Original Article

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Exploring the Effect of Lutein on TGF-β/SMAD2 Signalling Molecule Gene Expression in Lung Cancer Cells: An In-vitro Study

Oncology Section

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ABSTRACT

Introduction: Lung cancer is one of the most prevalent and deadly forms of cancer worldwide, accounting for a significant number of cancer-related deaths. Despite advances in diagnosis and treatment, the prognosis for lung cancer remains poor, emphasising the need for alternative and complementary therapeutic approaches. Transforming Growth Factor-beta (TGF-β) is a multifunctional cytokine that plays a crucial role in cell growth, differentiation, apoptosis and immune regulation. Suppressor of Mothers against Decapentaplegic (SMAD), a well known transcription factor, plays an essential role in carcinogenesis. Lutein, a naturally occurring carotenoid. Recent studies have suggested that lutein possess anticancer properties and could potentially be used as a therapeutic agent against various types of cancer.

Aim: To assess the effect of lutein on TGF-β/SMAD2 gene expression in lung cancer cells.

Materials and Methods: The present in-vitro study was carried in the Cancer and Stem Cell Laboratory Facility, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from April 2023 to May 2023. The lung cancer cell line (A-549) was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cell viability of lutein-treated lung cancer cells was assessed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cell morphology was studied using a phase contrast microscope. The gene expression of TGF-β/SMAD2 was analysed using real-time Polymerase Chain Reaction (PCR), and the results were presented graphically as fold change. All data obtained were analysed by one-way Analysis of Variance (ANOVA) followed by Student's t-test using PRISM software version 4.

Results: The findings suggest that lutein had good cytotoxic effects on lung cancer cells. The Half-maximal inhibitory concentration (IC50) value was found to be 5.14 μM/mL. Lutein inhibited the TGF-β and SMAD2 gene expression signalling pathway and induced apoptosis in A549.

Conclusion: Lutein significantly inhibited the TGF-β and SMAD2 gene expression signalling pathways, making it a potent anticancer drug. The downregulation of key genes involved in the TGF-β/SMAD2 pathway suggests a nuanced and intricate relationship between lutein and the regulatory mechanisms governing cellular signalling in lung cancer. These findings support the notion that lutein may exert a regulatory influence on the intricate balance of signalling cascades, offering a potential avenue for therapeutic intervention in lung cancer.

Keywords: Signalling cascade, Suppressor of mothers against deccapentaplegic, Transforming growth factor-beta

INTRODUCTION

Lung cancer is one of the most prevalent and deadly forms of cancer worldwide, accounting for a significant number of cancer-related deaths. Patient survival has not clearly increased despite the recent and noteworthy development of novel lung cancer therapy approaches, such as tyrosine kinase inhibitors and immunotherapies [1]. The prognosis for lung cancer remains poor, emphasising the need for alternative and complementary therapeutic approaches. TGF-β is a multifunctional cytokine that plays a crucial role in cell growth, differentiation, apoptosis and immune regulation. Dysregulation of TGF-β signalling pathways has been implicated in various diseases, including cancer. TGF-β signalling is mediated through the SMAD family of transcription factors, particularly SMAD2, which plays a key role in transmitting signals from the cell surface to the nucleus [2].

Lutein, a naturally occurring carotenoid, is primarily known for its role in promoting eye health. It plays significant roles in human health, particularly the health of eyes and brain, due to their antioxidant attributes. A good source of lutein is marigold flowers, *Tagetes erecta L*., Family Compositae [3]. Recent studies have suggested that lutein may possess anticancer properties and could potentially be used as a therapeutic agent against various types of cancer, including lung cancer [4]. However, the underlying mechanisms of lutein's anticancer effects, particularly its impact on TGF-β/SMAD2 signalling in lung cancer cells, remain poorly understood [5]. Due to their antioxidant properties, lutein and its isomers zeaxanthin and meso-zeaxanthin, which are frequently found in green leafy vegetables, avacados and eggs, play important roles in human health, notably the health of the eyes and brain [6,7].

The present study aims to explore the effect of lutein on TGF-β/SMAD2 signalling molecule gene expression in lung cancer cells. By investigating the changes in gene expression profiles induced by lutein treatment, insights can gained into the molecular mechanisms underlying lutein's potential anticancer properties. Furthermore, understanding how lutein modulates TGF-β/SMAD2 signalling may reveal novel therapeutic targets and provide a basis for developing lutein-based therapies for lung cancer. Various molecular biology techniques, including cell culture, lutein treatment, gene expression analysis and functional assays, are applied to test it. Lung cancer cell lines will be treated with different concentrations of lutein and gene expression changes will be analysed using techniques such as quantitative real-time Polymerase Chain Reaction (qPCR) and microarray analysis. Additionally, the authors will assess the functional consequences of lutein treatment on TGF-β/SMAD2 signalling by evaluating cell proliferation, apoptosis and migration assays. The results of the present study may contribute to the understanding of lutein's potential as a therapeutic agent for lung cancer and shed light on its mechanism of action through the modulation of TGF-β/SMAD2 signalling. Ultimately, this research could pave the way for the development of novel targeted therapies or combination treatments for lung cancer patients, improving their overall prognosis and quality of life.

MATERIALS AND METHODS

The present in-vitro study was carried in the Cancer and Stem Cell Laboratory Facility, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from April 2023 to May 2023, after approval from the Scientific Review Board (SDC/R 07/2023).

Study Procedure

Cell Line Maintenance: The authors acquired A-549 lung cancer cell lines from the NCCS, located in Pune, India. In T25 culture flasks supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics, Dulbecco's Modified Eagle Medium (DMEM) was used to cultivate these adenoma cancer cells. The cells were kept in a humidified environment with five percent CO2 at 37ºC. The cells were trypsinised and passaged, once they reached confluency.

Cell Viability (MTT) Assay: The cell viability of lutein-treated lung cancer cells was assessed by MTT assay. The study was done in triplicate to avoid manual errors. The assay relies on metabolically active cells reducing soluble yellow tetrazolium salt to insoluble purple formazan crystals. A-549 cells were seeded at a density of 5x103 cells/well in 96-well plates. After plating, the cells were starved for three hours at 37ºC in serum-free media, and they were then twice rinsed with 100 µL of the medium. The assay relies on metabolically active cells reducing soluble yellow tetrazolium salt to insoluble purple formazan crystals. A-549 cells were seeded at a density of 5x103 cells/well in 96-well plates. After plating, the cells were starved for three hours at 37ºC in serum-free media, and they were then twice rinsed with 100 µL of the medium. After starvation, cells were treated with different concentrations of lutein (1-10 µM/mL) for 24 hours. At the end of treatment, the medium from the control and treated cells was discarded, and 100 µL of MTT-containing DMEM (0.5 mg/mL) was added to each well. The cells were then placed in the CO2 incubator and incubated for four hours at 37ºC. After discarding the MTT-containing media, 1x PBS was used to wash the cells. Subsequently, the formazan crystals were dissolved in 100 µL of dimethyl sulfoxide and allowed to sit in the dark for an hour. The colour developed's intensity was then measured at 570 nm using a Micro Enzyme-linked Immunosorbent Assay (ELISA) plate reader. The control cells are the same as the treated cells with the exception that they are without lutein treatment. The number of viable cells was expressed as a percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability=(A570 nm of treated cells/A570 nm of control cells)×100. Furthermore, the IC50 was calculated. IC50 is the half-maximal inhibitory concentration and it represents the concentration of a drug required to inhibit cell viability by 50%.

Morphology Study: The MTT assay was used to determine the ideal doses (IC-50: 5.1 µM/mL) for more research. Using a phase-contrast microscope, analyse in cell morphology. After seeding 2×10^5 cells in six-well plates, the cancer cells were exposed to 5.1 µM/mL of lutein for 24 hours. The media was withdrawn from the cells at the conclusion of the incubation time, and they were once again cleaned with Phosphate Buffer Saline (PBS, pH 7.4). Using a phase-contrast microscope, the plates were examined.

Real Time PCR: The gene expression of TGF-β/SMAD2 was analysed using real-time PCR. Real-time PCR results were analysed by the comparative Threshold Cycle, {C(T)} method, and the 2 delta {C(T)} method was used for fold change calculation as described by Schmittgen and Livak, 2008. The total Ribonucleic Acid (RNA) was isolated by the standardised protocol using Trizol Reagent (Sigma). Two µg of RNA was used for complementary Deoxyribonucleic Acid (cDNA) synthesis using reverse transcription with a PrimeScript 1st strand cDNA synthesis kit (TakaRa, Japan). Using certain primers listed in [Table/Fig-1], the targeted genes were amplified.

Gene	Forward	Reverse	Tm
TGF-β1	ACACCAACTATTGCTTCAG	TGTCCAGGCTCCAAATG	53
SMAD2	GTCTCTTGATGGTCGTCTC	GGCGGAAGTTCTGTTAGG	60
GAPDH	CGACCACTTTGTCAAGCTCA	CCCCTCTTCAAGGGGTCTAC	58

[Table/Fig-1]: Real-time Reverse Transcriptase-PCR (RT-PCR) primer sequences.

GoTaq® qPCR Master Mix (Promega), which includes all of the PCR components and SYBR green dye, was used to run the PCR reaction. Real-time PCR was carried out using a Biorad CFX96 PCR machine. Comparative CT analysis was utilised to analyse the data, and Schmittgen and Livak's 2-ΔΔCT technique was employed to calculate the fold change.

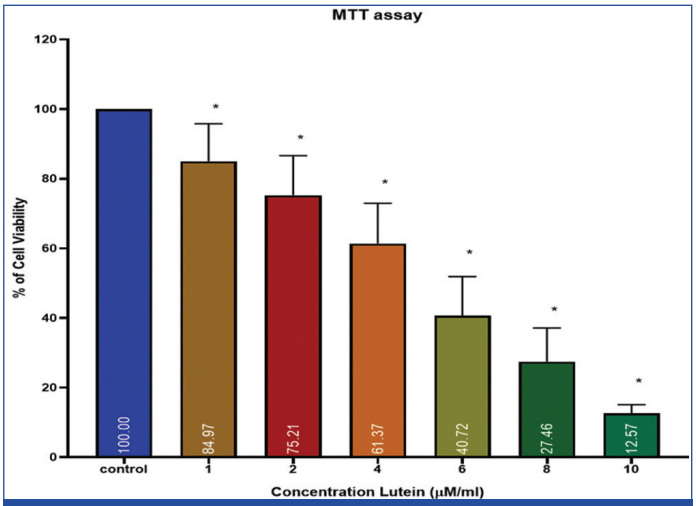
STATISTICAL ANALYSIS

All data obtained were analysed by one-way ANOVA followed by Student's t-test using PRISM software version 4, represented as mean±Standard Deviation (SD) for triplicates. The level of statistical significance was set at p-value<0.05.

RESULTS

Effect of Lutein on Cell Viability of Lung Cancer Cell Line

The MTT assay was utilised to evaluate the cytotoxic potential of lutein in lung cancer cells. For a full day, the cells were exposed to various lutein concentrations (10-120 µg/mL). At the 24-hour mark, the treatment dramatically reduced the viability of A-549 cancer cells compared to the control [Table/Fig-2]. As the concentration increased, the percentage of viable cells decreased progressively. A 50% growth inhibition was noted at a concentration of 5.14 µg/mL. So, for the upcoming trials, an IC50 dose of 5.14 µg/mL was taken into consideration.



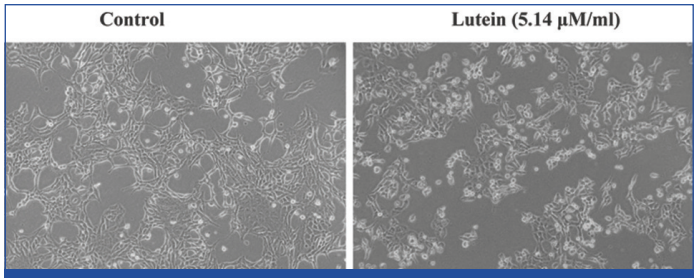
[Table/Fig-2]: The cytotoxic effects of lutein on lung cancer cells. Cells were treated with extract lutein (1-10 µM/mL) for 24 hours and cell viability was evaluated by MTT assay. Data are shown as means±SD (n=3).
*Compared with the control blank group, p-value<0.05.

The Effect of Lutein on Cell Morphology

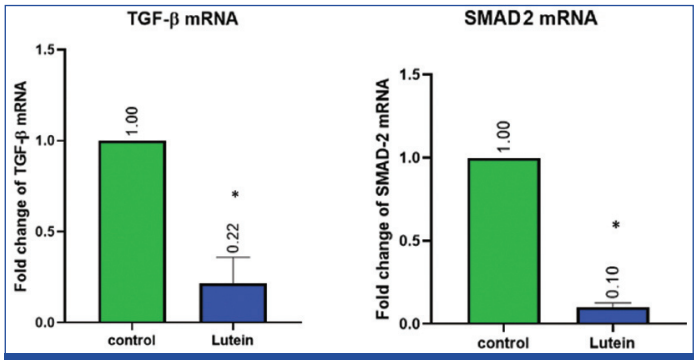
The morphological investigation of lutein-treated cells was done under an inverted phase-contrast microscope. After being exposed to lutein (5.14 µg/mL) for 24 hours, the A-549 cells were compared to the control group. The treated cells exhibited notable morphological alterations, such as shrinkage and decreased density, which are indicative of apoptotic cells [Table/Fig-3].

The Effect of Lutein on TGF-β/SMAD2 Gene Expression

The TGF-β/SMAD2 gene expression was assessed. Target gene expression is normalised to GAPDH mRNA expression and the results were expressed as a fold change [Table/Fig-4]. The fold change indicated a downregulation in the genes, indicating good anticancer potential.



[Table/Fig-3]: Effect of lutein on cell morphology of human lung cancer cell line (A-549). The cells exhibited cell shrinkage and cytoplasmic membrane blebbing.



[Table/Fig-4]: Effect of lutein (5.14 µM/mL) on TGF-β/SMAD2 gene expression in lung cancer cell line. Each bar represents mean±SEM of three independent observations.
*represents statistical significance between control vs lutein treatment groups at p-value<0.05 level.

DISCUSSION

Lung cancer is a type of cancer that begins in the lungs. It is one of the most common cancers worldwide and a leading cause of cancer-related deaths. There are two main types of lung cancer, Non-small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). NSCLC is more common, accounting for about 85% of all lung cancers, while SCLC is a more aggressive form. Early detection of lung cancer is crucial for successful treatment. Diagnosis often involves imaging tests like Computed Tomography (CT) scans}, biopsies and other procedures to determine the type and stage of the cancer [8,9].

Lutein is a carotenoid, a type of pigment found in certain fruits, vegetables and other plants such as marigold [10]. It is particularly abundant in dark green, leafy vegetables such as spinach, kale and collard greens. Lutein, along with its isomer zeaxanthin, is known for its role in supporting eye health. Few studies have explored its inhibitory effect on adipogenesis [11]. Lutein acts as an antioxidant, neutralising free radicals that can cause damage to the eyes [12,13].

The MTT assay is a commonly used colourimetric assay to measure the viability and proliferation of cells [4]. This assay is widely employed in cell biology and drug discovery research to assess the effects of compounds on cell viability. The MTT assay is based on the ability of living cells to reduce a yellow, water-soluble tetrazolium salt (MTT) to a purple, insoluble formazan product [13]. The absorbance of the resulting solution is then measured using a spectrophotometer. In the present study, cell viability was reduced upon treatment with increasing concentrations of lutein. This suggests that lutein has good anticancer potential due to a significant increase in cancer cell death. The IC50 dose was found to be 5.14 µM/mL.

Cell morphology assays are techniques used to study and analyse the physical characteristics and shapes of cells. These assays provide valuable information about the structure, size and appearance of cells, which can be indicative of various cellular processes, health and responses to experimental conditions. The A-549 cell line was observed under a phase contrast microscope post-treatment with lutein. The number of cells decreased after lutein (5.14 µM/mL) treatment, and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. This suggests that lutein has good proapoptotic effects and promotes cancer cell death.

The TGF-β/SMAD2 signalling pathway involves the activation of SMAD2 by phosphorylation and its subsequent translocation to the nucleus, where it regulates the transcription of target genes. TGF-β/SMAD2 signalling can regulate various target genes involved in cell cycle control, apoptosis, extracellular matrix production and immune response, among other processes. In lung cancer, the TGF-β/SMAD2 pathway can be dysregulated, leading to altered gene expression patterns that contribute to the development and progression of the disease [14,15]. Fold change in gene expression is a measure used to quantify the relative change in expression levels of a gene between two experimental conditions or groups. It provides information about the magnitude of the difference in gene expression. The fold change of TGF-β/SMAD2 gene expression in the lung cancer cell line indicates a downregulation of the genes. The dysregulation of these genes causes the progression of the disease. Therefore, the results suggest that lutein has good anticancer potential.

Studies on the anticancer effects of lutein have shown its potential against various types of cancers [14]. One study shows that lutein promotes growth inhibition of breast cancer cells through increased cell type-specific Reactive Oxygen Species (ROS) generation [15]. The results from a study by Zhang WL et al., show that lutein inhibits the Phosphatidylinositol 3-kinase/ Protein kinase B (PI3K/ AKT) signalling pathway and induces apoptosis in A549 [4]. The present study focused on the TGF-β/SMAD-2 gene expression, and the findings imply that lutein causes apoptosis in A549 and inhibits the TGF-β and SMAD2 gene expression signalling pathway. As a result, lutein may be employed as a powerful and natural anticancer medication. The Real-time Reverse Transcriptase-PCR (RT-PCR) primer sequences used in the study are given in [Table/Fig-1].

Limitation(s)

The present study has only investigated the TGF-β/SMAD2 signalling in lung cancer cells. Further exploration of other pathways and in-vivo studies using animal models are to be carried out in the future to warrant the use of lutein in lung cancer cells.

CONCLUSION(S)

The findings suggest that lutein had good cytotoxic effects on lung cancer cells. Lutein inhibited the TGF-β and SMAD2 gene expression signalling pathway and induced apoptosis in A549, therefore, it may be used as a potent natural anticancer drug. The ability of lutein to modulate TGF-β/SMAD2 signalling at the molecular level opens up possibilities for targeted therapeutic strategies aimed at disrupting or mitigating aberrant signalling in lung cancer. Lutein has significantly inhibited the TGF-β and SMAD2 gene expression signalling pathway. It is a potent anticancer drug. There is a need for further exploration into the precise molecular mechanisms underlying lutein's effects on TGF-β/SMAD2 signalling. In-vivo studies and clinical trials are required to further assess its anticancer potential.

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