 30 sec for (Wnt3a, CTNNβ1, SMAD2 and TGFβ1) respectively, and elongation at 72˚C for 30 sec; and a final extension step at 72˚C for 5 min. All PCR products were separated via 3% agarose gel electrophoresis, compared with the 50 bp DNA marker (50 bp DNA Ladder; cat. no DMO 12-R500; Gene DireX.) and stained with Ethidium bromide (DNA Gel Stain BioBasic, Canada.) before casting into the tray. Gels were visualized using a gel documentation system (UV Transilluminator UST-20M-8K; Biostep GmbH). Purified PCR products were sent to Macrogen Company, Korea, for the DNA sequence analysis.

Statistical analysis

After completing a normality test, the nonparametric Spearman correlation of the U-Mann Whitney test was used to assess the statistical differences in the serological data for the TGFβ1/SMAD2, CTNNβ1/Wnt3a, CYFRA21-1, and CEA between HC and NSCLC patients. The Mutation Surveyor software V5.1.2 was utilized for the purpose of analyzing the outcomes of Sanger sequencing. The software was programmed to automatically align the sample traces to GenBank references. Data were analyzed using GraphPad Prism 9.4 (GraphPad Software, Inc.). The receiver operating characteristic curve was employed to compute the area under the curve (AUC) for the concentration of CEA and CYFRA21-1 in patients with non-small cell lung cancer (NSCLC). A nonparametric Spearman correlation test was employed to determine the strength of the correlation between the expression levels of TGFβ1/SMAD2 and CTNNβ1/Wnt3a. Continuous data were evaluated by the Mann–Whitney test and expressed as median and percentile range (25–75%). The statistical significance was fixed at $p < 0.05$.

Results

Serum TGFβ1, SMAD2, Wnt3a, CTNNβ1, CEA and CYFRA21-1 concentrations in patients with NSCLC were significantly increased (P<0.001) compared with healthy individuals. the serum concentrations of TGFβ1, SMAD2, Wnt3a, and CTNNβ1 (data not shown). For NSCLC patients, CEA (median =23.50; IGR=18.0), CYFRA 21-1 (median, 12.50; IQR=11.0) were significantly increased (P<0.001) compared with healthy individuals CEA (median, 1.100; IQR=1.12), CYFRA 21-1 (median, 0.975; IQR=1.0) respectively; Fig. 1 A and B. Furthermore, CEA and CYFRA21-1 were revealed to be efficient biomarkers for NSCLC.

Compare CEA and CYFRA21-1 markers for NSCLC types (adenocarcinoma and squamous cell carcinoma) and gender (male and female); it is significantly higher in all of them p <0.0001. In both categories, CEA and CYFRA21-1 have a higher value in male and squamous cell carcinoma.

Correlation

There was a statistically non-significant positive and negative correlation between (CEA and CYFRA21-1 with other Biomarkers). Furthermore, there was a negative correlation between (CEA with CTNNβ1 and Wnt3a) and (CYFRA21-1 with TGFβ1 and Wnt3a) and a positive correlation between (CEA with TGFβ1 and SMAD2) is shown in Figure 2: A to H. Still, positive and high significance between (CEA and CYFRA21-1) is shown in Figure 2: I. Also, there was no significant between gender with CEA

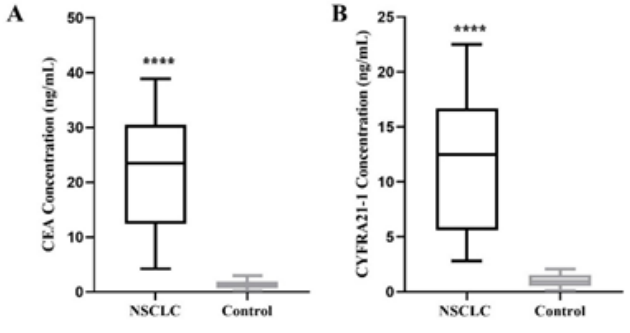

Figure 1. Comparison between Serum Biomarkers concentrations(ng/mL) in healthy individuals and patients with NSCLC. (A) CEA (B) CYFRA21-1. Significant changes were observed between NSCLC and Control. The comparison was performed using an unpaired Mann-Whitney test. *** P<0.001 vs Healthy individuals with NSCLS.

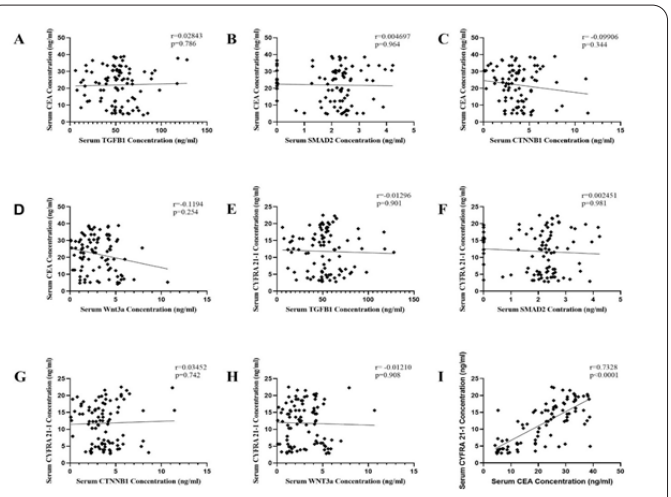

Figure 2. Correlation between serum-biomarkers: A) between SMAD2 and TGFβ1, B) between CTNNβ1 and TGFβ1. C) between Wnt3a and TGFβ1. D) between CTNNβ1 and SMAD2. E) between Wtn3a and SMAD2. F) between CTNNβ1and Wnt3a in NSCLC.

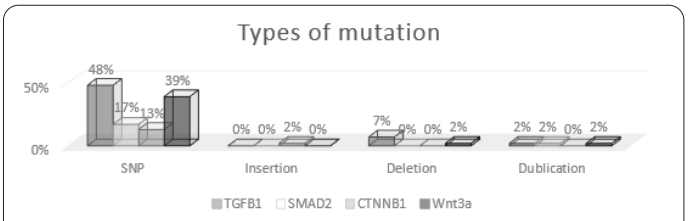

Figure 3. Show data about the rate of types mutation in NSCLC.

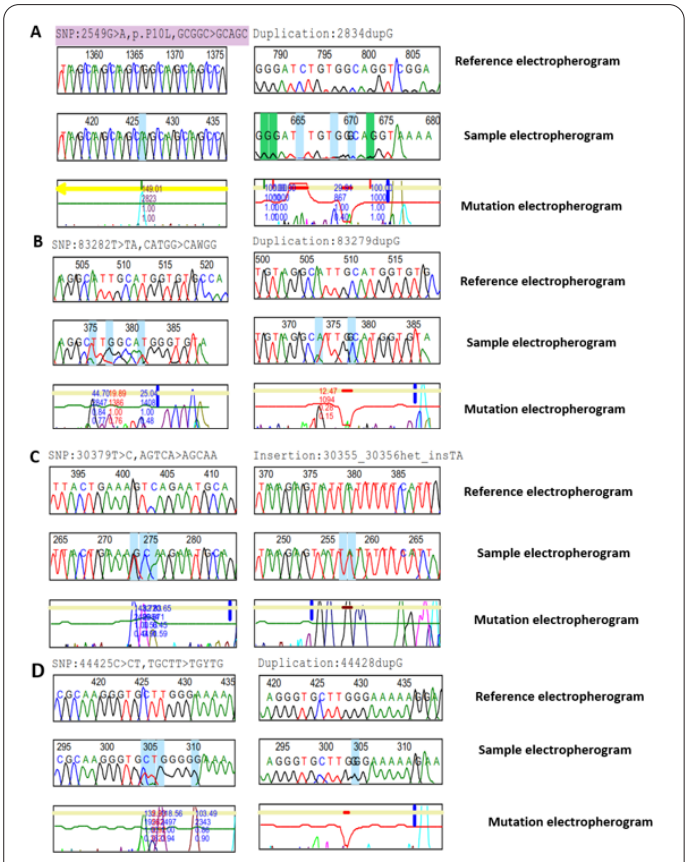

Figure 4. DNA sequence electropherograms showing nucleotide mutations in TGFβ1, SMAD2, Wnt3a, and CTNNβ1. (A) Homozygous substitution from G to A and homozygous duplication 2834 G in the TGFβ1 gene. (B) Heterozygous substitution from T to TA and homozygous duplication 83279 G in exon region, SMAD2 gene. (C) Homozygous substitution from T to C and heterozygous Insertion 30355_30356 TA in CTNNβ1 gene. (D) Heterozygous substitution from C to CT and homozygous duplication 44428 G in exon region, Wnt3a gene.

and CYFRA21-1 (r=0.150, p=0.482), (r=0.136, p=0.523) and types (r=-0.244, p=0.101), (r=-0.289, p=0.051) of NSCLC for CEA and CYFRA21-1 biomarkers, besides of positive correlation between gender and both tumour marker, but had a negative correlation between types of NSCLC for both tumour markers.

We have identified a total of 325 SNPs and three deletion/ one duplication in TGFβ1; in SMAD2, 23 SNP and one duplication, also Wnt3a 45 SNP, one duplication and one deletion; finally, in CTNNβ1, one insertion and 10 SNP /tandem-repeat polymorphisms in the four genes that participate in the remodelling and inflammatory, signalling pathway. Due to increasing life expectancy and the increased risk of cancer with ageing, lung cancer is common in elderly individuals. This study investigates the genetic polymorphisms of TGFβ1, SMAD2, Wnt3a, and CTNNβ1. A total of 325 mutations were detected across the four genes, as presented in Table 1. Figure 4 displays three electropherograms arranged in rows, representing the reference, sample, and mutation, respectively. A total of 243 mutations were documented in TGFβ1, while SMAD2 exhibited 24 mutations, CTNNβ1 had 11 mutations, and Wnt3a had 47 mutations. Three types of genomic mutations were recognised: Substitutions (C> CG, A>AT, T> TG, G> GA, A>AC, T>TA, T>G, G>A, and C> CT), duplication (GG) and deletions (C and G). The homozygous and Heterozygous variant mutation 2549G>A, 10P>L$120, 3924A>AG$18 and 4215C>T$121 on chromosome position 19:41858921, 19:41860296 and 19:41860587 has been previously reported in an external public database, dbSNP (https://www.ncbi.nlm.nih. gov/snp/), respectively.

The current investigation revealed that the mutation variant percentage was 100%, 100%, and 6%. However, it should be noted that the extant mutational variants of TGFβ1 have yet to be documented in external databases, as far as our current understanding extends.

The current findings have unveiled noteworthy distinguishing characteristics pertaining to Wnt3a, CTNNβ1, and SMAD2 which there were recorded unique mutations that have not been documented previously to the best of our knowledge. The analysis of the sequencing data of SMAD2 revealed two types of mutation: Substitution (A>AT, T>G, T>TA, T> TG, A>AC, T> TC) and duplication (G), and both homozygous and heterozygous variant mutations were identified. The heterozygous variant duplication and substitution, 83279dupG$4 and 83276A>AT$113 were observed in chromosome positions 18:45374786 and 18:45374789, respectively.

The investigation of the sequencing data revealed that Wnt3a had the highest rate of mutations when compared to SMAD2 and CTNNβ1. Furthermore, it is noteworthy that none of the mentioned mutations have been documented in any external database. This observation highlights the existence of three distinct categories of genomic mutations, namely substitutions (C>CG, T>G, C> CT, A>G, A>T, C> CA, A>AT, G>A, A>AG, T> TA, C>T, T>A), deletions (G), and duplications (G). The mutation (44427T>G$109) on chromosome position 1:228238649 is homozygous and has not been documented in external public databases. The current study reports a variant percentage of 71.4%

Table 1. Variants were identified in patients with NSCLC using Mutation Surveyor software.

Gene Name	Chromosome position	Mutation	Mutation genotype	Heterozygous or homozygous	Variants	Variant, %	External database
TGFβ1	19:41858609	Substitution	A>AT	Heterozygous	2237A>AT,114V>V/E$57	100%	Not Found
	19:41859201	Deletion	C	Heterozygous	2829delC$12	100%	Not Found
	19:41858921	Substitution	G>A	Homozygous	2549G>A,10P>L$150	100%	dbSNP:1800470
	19:41858653	Substitution	A>AC	Heterozygous	2281A>AC,99P>P/P$27	100%	Not Found
	19:41858685	Substitution	C>CG	Heterozygous	2313C>CG,89A>A/P$146	100%	Not Found
	19:41858921	Substitution	G>A	Homozygous	2549G>A,10P>L$128	100%	dbSNP:1800470
	19:41858659	Substitution	G>GT	Heterozygous	2287G>GT,97P>P/P$26	50%	Not Found
	19:41859201	Deletion	C	Heterozygous	2829delC$12	100%	Not Found
	19:41859205	Duplication	G	Heterozygous	2834dupG$4	100%	Not Found
	19:41858921	Substitution	G>A	Homozygous	2549G>A,10P>L$149	100%	dbSNP:1800470
	19:41858782	Substitution	C>CA	Heterozygous	2410C>CA,56K>K/N$25	25%	Not Found
	19:41858921	Substitution	G>GA	Heterozygous	2549G>GA,10P>P/L$103	100%	dbSNP:1800470
	19:41860296	Substitution	A>A	Homozygous	3924A>AG$18	100%	dbSNP:1800469
	19:41860587	Substitution	C>T	Homozygous	4215C>T$121	6%	dbSNP:1800468
	19:41860395	Deletion	G	Heterozygous	4023delG$10	6%	Not Found
SMAD2	18:45374786	Duplication	G	Heterozygous	83279dupG$4	33.30%	Not Found
	18:45374789	Substitution	A>AT	Heterozygous	83276A>AT$113	66.70%	Not Found
	18:45374787	Substitution	T>G	Homozygous	83278T>G$20	66.70%	Not Found
	18:45374783	Substitution	T>TA	Heterozygous	83282T>TA$25	66.70%	Not Found
	18:45374787	Substitution	T>TG	Heterozygous	83278T>TG$32	66.70%	Not Found
	18:45374789	Substitution	A>AT	Heterozygous	83276A>AT$45	66.70%	Not Found
	18:45374865	Substitution	A>AC	Heterozygous	83200A>AC,326E>E/D$15	16.70%	Not Found
WNT3A	1:228238647	Substitution	C>CG	Heterozygous	44425C>CG$88	28.60%	Not Found
	1:228238653	Substitution	A>G	Homozygous	44431A>G$115	60.00%	Not Found
	1:228238650	Duplication	G	Heterozygous	44428dupG$12	14.30%	Not Found
	1:228246795	Substitution	T>TA	Heterozygous	52573T>TA,230F>F/I$144	25.00%	Not Found
	1:228246949	Deletion	G	Heterozygous	52727delG$14	14.30%	Not Found
	1:228238437	Substitution	A>AG	Heterozygous	44215A>AG,132T>T/A$7	5.00%	dbSNP:150424650
	1:228238558	Substitution	C>CT	Heterozygous	44336C>CT,172A>A/V$12	5.00%	Not Found
	1:228238376	Substitution	T>TG	Heterozygous	44154T>TG,111F>F/L$7	6.20%	Not Found
	1:228238443	Substitution	G>GA	Heterozygous	44221G>GA,134A>A/T$7	5.00%	dbSNP:61743220
CTNNβ1	3:41266278	Substitution	G>GT	Heterozygous	30378G>GT$81	66.70%	Not Found
	3:41266279	Substitution	T>C	Homozygous	30379T>C$47	66.70%	Not Found
	3:41266280	Substitution	C>A	Homozygous	30380C>A$21	66.70%	Not Found
	3:41266255-3:41266256	Insertion	T	Heterozygous	30355_30356het_insTA$5	14.30%	Not Found
	3:41265990	Substitution	A>T	Homozygous	30090A>T$44	100%	Not Found
	3:41266202	Substitution	G>GA	Homozygous	30302G>GA,67E>E/K$5	5%	Not Found

for this mutation. Two genetic variations were detected on chromosome positions 1:228246949 and 1:228238650, namely the heterozygous deletion 52727delG$14 and the heterozygous duplication 44428dupG$12. The Heterozygous variant mutation 44215A>AG,132T>T/A$7and 44221G>GA,134A>A/T$7on chromosome position 1:228238437and 1:228238443 has been previously reported in an external public database, dbSNP (https://www.ncbi.nlm.nih. gov/snp/), respectively. The current investigation revealed that the proportion of the mutation in question was 5%. However, the unreported mutational variants of Wnt3a have not been documented in any external databases. The analysis of CTNNβ1 sequencing data has identified two distinct types of genomic mutations, namely substitutions (G>GT, T>C, C>CA, C>A, A>T, T>TG) and insertion (T). The homozygous variant mutation 30379T>C$47, located at chromosome position 3:41266279, has not been documented in external public databases. In the current study, the variant percentage of this mutation was found to be 66.7%. On the other hand, the heterozygous insertion 30355_30356het_insTA$5 was identified on chromosome positions 3:41266255-3:41266256, and its variant percentage in the present study was 14.3%.

In our study, the mutation rate for each biomarker, for TGFβ1 in adenocarcinoma and total NSCLC (54%,25/46) also for the total number of NSCLC (27%,25/93), for SMAD2 mutation (20%,9/46) also for a total number of NSCLC (10%,9/93), also for CTNNβ1 in adenocarcinoma and total NSCLC (15%,7/46) also for the total number of NSCLC (8%,7/93), Finally for Wnt3a in adenocarcinoma and total NSCLC (43%,20/46) also for the total number of NSCLC (22%,20/93). Also, in adenocarcinoma, the rate of mutation types for TGFβ1, SMAD2, Wnt3a, and CTNNβ1, Figure 3 detail about types of mutation. Also, have data about types of mutation rates in NSCLC.

TGFβ1, SMAD2, Wnt3a, and CTNNβ1 mutation in amino acid, for Example (Valine to Glutamic Acid, Asparagine to Lysine, Histidine to Glutamine, Methionine to Valine, Tyrosine to Cysteine, Aspartic Acid to Lysine, Alanine to Glycine, Proline to Alanine, Alanine to Glycine), (Alanine to Threonine, Threonine to Glycine, Alanine to Cysteine, Threonine to Cysteine),(Cysteine to Glycine, Threonine to Glycine, Alanine to Glycine), (Glycine to Threonine, Threonine to Cysteine, Cysteine to Alanine, Alanine to Cysteine) respectively.

Comparison of serum TGFβ1, SMAD2, CTNNβ1, and Wnt3a expression levels

The expression levels of TGFβ1, SMAD2, CTNNβ1, and Wnt3a in mutant adenocarcinomas of non-small cell lung cancer (NSCLC) serum were observed to be significantly higher expression serum levels compared to other non-mutant adenocarcinomas. The present study reports a significant upregulation of TGFβ1, SMAD2, CTNNβ1, and Wnt3a expression levels in serum samples of mutant adenocarcinomas of (NSCLC) when compared to non-mutant adenocarcinomas.

All results evaluating the TGFβ1, SMAD2, CTNNβ1, and Wnt3a gene expression in NSCLC tissues were collected and presented in Table 1. The relationship between gene mutations and the generation of biomarkers in adenocarcinoma in NSCLC is quite significant, with high mutations corresponding to high biomarker production.

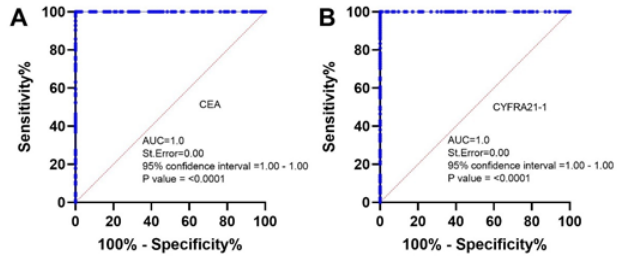

Figure 5. ROC curves of parameters for predicting NSCLC. A) Serum CEA level for predicting NSCLC, and B) CYFRA 21-1 for NSCLC.

Utilising ELISA, the TGFβ1, SMAD2, CTNNβ1, and Wnt3a expression levels in peripheral blood were found. In addition, no alterations were found in adjacent normal tissue in the lung cancer patient's healthy areas. Additionally, there was no mutation seen in NSCLC squamous cell carcinoma.

The present study revealed a statistically significant elevation in the serum concentrations of CEA and CYFRA21-1 among patients diagnosed with non-small cell lung cancer (NSCLC) as compared to the HC group of healthy individuals (P<0.001). Moreover, it has been demonstrated that CEA and CYFRA21-1 serve as effective biomarkers for non-small cell lung cancer (NSCLC). (AUC, 1.0; Fig. 5).

Discussion

The current investigation revealed a statistically significant elevation in the serum levels of CYFRA21-1 and CEA between NSCLC and HC groups. These results supported using these markers in clinical practice by observing a link between serum CYFRA21-1 and CEA levels in patients with NSCLC (32). The prognostic potential of NSCLC patients can be evaluated through the utilization of serum CYFRA 21-1 and CEA. The combined detection of both tests is likely to be more dependable (33). this interaction can be valuable in NSCLC as a predictive factor. The identification of predictive and prognostic factors in tumour development is crucial in determining the most efficacious therapies for cancer.

CYFRA21-1 and CEA do not correlate with SMAD2 and CTNNβ1. On the other hand, CEA has a negative correlation with CTNNβ1 and Wnt3a. There is also a negative relationship between CYFRA21-1, TGFβ1, and Wnt3a. Interestingly, the serum concentrations of CEA, CTNNβ1, and Wnt3a, as well as CYFRA21-1, TGFβ1, and Wnt3a, were negatively correlated. However, they share the same signal transduction pathway—the non-significant difference between CYFRA21-1 and CEA with other biomarkers. Explaining the biological basis for these correlations is difficult, and they may be incidental. However, because these potential biomarkers of NSCLC showed significant changes in serum levels during carcinogenesis, there may be an underlying pathophysiological relationship between them. Furthermore, CYFRA 21-1 is more abundant in lung squamous cell carcinoma than in adenocarcinoma (32). However, CYFRA 21-1 and CEA exhibited considerably higher expression in squamous cell carcinoma than adenocarcinoma in this study with highly significant (p<0.0001). Also shown in the reference CYFRA21-1 which is overexpressed in tumors of epithelial origin (34). Maybe have depending on the effect of the physiological mechanisms

between these biomarkers and the anatomy of organs.

Recent studies suggest an interaction between Wnt/b-catenin and TGFβ/SMAD signaling in controlling gene transcription and cell phenotype (35-37). The protein's signal peptide is coded for by exon 1 of the TGFβ1 gene. This cytokine is secreted into the extracellular media, and its production may be affected by alterations in its DNA sequence (38). In our study, the TGFβ1 mutation gene effect on adenocarcinoma gene expression was higher than the non-mutant gene. Also, other studies show variations in the TGFβ1 gene can affect cytokine expression in patients with cancer and have an important influence on the development of tumors (39). The role of TGFβ1 is crucial in numerous cellular processes and significantly impacts the immune system and the behavior of cancer cells (40). TGFβ1 is an innovative molecule that inhibits cell proliferation early in numerous cancers. In addition, TGFβ1 enhances late-stage cancer progression (41). Maybe this mutation by the oncogene factor causes the blood vessel to help grow the tumour cells.

Nevertheless, this result suggests that TGFβ1 SNP, especially rs1800468, rs1800469, rs1800470 and other SNP mutations that have not been recorded before, might play an essential role in developing NSCLC, especially adenocarcinoma. However, TGFβ1's function may be too complicated for a single variation impact, and gene-gene or gene-environment interactions may explain the association between TGFβ1 and adenocarcinoma (42). Although, in our study, all types of SNP in TGFβ1 associated with aggressiveness and metastasis also agree with another study, Chen X (43) similar pattern of results was obtained in line with previous studies; the study population's susceptibility to lung cancer may be raised by the presence of the rs1800470 polymorphism.

Elevated levels of TGFβ1 in the bloodstream and increased expression in bodily tissues have been found to negatively affect a range of immune system disorders, carcinomas, and transplant procedures. In addition, individuals with gene and regulatory region polymorphisms that increase TGFβ1 expression are more likely to develop related disorders. Therefore, SNPs that impact the expression levels of TGFβ1 are frequently employed as indicators of heightened susceptibility to disease (40). Therefore, this study aimed to elucidate the underlying mechanism accountable for the modified expression of TGFβ1 due to this single nucleotide polymorphism.

The previous study investigates the potential correlation between TGFβ1 polymorphisms and the presence or absence of metastasis in patients diagnosed with gastric adenocarcinoma, which demonstrated a considerable increase in metastasis (type IV) individuals compared to non-metastatic patients (38). When comparing our results to previous studies, it must be pointed out that stages III and IV of adenocarcinoma are present in NSCLC but absent in squamous cell carcinoma and adjacent normal tissue of the tumour; Our study did not incorporate any additional stages. Moreover, the disparate effect of the TGFβ1 genotype on non-metastatic and metastatic ailments could be elucidated by the discrete biological function of TGFβ1 signaling contingent on the tumour stage.

In contrast, an additional investigation conducted by Chen et al. concluded that the TGFβ1 (rs1800470) and (rs1800469) polymorphisms were not associated with lung cancer development and revealed the (rs1800469) polymorphism diminishes the danger of lung cancer growth in patients with NSCLC (39, 44). However, in our study, (rs1800470), (rs1800469) and (rs1800470) polymorphisms have been related to the high expression of TGFβ1 in adenocarcinoma for NSCLC in stages III and IV.

In addition, elevated TGF1 expression is not always consistent with increased cytokine-initiated signaling. This study's interesting finding was that TGFβ1 gene expression has an effect on adenocarcinoma presence in NSCLC. Tsushima, H. et al. suggest that plasma TGFβ1 levels may reflect overexpression of the gene in colon cancer tissues and are associated with disease progression (45). Furthermore, these results suggest that TGFβ1 also promotes the accumulation of extracellular matrix glycoproteins and cell adhesion proteins, which might enhance the metastatic potential of the tumour (45). Moreover, the upregulation of TGFβ1 expression could be crucial in advancing cancer due to its potential to promote angiogenesis and suppress immune surveillance mechanisms responsible for tumour recognition and destruction.

In the previous study in different organ tumors with the rs1800469 and rs1800470 TGFβ1 polymorphisms in Wilms Tumor patients, according to our findings, the rs1800469 and rs1800470 polymorphisms may function as indicators related to susceptibility and the clinical manifestation of this condition. (46). Also, our study confirms these results by mutant gene effect on high excretion of biomarkers TGFβ1 in adenocarcinoma NSCLC, but for squamous cell carcinoma, no mutation.

The present study highlights that gastric adenocarcinoma patients who are prone to metastases exhibit an increased incidence of single nucleotide polymorphisms (SNPs) in TGFβ1, which are linked to reduced TFGβ1 production (38). When comparing our results to previous studies, it must be pointed out that TGFβ1 mutant genes in adenocarcinomas have higher production than non-mutant ones. We believe there will be more reports of concurrent mutations in driver genes in the future, the clinical discovery of numerous oncogene mutations may aid in the determination of the best therapy plan. This difference between organs causes differential expression of biomarkers outcome.

Finally, we obtained evidence of a correlation between adenocarcinoma in NSCLC and the single nucleotide polymorphism SNP. The results strongly imply that we have evaluated the association of two polymorphisms, including TGFβ1 (rs1800469) and (rs1800471), with pancreatic cancer risk in the Iranian population (47). Also, this study shows that high expression in the last stages of NSCLC increases the concentration in the blood and the high mutation rate in TGFβ1 NSCLC patients. Because TGFβ1 helps to grow tumors in late stages, this may cause more mutation and uncontrol the growth of the cells. And all mutations in TGFβ1 adenocarcinomas do not have the mutation found in squamous cell carcinomas. And if negative, adjust the tumour sample (control tissue). This is the first study to examine the association between the rs1800468, rs1800469, and rs1800470 polymorphisms of the TGFβ1 gene and susceptibility to adenocarcinoma in NSCLC. The study revealed the genotype or allele frequencies of this particular SNP between individuals with NSCLC and the HC group. Additionally, a significant difference was observed between the adenocarcinoma with squamous cell carcinoma and a healthy adjusted tumor sample.

The gene that encodes Smad2 is located at 18q21. It has also been proposed as a putative tumour suppressor target for 18q LOH. Mutation of Smad2 occurs at a very low frequency in 2% Non-Small Cell Lung Cancer (NSCLC) (12, 48).

Numerous prior investigations have indicated a potential correlation between the expression level of SMAD2 in cancer cells and the development and prognosis of tumors in patients diagnosed with gastric carcinoma, glioma, breast cancer, colorectal cancer, and esophageal squamous cell carcinoma. However, the prognostic significance of SMAD2 expression in lung cancer cells remains largely unknown (49). Furthermore, there exists a notable paucity of empirical data regarding the prognostic implications of Smad2 expression in stromal fibroblasts in the context of lung cancer.

Furthermore, SMAD2 has a critical role in TGFβ induced apoptosis of prostate epithelial cells activated by TGFβ1 (13). Ying, Z. et al. suggest that in highly metastatic NSCLC cells with intact TGFβ signaling, unknown molecules yet to be identified may act to interfere with the function of SMAD2, particularly following its activation by phosphorylation, such that the suppressive effect of SMAD2 on cellular survival is inhibited (49).

It is valuable that the SMAD2 gene is frequently subject to genomic deletion or loss-of-function mutations in certain cancer types, including colorectal and pancreatic cancers. The occurrence of genomic modifications of SMAD2 is infrequent in several cancer types, such as breast and lung cancer. (3). SMAD2 has also harbored mutations in colorectal and Lung tumors (14). Smad2 is a crucial constituent situated downstream of the TGFβ signaling cascade. The expression of SMAD2 is significantly higher in colorectal cancer tissue in comparison to the normal mucosa of the colon. Additional research indicates that it is essential in promoting tumour progression (50). Overexpression of the SMAD2 in NSCLC may be related to the expression of TGFβ1 mutation because TGFβ1 regulation effect on SMAD2 massager to the nucleus effect.

In our study, SMAD2 genetic expression supports the tumour cell in stages III and IV by activating TGFβ1. However, in another study, the evidence that SMAD2 maps to a tumour suppressor locus at 18q21 and is mutated in colorectal cancers provides the possibility that SMAD2 is a tumour suppressor gene in sporadic colon cancers (51).

The protein -catenin is encoded by the CTNNβ1 gene, which is involved in cell-cell adhesions, and is typically expressed at the membrane of epithelial cells (52). Mutations in the β-catenin gene (CTNNβ1) affecting the amino-terminal region of the protein make it refractory to regulation by APC (53). Alterations in the gene encoding β-catenin (CTNNβ1) have been noticed in numerous human malignancies, which comprises lung cancer (20, 54). Most mutations were noted in Exon 3 of the CTNNβ1 gene, with missense mutation being the most common (19, 55). Mutations in the β-catenin gene appear uncommon in lung cancer. However, a case of lung cancer mutation exon 3 of CTNNβ1 involved lung adenocarcinoma progression was recorded (56). In the present study, we identified mutations in exon 3 of CTNNβ1 lung adenocarcinomas.

In our study, Exon 3 mutation of the adenocarcinoma 13% SNP and insertion 2% of adenocarcinoma in NSCLC. That the β-catenin gene is mutated in a subset of lung cancer and that some lung cancer cell lines express lower amounts of β-catenin protein despite substantial expression. Exon 3 of the β-catenin gene encodes the NH2-terminal regulatory domain, and its mutation or deletion has been identified as inducing stabilization of β-catenin in various human malignancies (18). Patients with low-grade, early-stage endometrial cancer who have CTNNβ1 (encodes -catenin) mutations are at a higher risk of recurrence. β-catenin protein translocation from the membrane to the nucleus and stimulation of Wnt/β-catenin signaling when mutation takes place in CTNNβ1 exon 3 which it is useful detection of endometrial carcinomas that occur as a result of CTNNβ1 mutation. (52). The findings suggest that the CTNNβ1 mutation-induced persistent activation of the Wnt signaling pathway plays a role in the advancement and/or onset of a particular type of lung carcinoma, with a preference for adenocarcinoma. Zhou, C. study show (5.3%) of patients with CTNNβ1 mutations were identified (54). Oncogenic activation of CTNNβ1 by interstitial deletion of exon 3 has been reported in colorectal and hepatocellular carcinomas (56). According to Shigemitsu et al. (2001), lung cancer seldom harbors a CTNNβ1 mutation (18). Another study was conducted on CTNNβ1-mutated non-small cell lung carcinomas tested by next-generation sequencing for mutations in exon 3 of the CTNNβ1 gene (2.48%). Among lung tumors, CTNNβ1 mutations have only previously been reported in adenocarcinomas (57). They may have different mutation rates related to aggressive tumors and stages of cancer. Different mutation rates depend on types of cancer organs and stage of cancer cells.

β-catenin is degraded after the conjunction of serine-threonine phosphorylation sites for GSK-3β that is expressed by Exon 3 of CTNNβ1. Missense mutations represent the predominant type of CTNNβ1 mutation (58).

Besides colon cancer, in various human tumors, including liver, endometrium, ovary, prostate, stomach, and anaplastic thyroid cancer, the β-catenin gene is activated via point mutation or deletion exon 3 that codes a regulatory element required for protein degradation (18). However, the results of this analysis show that Exon3 mutations are common in non-small cell lung cancer (NSCLC).

The Human Wnt3a gene located on the chromosome and the canonical Wnt (1q42.13) has been regarded as activating the β-catenin accumulation signaling pathway (24, 59). The Wnt3a protein is a member of the Wnt family that triggers the activation of the canonical Wnt signaling pathway (22). Wnt3a, as a canonical Wnt ligands member, regulates cellular functions such as cell proliferation, differentiation, self-renewal, and motility. Furthermore, to further support the regulatory role of Wnt3a in cell apoptosis, Wnt3a, as a canonical Wnt ligand, is highly expressed in various cell types, including human scirrhous gastric carcinoma, prostate cancer, breast cancer, and epithelial cell lines (1). In addition, Wnt3a upregulates transforming growth factor TGFβ signaling through SMAD2 in a b-catenin-dependent way(60). Research on human Wnt3a has primarily centered on its significant involvement in human malignancy. The elevated expression of Wnt3a in various tumors has been associated with a poorer prognosis. (61).

Also, our study has more Mutation single nucleotide polymorphism Wnt3a 39% SNP,2% deletion, and 2% duplication of adenocarcinoma in NSCLC patients. A study conducted by Lisha Qi and colleagues involved an examination of Wnt3a expression in a diverse range of colon cancer tissue samples, with the aim of elucidating

its involvement in the progression of colon cancer (22). He, Q. has revealed a novel regulatory role of Wnt3a on tumour cell proliferation during serum deprivation (17). An additional study indicated that 88.2% of the participants exhibited affirmative Wnt3a expression. The expression of Wnt3a was found to be significantly upregulated in samples exhibiting advanced clinical stages and metastasis/recurrence (22). Furthermore, a separate investigation initially examined the expression of Wnt3a in numerous tissue samples of colon cancer to ascertain its function in the advancement of colon cancer. Our findings indicate a noteworthy association between the expression of Wnt3a. This outcome is in line with a recent investigation on colorectal cancer, which demonstrated a marked upregulation of Wnt3a in both primary and metastatic lesions (22). Studies on human Wnt3a have focused primarily on its key role in human malignancy, and its high expression in many kinds of tumors was correlated with a worse outcome. The present collection of literature on the correlation between Wnt3a and hepatocellular carcinoma (HCC) is restricted, and further investigation is necessary to determine its prognostic significance. Therefore, Pan, L.H. *et al.* study's objective was to investigate alterations in the expression of oncogenic Wnt3a in cancerous tissues and to explore its clinical utility as a novel molecular marker for HCC prognosis (61).

To conclude, the utilization of circulating oncogenic Wnt3a as a marker has been validated in a group of patients diagnosed with hepatocellular carcinoma (HCC) or chronic liver diseases (24). There was no statistically significant correlation observed between Wnt3a expression and variables such as gender, age, and tumour size. In the current study, the expression of serum in low circulatory expression was 17% positive for NSCLC. The different expressions related to cancer cell stage and the detection methods.

High expression of Wnt3a in different kinds of tumors was related to a worse outcome. The findings are promising, and the initial evidence confirmed that Wnt3a is one of the key molecules in the Wnt/β-catenin pathway in Adenocarcinoma and NSCLC generally.

The results of this study showed that the genetic differences of TGFβ1/SMAD2 and Wnt3a/CTNNβ1 might play important roles in how NSCLC grows and forms new blood vessels. These results may help us learn more about how NSCLC angiogenesis works at the molecular level and may help us find a new way to treat NSCLC. In this investigation,

CYFRA21-1 and CEA levels in NSCLC were statistically more significant than in controls. CYFRA21-1 and CEA have a higher value in mele and squamous cell carcinoma and high significance between gender and types of NSCLC patients—statistically non-significant correlation between CYFRA21-1 and CEA with all biomarkers. The findings of the present study indicate that genetic variations and elevated levels of the cytokines under investigation may exert a substantial impact on angiogenesis and the pathogenesis of non-small cell lung cancer (NSCLC). We need more understanding mechanisms of the biomarker's mutation signaling in NSCLC lung carcinogenesis.

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Author contributions
Hemn Abdalla Omer: Conceptualization, data curation, methodology, formal analysis, project administration, writing, original draft, and writing review and editing. Kawa Amin: Conceptualization, methodology, data curation, formal analysis, visualization, resources, software, supervision, and writing—review and editing.

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