RESEARCH ARTICLE

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An Integrative Approach to Lung Cancer Therapy: Linking the TGF-β (+869 C/T) Polymorphism to a Structurally Validated Natural Inhibitor

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Abstract

Objective: This study aimed to create a genetic and clinical roadmap for the TGF-β pathway in an Iraqi population, focusing on the TGF-β1 (+869 C/T) polymorphism. **Methods:** A structure-guided approach was used to identify a natural compound, quercetin, as a potential inhibitor of its primary receptor, ALK5. The clinical aspect evaluated the biochemical profile of each patient by measuring serum levels of TGF-β and IL-17, while assessing the genetic predisposition of each individual using ARMS-PCR to genotype the TGF-β1 (+869C/T) polymorphism. Following the evaluation of clinical findings, a molecular docking study was conducted to investigate how quercetin interacts with the ALK5 kinase domain (PDB: 1VJY). **Result:** The study demonstrated a two-fold signature of risk among the patients. First, the levels of serum TGF-β and IL-17 were significantly elevated ($P < 0.05$) in lung cancer patients compared to healthy individuals. Second, patients exhibited a genetic predisposition to lung cancer—specifically, those with the "TT" genotype had a higher risk of developing the disease than individuals with the protective "CC" genotype. Furthermore, the model illustrated that quercetin forms key hydrogen bonds with the ALK5 kinase hinge residues Glu245 and Lys232, which are essential for inhibitory binding. **Conclusion:** This study established a strong connection between the activity of the TGF-β pathway, the +869C/T polymorphism, and lung cancer susceptibility in the Iraqi population. Additionally, it provided conclusive structural evidence supporting quercetin as a highly promising natural inhibitor of ALK5.

Keywords: Lung cancer- TGFB1 Polymorphism- ALK5 / TGF-β Receptor 1- Molecular docking

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide and the second most frequently diagnosed malignancy in both men and women in the United States [1, 2]. This formidable foe is not homogeneous but is divided mainly into non-small cell (NSCLC) and small cell lung cancer, histologic variants that dictate how we treat this disease [3]. Although the relationship between tobacco smoking continues to be a major factor (accounting for approximately 80-90% of all lung cancer deaths) there are many other factors such as radon exposure that contribute significantly to the total burden of this disease [4]. What may be most remarkable is how dramatically the face of lung cancer is changing. The long-standing areas where male lung cancer incidence has been higher than female have begun to shrink, as the rate of female lung cancer incidence continues to grow. One of the most notable trends in this area is the increasing rate at which adenocarcinoma is becoming more frequent than squamous cell carcinoma [5, 6].

Cancer progression may be understood from an analysis of the tumor's immune microenvironment and how cytokine systems govern this immune environment to produce an anti-cancer response; there are two primary ways this occurs: T helper 1 (Th1) cytokines such as IL-2 and IFN-γ are responsible for a localized, cell-mediated attack on cancer cells whereas T helper 2 (Th2) cytokines including IL-4 and IL-10 result in a systemic or humoral response to fight the cancer [7].

Increasingly, the evidence supports a particular conclusion: It is the aggressive, cell-mediated approach not the humoral which has been proven to be the best defense against the long term chronic inflammation that will ultimately allow for cancer to develop [9]. The study also supports this claim by identifying "smoking gun" examples namely high levels of pro-inflammatory agents (such as IL-6 and IL-8) in the sera of smokers, whose

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elevation has been strongly associated with an increased risk of developing lung cancer [9, 10].

While the fundamental role of TGF-β is well understood, the genetic variations that regulate its function in specific populations is not clear. Single nucleotide polymorphisms such as the TGFB1 +869 C/T variant can provide an increased susceptibility for cancer by their ability to modify gene and protein function [11, 12]. The knowledge gained from this is difficult to translate into therapy due to the dramatic duality of the pathway; a blunt attempt to inhibit it could risk silencing critical tumor-suppressive effects [13]. In our quest to find more elegant cancer therapies, we turn to the natural products that provide the most organic route to the selective inhibition of the TGF-β pathway [14-16].

To realize the therapeutic capabilities of quercetin (a flavonoid which has shown efficacy against cancer), we will need to go from understanding what quercetin does as an agent, to how quercetin acts at the molecular level. The main objective of this research project is to determine if quercetin directly engages the ALK5 domain. If such physical docking occurs, this would be the basis for a valid therapeutic approach.

The Aim of this study was to create a pathway to translate a population-based lung cancer problem into a molecular-based solution. In doing so, we are addressing two critical gaps simultaneously: First, by systematically characterizing the clinical and genetic landscape of TGF-β pathway dysfunction within an Iraqi lung cancer cohort, with focus upon the +869 C/T polymorphism and subsequent biochemical outcomes; Second, through computational validation of quercetin as a structural inhibitor of ALK5. Using our methods, we plan to provide a direct mechanistic connection between the genetically inherited risk and a pharmacologic countermeasure that can be targeted against the molecular defect, thereby providing a foundation for the next generation of precision medicine-based interventions in lung cancer that will utilize more advanced and accurate targeting strategies.

Materials and Methods

This study included 185 LC patients, ranging in age from 20 to 70, were involved in this study; they were receiving treatment at the Medical City Department's Oncology Teaching Hospital in Baghdad. Every patient was informed of the goal of these studies and consented to the protocol. For comparison, 180 healthy participants were included in the current study. The participants had been divided into two study groups, with each group consisting of:

- Group (1): 180 healthy people as control.
- Group (2): 185 LC patients as second group.

Inclusion and exclusion criteria

Individuals between the ages of 20 and 70 who had been diagnosed with lung cancer were included in the trial; individuals whose disease had progressed to other organs were not.

Blood samples

Each participant had 5 ml of venous blood drawn in order to evaluate the levels of many cytokines and the TGF-B (+869 C/T) polymorphism.

Extraction of DNA

For DNA extraction, two milliliters of venous blood were drawn from each participant or subject and placed in an EDTA tube. The salting out approach was used to isolate DNA [17].

PCR Premix kit

According to the Korean company BIONEER's accompanying paper, an AccuPower® PCR PreMix kit was used for ARMS-PCR technology research.

Primers

The TGF-B1 (+869 C/T) mutant gene was identified using three of the unique primers, where the antisense was:

Forward inner primer (T allele): 235 GGGCTGCGGCTGCTTCT 251 (163bp).

Reverse inner primer (C allele): 269 AGCAGCGGTAGCAGCATCG 251(179bp).

Forward outer primer (5' - 3'): 91 GAGGACCTCAGCTTTCCCTCG 111 (306bp).

Reverse outer primer (5' - 3'): 396 CCGCAGCTTGGACAGGATCT 377

The recognition of TGF-B1 (+869 C/T)

The TGF-B1 (+869 C/T) gene was identified using the polymerase chain reaction technique of the ARMS-PCR replication system, and a minor deviation from the protocol was noted in the master mix preparation. In compliance with the BIONEER protocol, the ARMS-PCR technology investigations were carried out utilizing a kit called AccuPower®. Specific C and the antisense primer were used to identify the C allele, while specific T and the antisense primer were used to identify the T allele. The optimal TGF-B1 detection and recognition conditions (+869 C/T) were shown in Table 1.

Molecular docking study

To investigate the binding dynamics of our selected compounds, Molecular Operating Environment (MOE), version 2024, for all computational modeling and docking simulations was used. Given the computationally intensive nature of the induced fit protocol, these tasks were performed on a high-performance computing workstation. The structural basis of our study was derived from the high-resolution crystal structure of the human TGF-β Receptor I (ALK5) Kinase Domain Inhibitor Complexed

Table 1. The Most Appropriate Conditions for Recognition

Phase	Tm (0C)	Time	No. of cycle
Initial denaturation	94	3 min	1 cycle
Denaturation	94	45 sec	35 cycle
Annealing	56	1 min	
Extension	72	45 sec	

With SB-431542 (ID: 1VJY), obtained from the RCSB Protein Data Bank. The above-described crystal structure served as the primary source of our structural data; we used this structure to prepare the receptor for docking via the QuickPrep module within MOE. Preparation of the receptor for docking included removal of all solvent molecules, correction of all structural anomalies, and addition of hydrogen atoms as described by the Amber10: EHT force field. Finally, we performed a restrained energy minimization to resolve steric clashes, producing a chemically optimized receptor model.

Prior to running the docking simulations, the three-dimensional structures of our reference compound, quercetin (PubChem CID: 5280343) and our reference inhibitor, SB-431542 (PubChem CID: 9915831) were downloaded from PubChem and then minimized with respect to energy using the MMFF94x force field, prior to entering the docking simulation in a low energy state. To validate the entire computational method, we utilized a re-docking procedure that consisted of docking the previously minimized SB-431542 ligand into the active site of its native receptor. Success of this validation was determined by comparing the calculated root mean square deviation (RMSD) between the predicted pose of SB-431542 and the known experimental pose of the SB-431542/ALK5 complex. A comparison of the RMSD value to < 2Å indicated a correct representation of the known binding mode and validated the docking protocol. Once our protocol had been validated, we completed a comparative study that included a two-stage induced fit docking (IFD) process that provided a more detailed representation of the receptor's inherent flexibility. The Triangle Matcher algorithm produced a total of 100 possible binding orientations for the ligands. The top twenty possible binding orientations were further analyzed during a more rigorous refinement stage. As part of the IFD process, the Induced Fit protocol allowed for the movement of the receptor's side chain atoms and their adaptation to the ligand, providing a more accurate representation of the binding event. Finally, the binding affinities of the final refined binding orientation of each ligand were estimated by using the GBVI/WSA dG scoring function (ΔG). For our final analysis, we selected the top-ranked pose for each compound to meticulously examine the key intermolecular interactions, such as hydrogen bonds and hydrophobic contacts, that defined its binding.

Statistical analysis

PCR product data were compared using Fisher's at P <0.05 to identify which averages differed significantly using the SPSS statistical software. J. H. Abramson developed the Compare 2 Ver.3.04 program between 2003 and 2017 to assess genotypes, allele frequencies, odds ratios (OR), and confidence intervals (CI) [16]. Per the website www.had2know.com, the results were examined using the Hardy-Weinberg equilibrium rule.

Table 2. Number and Percentage of LC Patients and Control According to Sex

Sex	Control (n=180)		Patients (n=185)	
	No.	(%)	No.	(%)
Male	135	75%	136	73.5%
Female	45	25%	49	26.5%
Total	180	100%	185	100%

Results

Socio-demographic properties

The highest rate of LC was recorded in the male group, reaching 73.5%. While, the low rate of LC was reported in the female group, reaching 26.5%, as shown in Table 2.

The highest incidence of LC was identified in the 61-70-year group. While, the low rate of LC was identified in the 20-30-year group. The average age for LC individuals was 59.17±3.85 years, as shown in Table 3.

Cytokines

Table 4 shows the levels of various interleukins in LC participants. TGF-β levels indicated a significant (P < 0.05) elevated levels in patients (34.68± 4.09 pg/ml) compared to the healthy participants (10.74±1.13 pg/ml). Serum IL-4 concentrations in LC patients were significantly (P < 0.05) higher (79.31 ±5.42 pg/ml) than in healthy participants (48.11 ±3.95 pg/ml).

Molecular study

The current investigation found that the homozygous polymorphism (CC) genotype was related with a lower risk of developing lung cancer, whereas the TT genotype was associated with an increased risk among Iraqi patients. Where, the frequency of CC, CT and TT genotypes among patients was 28.1%, 17.3% and 54.6% respectively, compared with 47.8%, 23.7% and 28.3% respectively, among controls (Table 5). Statistically, there was a significant difference in the frequency of CC (OR=0.35, 95%CI=0.21-1.68, P=0.032) and TT genotype (OR= 0.257, 95%CI=0.44-1.13, p= 0.001) between patients and controls. TGF-β (+869 C/T) PCR optimization for +869 C / T detection was shown in (Figure 1). The temperature

Table 3. Number and Percentage of LC Patients and Control According to Age

Age (year)	Control (n=180)		Patients (n=185)	
	No.	(%)	No.	(%)
20-30	31	20.0%	9	2.3%
31-40	25	18.75%	15	7.1%
41-50	21	13.75%	23	16.5%
51-60	65	25.0%	49	34.1%
61-70	38	22.5%	89	40.0%
Total	180	100%	185	100%

Table 4. Some of Cytokines Levels in Studied Groups

Groups/ Parameter	Control (180)	Patients (185)	P-Value
TGF-β pg/ml	10.74±1.13	34.68± 4.09*	0.001
IL-17 pg/ml	48.11 ±3.95	79.31 ±5.42*	0.001

Table 5. The Genotype Frequencies for TGF-β (+869 C/T) in Subjects

	Genotype	LC samples (185)	Control samples (180)	(95%CI) OR	P value
TGF-β (+869 C/T)	CC	52 (28.1%)	86 (47.8%)	0.35 (0.21-1.68)	0.032*
	P.F	44.57%			
	CT	32(17.3%)	43 (23.7%)	1.13 (0.54-1.16)	0.126 NS
	E.F	16.55%			
	TT	101(54.6%)	51 (28.3%)	0.257(0.44-1.13)	0.001*
	E.F	38.84%			

*(P≤0.05); EF, Etiological faction; PF, Preventive faction

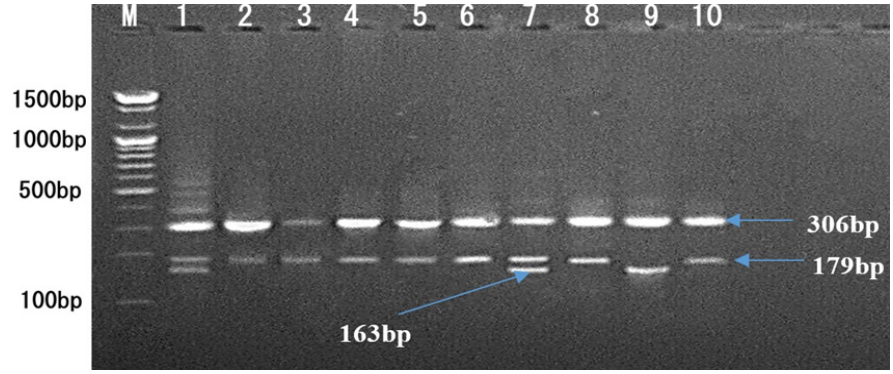

Figure 1. Representative Agarose Gel Electrophoresis of ARMS-PCR Products for the TGF-β (+869 C/T) Polymorphism. The analysis reveals three distinct genotypes based on the pattern of amplified DNA fragments. C common control band is visible at 306 and 179bp in all successfully amplified samples. The allele-specific primers produce (306, 179 and 163) bp band for the CT allele and 163 bp band for the T-allele.

at which annealing was performed was 62°C. These appropriate conditions were identified by the development of the thickest DNA fragment at the specified size, while reducing unspecific fragment.

Molecular docking results

In order for the results from the in silico study to be reliable, the authors initially tested the methodology of their in silico studies by validating the computational protocol with a redocking experiment on SB-431542 bound to the ALK5 kinase domain (PDB: 1VJY) using an induced-fit docking approach. The authors were able to reproduce the experimental binding mode of SB-431542 to the ALK5 kinase domain with a RMSD of 0.5313 Å. The authors state this is acceptable since it is less than the threshold of <2.0 Å established for induced-fit docking models; they also compared this to a rigid-receptor docking model with a RMSD of 2.6289 Å. Following the validation of the computational protocol the authors used the GBVI/WSA dG scoring function to determine the binding affinities of the reference synthetic inhibitor SB-431542, and the natural compound Quercetin. They

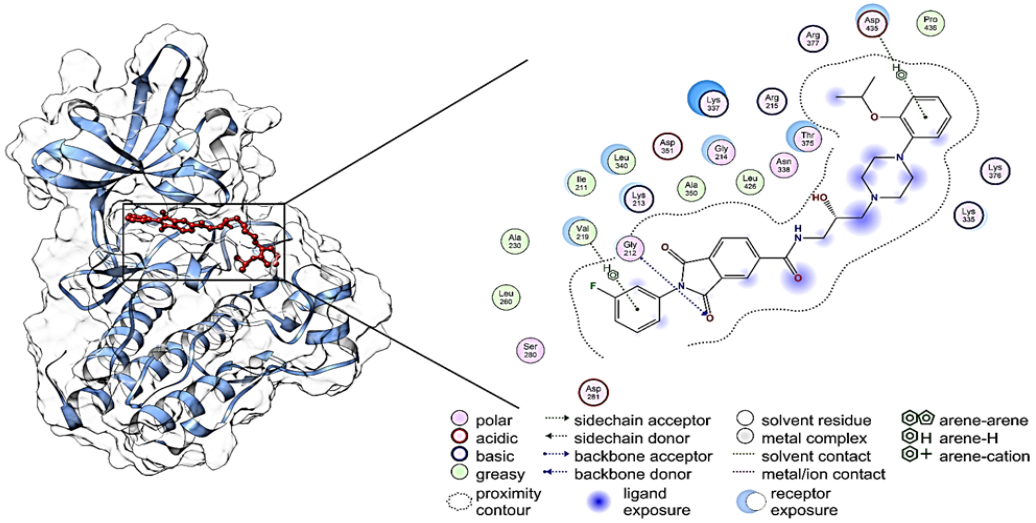

Figure 2. Binding Pose and Interaction Profile between Kinase Domain of the Human TGF-β Receptor 1 (PDB ID: 1VJY) and Inhibitor SB-431542.

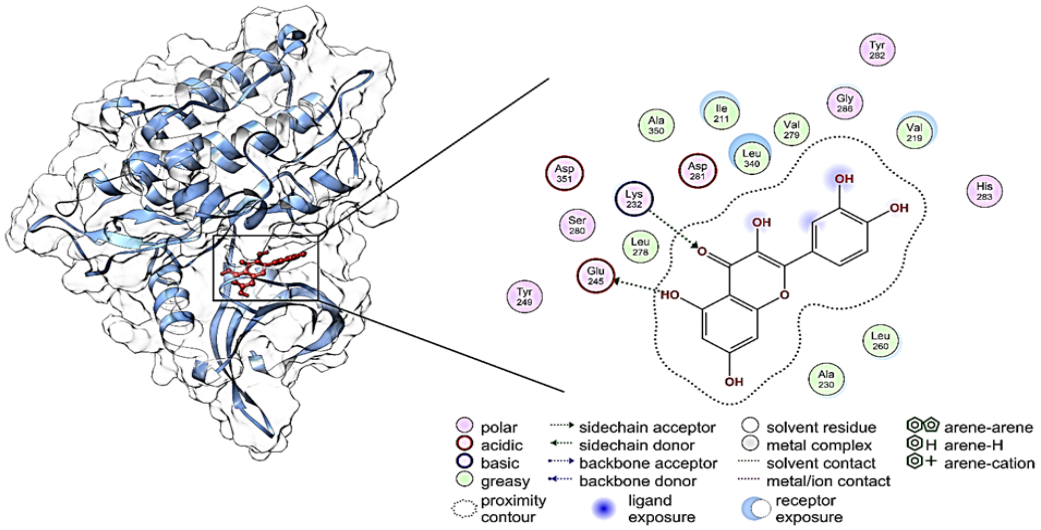

Figure 3. Binding Pose and Interaction Profile between Kinase Domain of the Human TGF-β Receptor 1 (PDB ID: 1VJY) and Quercetin.

Table 6. Molecular Docking Results for SB-431542 and Quercetin against the ALK5 Kinase Domain. The Docking Score (S) represents the final score calculated by the GBVI/WSA dG scoring function and is reported in kcal/mol.

Compound	Protein	Ligand	Receptor	Interaction	Distance (Ao)	E (Kcal/Mol)	S (doccking score)	RMSD
SB-431542	1VJY	O5	CA GLY 212 (A)	H-acceptor	3.48	-0.5	-9.1643	2.6289
		6-ring	CG1 VAL 219 (A)	pi-H	4.07	-0.6		
		6-ring	CA ASP 435 (A)	pi-H	4.29	-0.9		
Quercetin		O3	OE2 GLU 245 (A)	H-donor	2.85	-0.5	-7.1869	0.5313
		O4	NZ LYS 232 (A)	H-acceptor	3.37	2.1		

found SB-431542 had a docking affinity of -9.1643 kcal/mol and Quercetin had a docking affinity of -7.1869 kcal/mol. Additionally, when analyzing the structural binding poses of both compounds, the authors found that Quercetin occupied the same ATP-binding site as SB-431542. Specifically, the authors identified that Quercetin formed two critical hydrogen bonds with hinge region residues Glu245 (E245) and Lys232 (K232) of the ALK5 kinase domain at a distance of 2.85 Å and 3.37 Å, respectively as indicated in Figure 2, 3 and Table 6.

Discussion

Based on the study's results, as indicated in Table 2, the great majority of the patients (77.6%) were all men. In support of this conclusion, Hu et al. [18] and Khue et al. [19] found that men made up the great majority of study participants. In the province of Al-Najaf, lung cancer was also found to be more common in men than in women, with 67% and 33%, respectively, in many earlier studies [20]. The current study's frequency of lung cancer in people aged 56–65 years was 31.6%, which was almost the same as in another study, and 27% for people aged 46–55 years [21], in contrast, [22] they found that people over 70 had a higher incidence of lung cancer due to changes in lifestyle that might have been caused by exposure to lung cancer germs.In addition, a number of smoking samples in the current study seventy-eight samples total amount to 65%, which means that men are more likely than women to develop lung cancer, especially in groups older than fifty. This could be due to a higher percentage of men smoking compared to women, as well as other risk factors that men were more exposed to than women, such as cigarette smoking.

The highest incidence of LC was identified in the 61-70-year group. While, the low rate of LC was identified in the 20-30-year group. The average age for LC individuals was 59.17±3.85 years, as shown in Table (3).

According to the research's age-related findings, as indicated in Table 2, the majority of the patients examined are older than 59. The findings of Qian and Hou [23], which indicate that most of the subjects in the study were older than 50, are in line with this result. In addition, Weiss et al. [24] found that the majority of study participants were at least 60 years old. In another study, Tong et al. [25] discovered that a majority of the sample was in the age range of 51 to 60 years. Table 3 of the current study also indicated that most of the age groups affected by lung cancer were 51-60 years and 61-70 years, while Siegel et al. [22] they showed more incidence of lung cancer in those aged over 70 years, because of changes in the style of life that may be reflected the exposure to lung cancer infection.

High TGF-β levels have been linked to lung cancer in previous research, and survival among individuals with non-small cell lung cancer (NSCLC) has been found to be correlated with TGF-β protein expression [26-28]. The tumorigenesis of the patients in these investigations

seems to be influenced by TGF-β. Patients with more advanced tumor stages had higher levels of TGF-β protein expression [28, 29]. Furthermore, if the TGF-β protein expression was higher than normal, patients seem to have a higher chance of receiving a diagnosis of lymph node metastases [30, 31]. The findings demonstrated that, when compared to the seemingly healthy control group, there were significant ($p \leq 0.05$) differences in the levels of IL-17 among patients with LC. One pro-inflammatory cytokine that contributes to the development and spread of tumors, the promotion of non-small carcinoma growth, and the activation of angiogenesis is IL-17 [32]. Similar to this study, earlier research [33] found that patients with lung cancer had higher levels of IL-17 in their serum when compared to the healthy participants. This was due to the fact that IL-17 plays a crucial role in the development of the microenvironment that supports tumor growth and the progression of tumors, as well as its crucial role in the fibrosis of organs like the lung [34]. Additionally, [35] they showed that the amount of IL-17 in tumor tissues was much higher than that in lung tumor tissues close to and normal distant tissues. The results of the current study also agreed with the study of Al Araji and Baydaa [36] who that indicated that the serum levels of IL-17 among patient cases with lung cancer (0.2309 ± 0.06659) pg/ml were the presence of significant ($p \leq 0.05$) that compared with apparently healthy control group (0.1377 ± 0.0117) pg/ml because of the contributions of IL-17 in the progression of tumors and the formation of the microenvironment that provides it growth in addition to the pivotal role of IL-17 in the disease fibrosis in organs as lung. According to Ying et al. [37], TGF-β signaling has two roles in the formation of tumors. Early on, TGF-β signaling prevents the development and spread of human prostate cancers. In contrast, TGF-β functions as an oncogenic signal when cancer is advanced. Drugs that target TGF-β can greatly decrease tumor cell metastasis to bone [38], and the constitutive activation of the TGF-β pathway has been associated to bone metastasis in a range of malignancies, including lung cancer, according to a research by Dai et al. [39]. The effect of genetic variations on TGF-1 expression was the main focus of Sachidanandam et al.'s study [40]. The most prevalent type of polymorphism is single nucleotide polymorphism (SNP). They are distributed throughout the gene, but they are most frequently located in regions that are next to protein-coding genes, which are essential for gene expression. MicroRNA target sequence, alternative splicing, messenger RNA (mRNA) stability, signal peptide-mediated protein trafficking to the endoplasmic reticulum (ER), and amino acid-based protein activity can all be altered by SNPs [41]. Variation in the genetic of TGF-β1 (single nucleotide polymorphism) could have a possible role as a predicator for accuracy for overall survival in patients with non-small cell lung cancer [42]. The research conducted by Ren et al. [43] and Kang et al. [44] suggested that TGF-β1 T+869C and C-509T protected against lung. Lung cancer risk factors were also identified by other investigations as the TGF-β1 T+ 869C and C-509T polymorphisms. Although the docked position of Quercetin was slightly different than that of SB-431542 (the synthetic benchmark), the comparative docking scores indicate Quercetin has substantial potential to physically interact with the ALK5 receptor. According to the authors, the engagement of Glu245 and Lys232 in hydrogen bonding is key to understanding how Quercetin acts as an ATP competitor, since both are part of the kinase hinge area. Since the authors describe Quercetin's interaction with the TGF-β pathway as being competitively inhibitory to ATP binding, this interaction explains why Quercetin can inhibit the TGF-β pathway's oncogenic signaling. In addition, the authors relate the molecular basis of Quercetin's ability to inhibit TGF-β signaling to the study's clinical results, which indicate the presence of a molecular therapeutic strategy for treating TGF-β hyperactivity due to genetic predisposition. Nonetheless, the authors point out that there are limitations to the clinical use of Quercetin at present, primarily due to its poor bioavailability and extensive first pass metabolism. Therefore, the authors advocate for the development of new pharmaceutical formulations (e.g., nanoparticles, liposomes) to protect the effectiveness of Quercetin and provide a basis for translating Quercetin from a theoretical model to a viable treatment for humans.

In conclusions, the current study made a crucial connection between population-based lung cancer risk and targeted molecular treatments. The clinical evidence on one end of the bridge shows there is a clear population-based signature of risk for Iraqi patients (high levels of TGF-β/IL-17 & high-risk TT genotype). The molecular blueprint on the opposite end of the bridge, demonstrates that the natural product Quercetin docks with and blocks the action of ALK5 which drives the malignant effect of TGF-β. What is being described here is much more than simply identifying a relationship (correlation), but rather, defining a structural pathway from the identification of a clinical issue to a potential treatment. The finding links what makes a patient susceptible to lung cancer to how molecular inhibition occurs. As such, the results define the critical scientific basis needed for future studies to develop these new treatments. The findings of this study establish a scientifically based rationale for the transformation of a well-documented natural product into a valid drug candidate to be used as a weapon against lung cancer.

Author Contribution Statement

N. B. A, M. A. H., R. H. Y., and M. S. J. conceptualized the study and drafted the manuscript. N. B. A. and R. H. Y. responsible for running experiments, while N. B. A and M. A. H. collected the data. N. B. A, M. A. H., R. H. Y., and M. S. J. were responsible for data extraction and analysis. All authors read and approved the final manuscript.

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