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Short Communication

PTEN-Akt/mTOR Expression Level in A549 Lung Cancer Cells in Response to Cisplatin, Carotenoids, and their Combination

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ABSTRACT

The negative impacts of cisplatin often lead to undesirable side effects. Moreover, the issue of resistance has been recognized as a substantial hurdle in achieving effective therapeutic results. Multiple studies have emphasized the reliance of particular tumors, such as lung cancer, on the PI3K/Akt/mTOR pathway. Cisplatin is known to initiate Akt regulation in cancer cells, which ultimately makes these cells resistant to apoptosis. Despite the existence of specific inhibitors tailored to intervene in the PI3K/Akt/mTOR pathway, the continuously evolving epigenetic environment of this cascade plays a role in the development of resistance in cancer cells against these inhibitors. Antioxidants, exemplified by carotenoids, have garnered attention due to their versatile roles. They function as pro-oxidants, initiating apoptosis in cancer cells, while also acting as antioxidants that promote the restoration of normal cell function. This study aimed to examine the influence of bixin and fucoxanthin, both separately and in conjunction with cisplatin, on the transcriptional levels of PTEN, Akt/mTOR, and the tumor suppressor p53 in A549 cell lines. Cell viability was assessed using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Drug combinations were performed in accordance with the Chou-Talalay theorem. The Real-Time Quantitative-Polymerase Chain Reaction (RT-q-PCR) method was employed to evaluate the expression levels of the PI3K/Akt/mTOR genes. The ongoing study furnished proof that carotenoids, particularly bixin and fucoxanthin, showcase anticancer attributes and have the potential to complement cisplatin chemotherapy in lung cancer cells. This potential is realized by influencing the modulation of PTEN and the decrease in Akt and mTOR expression.

Keywords: lung cancer, cisplatin, carotenoids, PTEN, Akt/mTOR, p53

Introduction

Cancer represents a significant public health concern, particularly in developing nations.[1–3] Among the various types of cancer, lung cancer carries the highest mortality rate and is ranked as the third most prevalent cancer in multiple countries.[4] Even after undergoing surgery, lung cancer patients remain at risk of recurrence. As a result, adjuvant chemotherapy, such as cisplatin-based regimens, is often administered to stage 2 and 3 patients.[5] However, chemotherapy drugs commonly induce toxic effects in patients. For instance, a study reported that 89% of patients receiving cisplatin and vinorelbine chemotherapy experienced toxicity levels of 3-4, with 49% requiring hospitalization.[6]

Cisplatin, also known as cis-diamine dichloroplatinum (II), is a chemotherapeutic agent based on platinum metal. Once inside the cells, it undergoes activation and forms complexes that interact with cell membranes and components within the cytoplasm. Therefore, the toxicity of cisplatin is not solely dependent on its DNA binding ability but also on its interactions with cytoplasmic proteins.[7] Although cisplatin and its analogs have demonstrated effectiveness in treating various types of cancer, they are associated with side effects and the development of cisplatin resistance.

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The mechanism of cancer and tumor resistance to cisplatin includes: (i) pre-target resistance, namely inhibition of cisplatin binding to DNA by reducing cellular accumulation or increasing binding to cytoplasmic components; (ii) target resistance, i.e. tolerating or repairing cisplatin-bound DNA; (iii) post-target resistance through altered signaling in response to DNA damage by cisplatin; and (iv) off-target resistance, i.e. through a mechanism that does not involve cisplatin-initiated signals directly but allows cells to prevent cisplatin-induced cell death.[8,9]

The two primary pathways involved in cell survival, Akt and extracellular signal-regulated kinase 1/2 (ERK1/2), exhibit relatively higher regulation and activity levels in cancerous tissues, including lung cancer.(10) Cisplatin has the ability to trigger Akt regulation in cancer cells, resulting in resistance to apoptosis.[11] Downstream signaling of Akt, which includes the mammalian target of rapamycin (mTOR) pathway, plays a crucial role in modulating protein synthesis, cell division, and the cell cycle. Some tumors, including lung cancer, depend on the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway.[10,12] Moreover, the PTEN-PI3K axis, involving the phosphatase and tensin homolog deleted on Chromosome 10 (PTEN), also plays a role in regulating cellular activity. Therefore, targeting and modulating this axis is an important aspect of cancer therapy.[13]

Despite the existence of various inhibitors designed to target the PI3K/Akt/mTOR cascade in cancer cells, resistance mechanisms have emerged as a result of genetic alterations.[10] Consequently, novel strategies are required to effectively target the genes and proteins involved in this cascade, including the exploration of agents derived from natural products.(10,14) The relatively low toxicity of many natural products allows for their selective use as anticancer agents while preserving normal cells. Therefore, natural products hold great potential as adjuvants in cancer chemotherapy.

Antioxidant, such as carotenoids, have garnered interest due to their unique dual functionality as both antioxidants and pro-oxidants, which

can induce apoptosis in cancer cells by generating reactive oxygen species (ROS).[9,15] Fucoxanthin, a carotenoid, has been found to inhibit the growth of non-small-cell lung carcinoma (NSCLC) cells, promoting apoptosis and inhibiting cell metastasis through the upregulation of p53, p21waf1/cip1, PUMA, and fas, while downregulating Bcl-2.[16] Furthermore, fucoxanthin enhances the sensitivity of lung cancer cells to gefitinib, reducing the necessary chemotherapy dosage to suppress cell growth.[16] Additionally, recent research has shown that bixin is a more effective inhibitor of A549 and HeLa cell proliferation compared to cisplatin.[9] However, no study to date has investigated the expression of PTEN, Akt/mTOR, and the tumor suppressor p53 in A549 NSCLC cell lines in response to carotenoids, specifically fucoxanthin and bixin. Moreover, no study has examined the combined effects of these carotenoids with cisplatin, which is a potential chemotherapy with known adverse effects and resistance issues.[9,15] Carotenoid fucoxanthin has been reported to inhibit non-small-cell lung carcinoma (NSCLC) cell growth, exhibits apoptosis and inhibition of cell metastasis through the upregulation of p53, p21waf1/cip1, PUMA, fas, and downregulation of Bcl-2.[16] In addition, fucoxanthin increases the sensitivity of lung cancer cells to gefitinib by reducing the amount of chemotherapeutic required to suppress cell growth.(16) Additionally, recent research has shown that bixin is a more effective inhibitor of A549 and HeLa cell proliferation compared to cisplatin.[9] However, there is paucity of information on research involving the expression of PTEN, Akt/mTOR, and the tumor suppressor p53 in A549 NSCLC cell lines in response to carotenoids, specifically fucoxanthin and bixin, two profound carotenoids with anticancer properties.

Materials and Methods

Chemical and cell lines

The lung cancer cell line A549 was obtained from American Type Culture Collection. Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax (Cat. No. P04-01550), 10% fetal bovine serum (FBS) (Cat. No. P30-3306), Penicillin-Streptomycin (Cat. No. P06-07100), and Trypsin 0.5%/EDTA 0.2% (Cat. No. P10-024500) were obtained from PAN-Biotech, Aidenbach, Germany. Trypan blue 0.4% solution (Cat. No. 12788) and 10X Phosphate Buffered Saline (PBS) (Cat.No. 78529) were purchased from Sisco Research Laboratories Pvt. Ltd.(SRL), Mumbai, India. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Cat. No. BA0004) was obtained from Assay Genie, Dublin, Ireland. Cisplatin, cis-diamine dichloroplatinum (II), fucoxanthin, and bixin were obtained from Sigma Aldrich, Darmstadt, Germany. All laboratory plastic ware was obtained from Wuxi NEST Biotechnology Co., Ltd, Jiangsu, China.

Culture of cancer cells

The A549 cancer cell line was grown in DMEM with Glutamax, supplemented with 10% FBS, 500 U/mL penicillin, and 500 g/mL streptomycin. The cell was cultured in 75 cm^3 cell culture flask at 37 ºC with continuous 5% CO_2 supply. Fresh media was added after sub-culturing cells every 3 days.[17]

Cytotoxicity assay

To determine the inhibitory concentration (IC) or effective dose (ED) required to hinder cell growth, a cytotoxicity assay was employed. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was utilized to assess dose-dependent cytotoxicity. A total of 5×10^4 cells were seeded into each well of 96-well plates and incubated for 24 hours. Subsequently, cisplatin, bixin, and fucoxanthin were added to the culture media, followed by an additional 48-hour incubation. After the incubation with the test compounds, 100 µL of 0.5 mg/ml MTT solution was added to each well and allowed to incubate for 3 hours, enabling viable cells to generate formazan crystals. The formed crystals were then dissolved in 100 µL of development solution and incubated for 15 minutes on a shaker. The absorption of the formazan was measured at 570 nm using the Nanoquant Infinite M200 Pro (Tecan, Switzerland). Cell viability was further confirmed using trypan blue dye solution.[17]

Drug combinations

In the present study, a combination design was employed, following the method developed by Chou[18], using constant ratios of the tested substances. The chosen ratios were based on previous studies and consisted of a combination of carotenoid and cisplatin at their respective IC_{50} values. These combinations were applied to the 96-well plates, with each well undergoing a two-fold dilution. The cytotoxic activity of the combinations was evaluated using the MTT assay, as described earlier. To assess drug interactions, the combination index method (CI) was utilized, following the median effect principle. The median-effect equation was employed to calculate Dx, the dose of a drug that inhibits 'x' percent of cells:

Combination Index (CI): $CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$

To analyze the combination effect and calculate the combination index (CI), CompuSyn Ver.1.0, developed by Ting-Chao Chou and Nick Martin, was employed, similar to our previous study.[17–19] Cell viability was validated using trypan blue dye solution.

Drug treatment for the RNA isolation

A549 cells were subjected to individual treatments with cisplatin, fucoxanthin, and bixin, as well as combinations of cisplatin with either fucoxanthin or bixin. The combinations were formulated using a constant ratio of the combined drugs that exhibited synergistic interaction, which are Combination Cisplatin and Bixin (1 : 1) and Combination Cisplatin and Fucoxanthin (16 : 1).[9] In brief, a total of 3×10^5 cells were cultured in each well of 6-well plates for 24 hours. Subsequently, either the compound alone or a combination of compounds was added to the cells as per the experimental design. The cells were then incubated for 48 hours at 37 ℃ with a constant 5% CO2 supply. Following the incubation period, RNA was extracted from both the treated and untreated cells.

RNA isolation

Total RNA was extracted from both the treated and untreated A549 cells using the Total RNA Mini Kit (Blood/Cultured Cell) from Geneaid Biotech, Ltd, New Taipei City, Taiwan, following the manufacturer's instructions (Cat. No. RB 100). The concentration and purity of the extracted RNA were assessed using a Nanodrop spectrophotometer from Thermo Fisher Scientific.

Real-Time Quantitative-Polymerase Chain Reaction (RT-qPCR)

A 100 pg/mL of RNA from each treated cells was reverse transcribed using the SensiFAST™ SYBR® No-ROX One-Step kit (BioLine, London, United Kingdom, Cat. No. BIO-72001) following the quantitative PCR with a total reaction volume of 20 µL. The gene expression of the Akt1, PTEN, mTOR, and p53, was amplified using the following primers: Akt1 F: 5'-TTCTGCAGCTATGCGCAATGTG-3', R: 5'-TGGCCAGCATACCATAGTGAGGTT-3', PTEN F: 5'-GGTTGCCACAAAGTGCCTCGTTTA-3', R: 5'-CAGGTAGAAGGCAACTCTGCCAAA-3', mTOR F: 5'-AGTGAATGACATCTTCACGTTTG-3', R: 5'-GTGCTGAGTTTGCTGTACCCATGT-3', p53 F: 5'-CCCTCCTCAGCATCTTATCCG-3', R: 5'-CAACCTCAGGCGGCTCATAG-3', and GADPH F: 5'-TGCACCACCAACTGCTTAGC-3', R: 5'-GGCATGGACTGTGGTCATGAG-3'. Reverse transcription was performed at 45 ℃ for 10 min, followed by 95 ℃ for 2 min, and 40 cycles of denaturation at 95 ℃ for 5 s, annealing at 60 ℃ for 10 s, and elongation at 72 ℃ for 5 s. Relative gene expression was normalized to the expression of GAPDH that is not significantly changed and the relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method (Livak method).

Data analysis

The Ct values obtained from the RT-qPCR analysis were subjected to a relative quantitative approach, comparing the treated and untreated cells. To normalize the data, the expression of the reference gene GAPDH was used for both groups of cells. The RT-qPCR experiments

were conducted in triplicate, with two technical replicates for each repetition, resulting in a total of three independent repeats. Statistical analysis was performed using SPSS software to determine significance, with a threshold set at $p<0.05$. The data are presented as mean ± standard deviation. The relative quantification diagram was generated using GraphPad Prism 8.0.1 software.

Results and Discussion

The PI3K/Akt/mTOR pathway and the PTEN-PI3K axis are important signaling cascades implicated in tumor development. However, these pathways have been shown to exhibit resistance to commonly used chemotherapeutic agents, including cisplatin. In our previous study, we demonstrated that fucoxanthin and bixin have the ability to suppress the growth of lung and cervical cancers and enhance the sensitivity of lung cancer cells to cisplatin.[9] Nevertheless, the precise mechanism by which fucoxanthin and bixin affect the PTEN-PI3K/Akt/mTOR signaling pathway and PTEN itself remains unclear. In the present study, we examined the gene expression profile of A549 cells treated with fucoxanthin, bixin, and cisplatin, either individually or in combination. Our objective was to elucidate whether fucoxanthin and bixin can reverse cisplatin chemoresistance by modulating key proteins such as Akt, which is known to be overexpressed in chemo resistant cancer cells and induced by cisplatin. Additionally, we investigated the expression of p53, a protein localized in the cell nucleus that inhibits tumor formation by restraining excessive or uncontrolled cell growth and proliferation in A549 cancer cell lines.[20]

p53 and Akt expression level

p53 is known to be activated in response to cellular stress and DNA damage.[21] Cisplatin, an anti-cancer agent, can form cross-links with the purine base of the DNA molecule.[22] As shown in Figure 1, the expression p53 was found to be highest in cells treated with cisplatin compared to those treated with carotenoids alone or their combinations. This observation suggests that cisplatin induces more DNA damage, leading to increased p53 expression. At the protein level, p53 activation indirectly influences the modulation of the caspase apoptotic pathway by localizing to mitochondria and interacting with Bcl-2 family proteins, ultimately inducing mitochondrial outer membrane permeability. The subsequent release of cytochrome c and activation of procaspase-3 lead to caspase-3 activation and apoptosis.[23] Previous studies have reported that fucoxanthin acts as a competitive inhibitor of mortalin, a protein that binds to and inactivates p53.[24,25] The formation of the fucoxanthin-p53 complex allows p53 to translocate to the nucleus, where it becomes activated in cancer cells.[24]

It is well-known that both fucoxanthin and bixin possess strong antioxidant properties. Interestingly, many antioxidants derived from carotenoids can also exhibit pro-oxidant activity by increasing the production of Reactive Oxygen Species (ROS) in cancer cells, leading to cell damage and triggering the activation of p53.[15,26,27] In the present study, the combination of cisplatin with either fucoxanthin or bixin was able to mitigate the cell damage induced by cisplatin in A549 lung cancer cell lines, as evidenced by the reduction in p53 expression (Figure 1). This protective effect could be attributed to the balanced antioxidant-pro-oxidant properties of the carotenoids present in the combination treatment.

The activation of Akt is initiated by the phosphorylation of threonine 308, which subsequently regulates various target proteins involved in critical cellular processes such as cell growth, proliferation, cell cycle progression, transcription, protein synthesis, cell survival, and glucose metabolism. The activation of Akt plays a significant role in tumor development and has been implicated in the inhibition of apoptosis through the inactivation of pro-apoptotic proteins.[28] Previous research has indicated that Akt can be activated in response to DNA damage, including damage induced by cisplatin.[11]

In the present study, it was observed that cisplatin administration resulted in the highest expression of Akt in A549 lung cancer cells, suggesting that cisplatin-induced DNA damage activates Akt and potentially influences cancer cell development. Conversely, treatment with fucoxanthin or bixin alone significantly reduced Akt expression in A549 lung cancer cell lines compared to untreated cells. This indicates that both carotenoids have potential as anticancer agents by inhibiting Akt, which may reduce the likelihood of apoptosis resistance. Furthermore, the antioxidant properties of carotenoids contribute to the prevention of DNA damage and subsequent Akt activation. Interestingly, the presence of fucoxanthin and bixin also led to a decrease in Akt expression induced by cisplatin. The reduction in excess DNA damage may also contribute to the lower level of p53 expression observed in cells treated with carotenoids.

PTEN and *mTOR* genes expression

PTEN plays a crucial role in inhibiting tumor cell growth and enhancing sensitivity to apoptosis. Alterations or loss of PTEN function have been implicated in tumorigenesis and tumor progression.[29,30] Additionally, PTEN activity is known to downregulate the PI3K/Akt/mTOR pathway.[31] In the present study, it was observed that PTEN expression was significantly reduced in cells treated with cisplatin compared to untreated cells, suggesting a potential loss of PTEN function and the development of resistance to apoptosis. Conversely, treatment with fucoxanthin and bixin resulted in a significant increase in PTEN expression, rendering the cells more susceptible to apoptosis. Moreover, cells treated with a combination of cisplatin and either fucoxanthin or bixin exhibited higher PTEN expression compared to cells treated with cisplatin alone (Figure 2).

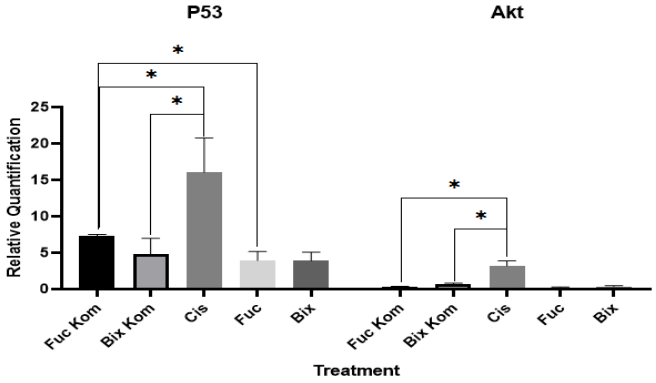

Figure 1: Relative Quantification of p53 (left side) and Akt (right side) genes expression of A549 cells treated with Fucoxanthin and Cisplatin in Combination (Fuc Kom), Bixin and Cisplatin in Combination (Bix Kom), Cisplatin (Cis), Fuc (Fucoxanthin), and Bix (Bixin). * Indicates a significance difference ($p < 0.05$)

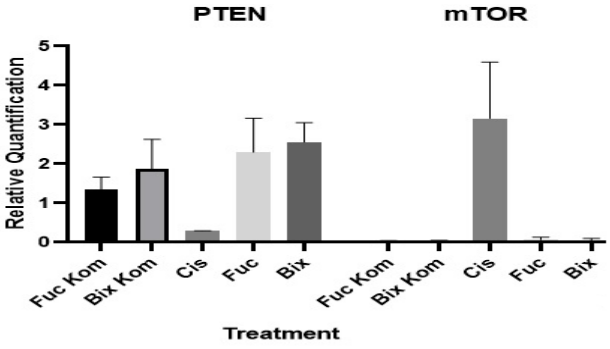

Figure 2: Relative Quantification of PTEN and mTOR genes expression of A549 cells treated with Fucoxanthin and Cisplatin in Combination (Fuc Kom), Bixin and Cisplatin in Combination (Bix Kom), Cisplatin (Cis), Fuc (Fucoxanthin), and Bix (Bixin).

mTOR, a serine/threonine kinase, comprises two complexes known as mTORC1 and mTORC2. mTORC1 phosphorylates key effectors such as 4EBP1, S6K, and SREBP, leading to enhanced protein translation, lipid synthesis, and lysosomal biogenesis. On the other hand, mTORC2 is sensitive to extracellular growth factors and activates downstream targets to promote signaling cascades, cytoskeleton growth, cell migration, and inhibit apoptosis.[32] Activation of mTORC2 occurs through the phosphorylation of PIP2 to PIP3 on the plasma membrane,

a process that can be hindered by PTEN.[33-35] Moreover, phosphorylated Akt can also activate mTOR, resulting in dysregulated apoptosis, proliferation, and cell motility.[11] Figure 2 demonstrates that the highest expression of mTOR was observed in cells treated with cisplatin. The loss of PTEN function induced by cisplatin leads to PIP2 phosphorylation, subsequently triggering mTOR activation. Treatment with fucoxanthin or bixin alone significantly suppressed mTOR expression. Although the combination of cisplatin with fucoxanthin or bixin also reduced mTOR expression, the decrease was not statistically significant compared to the administration of carotenoids alone.

Conclusion

The PI3K/Akt/mTOR pathway plays a crucial role in tumor development, beginning with the synthesis of PIP2 to PIP3 (phosphatidylinositol-3,4,5-triphosphate) in the cell membrane. Activation of Akt and PI3K signaling in cancer cells can be regulated by PTEN. The findings of this study provide evidence that carotenoids, specifically fucoxanthin and bixin, hold promise as potential anticancer agents and as adjuvants to cisplatin chemotherapy in lung cancer cells. These carotenoids exhibit their effects by activating PTEN, thereby reducing the levels of Akt and mTOR.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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ADRN: conceptualization, data collection, methodology, data analysis, and writing original draft, TFT: data collection, methodology, formal analysis, and writing - review and revised, FC: data collection, methodology, data analysis, and writing - review. All authors contributed to the article and approved the submitted version.

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