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Original Article

The effect of medicarpin on PTEN/AKT signal pathway in head and neck squamous cell carcinoma

Aysel Kalayci Yiğin, Huseyin Donmez[1], Mustafa Hitit[2], Sena Seven[3], Nadire Eser[4], Ercan Kurar[5], Mehmet Seven

Department of Medical Genetics, Cerrahpaşa Medical Faculty, Istanbul University-Cerrahpaşa, [3]Department of Pharmacognosy, Faculty of Pharmacy, Istanbul University, Istanbul, [2]Department of Genetics, Faculty of Veterinary, Kastamonu University, Kastamonu, [4]Department of Pharmacology, Faculty of Medicine, Kahramanmaras Sutcu Imam University, Kahramanmaraş, [5]Department of Medical Biology, Meram Medical Faculty, Necmettin Erbakan University, Konya, Turkey, [1]Division of Pharmacy and Optometry, Faculty of Biology Medicine and Health, University of Manchester, Manchester, UK

For correspondence: Dr. Aysel Kalayci Yiğin, Department of Medical Genetics, Cerrahpaşa Medical Faculty, Istanbul University-Cerrahpaşa, Istanbul 34098, Turkey. E-mail: akyigin@iuc.edu.tr

Submitted: 21-Apr-2021
Accepted in revised form: 03-Jun-2021
Published: 20-Dec-2021

Access this article online
Website: www.cancerjournal.net
DOI: 10.4103/jcrt.jcrt_641_21
Quick Response Code:

ABSTRACT

Background/Aim: We aimed to investigate the *in vitro* modulating effects of medicarpin on the PI3K/AKT signal pathway gene expressions in head and neck squamous cell carcinoma (HNSCC).

Materials and Methods: The effect of medicarpin on *PTEN* and other associated genes in the PTEN/AKT signal pathway was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, real-time quantitative polymerase chain reaction, and Western blot analysis in the SCCL-MT1 (HNSCC) and control (HEK-293) cell lines.

Results: The IC50 dose was 80 μM as a result of medicarpin treatment on HNSCC cells ($P = 0.0006$). It was found that *PTEN* and *AKT* gene expressions increased after the medicarpin administration while *PDK1* gene expression was decreased in SCCL-MT1 cells ($P = 0.0002$, $P = 0.0003$, and $P = 0.05$, respectively). Protein expression results showed that medicarpin-treated cells significantly increased in pAKT ($P = 0.024$), pPTEN ($P = 0.032$), and decreased pPDK1 ($P = 0.059$) in SCCL-MT1.

Conclusions: Our data show that medicarpin modulates HNSCC cells by increasing the *PTEN* and decreasing *PDK1* expressions. *PDK1* gene expression effects mTOR pathway which may increase *AKT* gene. Our study suggests that both medicarpin extracts combination with the HNSCC drug may be more effective in cancer treatment. Future prospective studies that integrate molecular and pharmacogenetic studies are crucial for revealing the mechanism and preventive medical efforts.

KEY WORDS: Head and neck squamous cell carcinoma, *in vitro*, medicarpin, PTEN/AKT signal pathway

INTRODUCTION

Cancer is a very common disease throughout the world with a high mortality rate.[1] Head and neck cancers, which make up about 4% of all cancers, are the sixth most common cancers in the world. It is a major cause of morbidity and mortality and responsible for >900,000 cases and 370,000 deaths annually worldwide.[2] Approximately 90% of head and neck cancers consist of head and neck squamous cell carcinomas (HNSCCs) with a 5-year survival rate only of ~50%. Although surgical techniques, chemotherapy, and radiation therapy have many developments, the overall survival rates in the patients with HNSCC have not significantly decreased over the last 10 years.[3]

Among the identified risk factors in HNSCC, smoking, consuming alcohol, HPV infections, and premalignant lesions increase tumor development. In addition to these risk factors, mutations of *EGFR*, *p53*, *p16*, *PTEN*, and *RB* genes or over activation of some signal pathways play a role in the pathogenesis of the disease.[4-6] In the literature, PI3K/AKT is the most altered signal pathway and approximately 80% of HNSCC contain genetic mutations in one or more genes of the PI3K/AKT pathway.[7,8]

Activation of the PTEN/AKT signal pathway leads to increased cell growth, survival, apoptosis, and cell migration which are critical in control of cellular processes. The *PTEN* gene is a tumor-suppressor gene involved in the PI3K/AKT signal pathway and its inactivation result in dysregulation of the signal pathway. Activation of the PI3K/AKT signal promotes its downstream targets and is also widely

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Cite this article as: Yiğin AK, Donmez H, Hitit M, Seven S, Eser N, Kurar E, *et al*. The effect of medicarpin on PTEN/AKT signal pathway in head and neck squamous cell carcinoma. J Can Res Ther 0;0:0.

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associated with chemotherapeutic resistance.[8] Therefore, the modulation of various molecular signalling pathways including PI3K/AKT can be considered a good strategy for complementary therapies.

Naturally occurring drugs have long been used in the ancient systems for the treatment of many diseases, and nontoxic phytochemicals are being considered complementary or an adjuvant for the effective treatment of many diseases especially cancer. Half of all the anti-cancer drugs approved internationally are either natural products or their derivatives and were developed based on knowledge gained from small molecules or macromolecules that exist in nature.[9,10] Medicarpin is a natural bioactive pterocarpan with a wide variety of biological activities.

In recent years, many new bioactivities of medicarpin have been discovered, such as anti-osteoporosis and bone protection, antiapoptotic, anticancer, antibacterial, and antifungal effects.[11-14] Although medicarpin has bioactivities reported in some cancer types, its effectiveness on HNSCC is not fully understood. In this study, we aimed to investigate the *in vitro* modulating effects of medicarpin on the PI3K/AKT signal pathway genes in HNSCC.

MATERIALS AND METHODS

Chemical and reagents

Cell culture and TRIzolTM reagents were procured from Invitrogen (Massachusetts, USA). Medicarpin (98.0%) were purchased from Hangzhou DayangChem (#CAS: 32383-76-9, Chine). SensiFASTTM cDNA Synthesis Kit, antibodies, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were bought from respectively Bioline (London, United Kingdom), Cell Signaling Technology (Danvers, USA) and Boster Biological Technology (California, USA). SCCL-MT1 (HNSCC) cell line was supplied by Professor Alan Eppstein's laboratory and HEK-293 (negative control) cell line was supplied by ATCC.

Cell culture

SCCL-MT1 and HEK-293 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO^2. Before experiments, cells were cultured in a drug-free medium for at least 7 days.

Cell viability assay

Cell viability was assessed by the MTT assay. Briefly, cells growing in suspension were harvested and again were seeded in a 96 well plate at a density of 5×10^3 cells per well and incubated with increasing concentrations (0–300 μM) of the appropriate compound for 72 h. In each control experiment, an equal volume of DMSO which is used to dissolve extracts or compounds was added to each well. After incubation, 20 μl MTT (2.5 mg/ml) was added to each well for 3 h. Then, the medium was removed, and formazan crystals were dissolved in 200 μl DMSO. Absorbance was measured at 540 nm using a microplate reader and triplicate experiments were conducted in each test. Percent viability was calculated as (Absorbance of treated sample/Absorbance of the nontreated sample) ×100.

Real-time quantitative polymerase chain reaction assay

Total RNA was extracted from cultured cells using TRIzol™ reagent at the room temperature for 5 min and centrifuged for 5 min (15,000 × *g*, 4°C). The supernatant was collected, and 0.1 ml chloroform was added and mixed at the room temperature for 5 min and the sample was centrifuged again for 15 min (15,000 × *g*, 4°C). An equal volume of isopropanol was added to the supernatant at the room temperature for 10 min, then centrifuged for 10 min (15,000 × *g*, 4°C). Nuclease-free water was used to dissolve the RNA pellet. The absorbance of the RNA sample was measured at 260 and 280 nm (OD260 and OD280), and the concentration of the RNA was calculated.

The extracted total RNA (1 μg) was reversely transcribed into cDNA using the SensiFAST™ (Meridian Life BioSciences, USA) cDNA Synthesis Kit. The settings for reverse transcription were 25°C for 10 min (primer annealing); 42°C for 15 min (reverse transcription); 85°C for 5 min (inactivation), and 4°C hold (or chill on ice). The cDNAs were stored at −80°C. Quantitative polymerase chain reaction (qPCR) mix for each gene was set up in 20 μl final reaction mix containing 10 μl SensiFAST™ (Meridian Life BioSciences, USA) SYBR No-ROX Kit, 5 pMol of forward and reverse primer, and 2 μl cDNA. Primer sequences are given in Table 1. The qPCR profile consisted of 95°C for 2 min (polymerase activation); 40 cycles of 95°C for 5 s and 72°C for 60 s. *B2M* and *RPL13A* were used as the normalizing genes.

Western blotting

The protein expression levels were analyzed using Western blot. The cells (3 × 106) were lysed on ice for 15 min in RIPA lysis buffer (50 mM Tris–HCl, 150 mM ethylenediaminetetraacetic acid, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 0.1% protease inhibitor

Table 1: Primers sequences used for quantitative polymerase chain reaction analysis

Gene	Type	Primer sequence (5'–3')
PDK1	Forward	CACGCTGGGTAATGAGGATT
	Reverse	ACTGCATCTGTCCCGTAACC
AKT	Forward	GCACCTTCCATGTGGAGACT
	Reverse	CCCAGCAGCTTCAGGTACTC
PTEN	Forward	ACCAGGACCAGAGGAAACCT
	Reverse	GCTAGCCTCTGGATTTGACG
	Reverse	AAGTTGGTGGGCCAGAATG
B2M	Forward	CAGCGTACTCCAAAGATTCA
	Reverse	GAATGCTCCACTTTTTCAAT
RLP13A	Forward	GGATCCCTCCACCCTATGACA
	Reverse	CTGGTACTTCCACCCGACCTC

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mixed. After centrifugation (15,000 ×*g*, 20 min, 4°C), the supernatant was collected, and total protein concentration was determined using the Bradford method. Equal amounts of proteins were separated on a 4%–12% polyacrylamide gel and electro-transferred on Polyvinylidene difluoride (PVDF) membranes. Immunodetection was performed using the LumiGLO Reagent and peroxide; Cell Signalling Technology (#7003, Danvers, USA) according to the manufacturer's instructions with the appropriate monoclonal antibodies. Densitometric measurement of immunoblots was performed using the ImageJ version 1.52s (Image analysis software, NIH). The β-actin was used as internal control. The band densities obtained as a result of densitometer analysis were normalized with B2M.

Statistical analysis

B2M and *RPL13A* gene reference genes were used for the normalization of qPCR data. qPCR analysis was conducted using the $^{\Delta\Delta}$CT method. On the other hand, *B2M* only was used in normalization in the Western blot. Results were expressed as mean ± standard deviation of triplicate independent experiments. The mean difference between the two treatment groups was compared by the Student's paired *t*-test. Statistical significance was considered when $P < 0.05$.

RESULTS

The effect of medicarpin on head and neck squamous cell carcinoma cell viability

The effect of different medicarpin doses on SCCL-MT1 cells was examined by using an MTT assay. First, the effective dose IC50 value treated with medicarpin was determined by applying different dose series (20, 40, 80, 160, and 320 μM) at different hours. After 1, 6, 24, 48, and 72 h, incubation with each compound, cell viability was measured. Medicarpin reduced cell viability ($P = 0.031$) in a concentration-dependent manner, giving the same pattern with the cell lines. IC50 was 80 μM for 48 h treatment of cells with medicarpin also showed dose-dependent viability [Figure 1]. The effect of medicarpin on HNSCC cell viability was observed to have an antiproliferative effect.

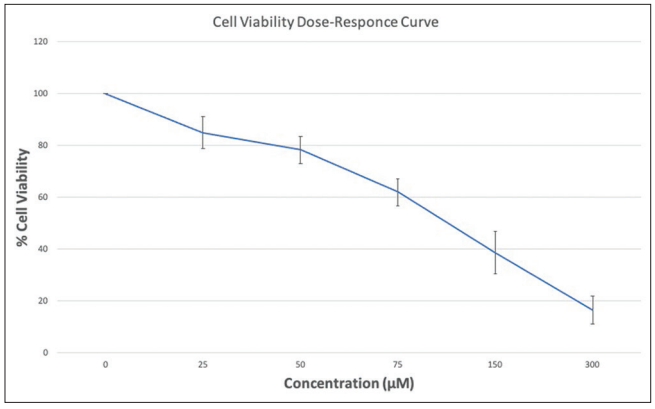

Figure 1: Effect on medicarpin on the viability of head and neck squamous cell carcinoma cells. Cells were treated with 25–300 μM medicarpin for 1 h, 6 h, 24 h, 48 h, and 72 h. Resistance index was expressed for (IC50 head and neck squamous cell carcinoma)

Modulation of PI3K/AKT pathway genes by medicarpin

PI3K/AKT signaling is an important pathway that has been shown to play a vital role in the viability of cancer cells. To investigate the effect of medicarpin on mRNA expression levels of *AKT, PTEN*, and *PDK1* genes in SCCL-MT1 for 48 h, real-time qPCR (RT-qPCR) was performed. As illustrated in Figure 2, the *AKT* and *PTEN* gene expression levels were significantly increased ($P = 0.00027$ and $P = 0.00038$, respectively) medicarpin-treated cells compared with the control group. *PDK1* mRNA gene expression level was decreased, but statistically less significant ($P = 0.0580$) compared to *PTEN* and *AKT* gene expressions.

Medicarpin efficiently activated PI3K/AKT signaling

To examine the role of this pathway in medicarpin-treated SCCL-MT1 cells, the protein expression levels of selected proteins and their levels of phosphorylation forms were evaluated using the Western blot analysis. Treatment with medicarpin for 48 h led to an increase in levels of pPTEN ($P = 0.024$), pAKT ($P = 0.032$), and pPDK1 ($P = 0.059$), and band densities of proteins are shown in Figure 3. The Western blot results showed that medicarpin increased the protein levels of total

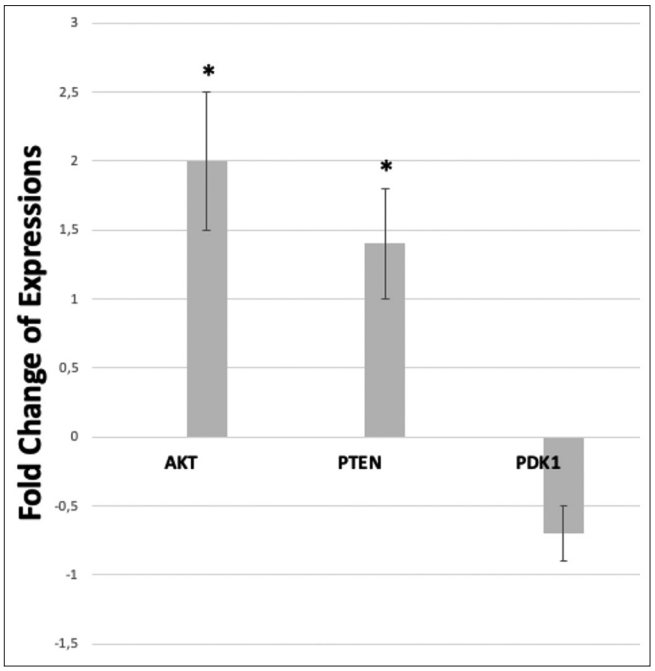

Figure 2: Effect of medicarpin in head and neck squamous cell carcinoma cells. Head and neck squamous cell carcinoma cells were treated with 80 μM medicarpin. PTEN/AKT signaling pathway genes were analyzed by the real-time quantitative polymerase chain reaction. All expression was normalized to *B2M* and *RLP13A* gene expressions. The expression ratio of gene control was normalized and data showed means ± standard deviation from three independent experiments. $P < 0.05$ compared to untreated head and neck squamous cell carcinoma cells

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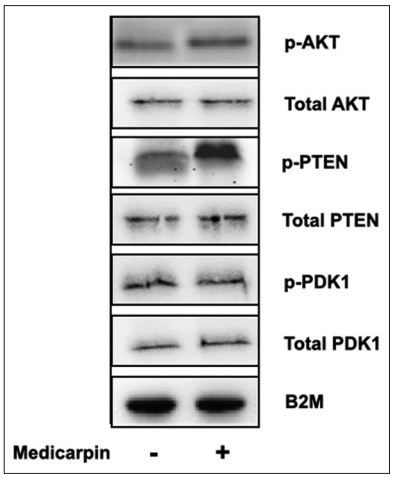

Figure 3: The protein levels of pPDK1, PDK1, pPTEN, PTEN, pAKT, AKT, and H2M in head and neck squamous cell carcinoma

PTEN ($P = 0.0067$) and total AKT ($P = 0.0033$). In contrast, it decreased the protein levels of total PDK1 ($P = 0.0531$), by affecting total PDK1 gene expression. These findings indicate that medicarpin treatment increased PTEN and AKT levels but caused a decrease in PDK1.

DISCUSSION

HNSCC is among the deadliest cancer types.[15] HNSCC occurs most often in men after their 50s, although the incidence among younger individuals is increasing. HNSCC is caused by a variety of factors including smoking, consuming alcohol, HPV infections, premalignant lesions, and alterations in some signal pathway genes.

Activation of the PI3K/AKT signaling pathway leads to increased expression of genes involved in cell growth, survival, migration, and apoptosis, which are critical to HNSCC development.[7] Despite the development of more advanced medical treatments, survival and management of disease still remain a problem. Conventional chemotherapy can often damage rapidly proliferating cells and prevent mitosis by blocking DNA synthesis and severe side effects. Recently, the use of complementary therapy with nature-derived drugs is come into prominence due to its decrease of particularly cytotoxicity and side effects in therapy.[16] Medicarpin, one of the flavonoid compounds, is also associated with a broad spectrum of therapeutic effects. Medicarpin has been used in complementary medicine for many diseases including osteoporosis, rheumatoid arthritis, and cancer.[11,12,14] The development of nature-derived agents through the activation of signal pathways including PI3K/AKT may be regarded as a good strategy for complementary therapies. The mechanisms in signal pathways of medicarpin have not been elucidated in detail.

We investigated the PI3K/AKT signaling pathway in HNSCC cells to understand the relationship between medicarpin and modulator effects. First, we report the effects of medicarpin on the SCCL-MT1 cell line. Previous research reported that isolated flavonoids from alfalfa leaf extracts and medicarpin were identified as potential antiproliferative agents on several cancer cell lines.[17] Our data also showed that some compounds contained in alfalfa leaves, such as medicarpin was able to induce a cytotoxic effect in HNSCC. Cytotoxic effects against different tumor cells were reported in the literature. For example, IC50 doses of medicarpin were 11 μM in colon carcinoma 26-L5 cells[18] and 83 μM in leukemia HL-60 cells.[19] These results were in agreement with our findings that IC50 was found as 80 μM in HNSCC, which demonstrated that medicarpin reduces cell viability in HNSCC.

We demonstrated that the PTEN/AKT signal pathway was affected after treatment with medicarpin in the HNSCC cell line. According to our results, when comparing HNSCC and control cells, there was a significant increase in the expression levels of *PTEN* and *AKT*. However, decreased expression level of *PDK1* in this pathway was statistically less significant compared to *PTEN* and *AKT* expressions. Similarly, protein expression levels had also supported mRNA gene expression level results.

The binding of growth factors to their receptor proteins activates PI3K/AKT pathway, thus activating a cascade of proliferative downstream signaling pathways to transmit proliferative signals into the nucleus. Medicarpin-treated HNSCC through PI3K/AKT led to increased mRNA expression of *PTEN* which is a tumor-suppressor gene in this pathway, thereby, downregulation of the *PTEN* gene block tumor cell viability. Medicarpin treatment also decreased the expression of cell viability and cell cycle progression gene like *PDK1*. Interestingly, medicarpin increased gene and protein expression levels of *AKT*. AKT has effect on more than one upstream and downstream genes including mTOR.

CONCLUSIONS

Our data showed that medicarpin modulates HNSCC cells by increasing *PTEN* and decreasing *PDK1* expressions. The integration of all these functions and connections makes the *PTEN* gene a central inhibitory pathway with a major impact on cancer, on the other hand, decreasing *PDK1* gene expression effects mTOR pathway which may the cause of increasing *AKT* gene. Finally, our study suggests that medicarpin extracts combination with HNSCC drugs may be more effective in cancer treatment. Future prospective studies that integrate molecular and pharmacogenetic studies are crucial for revealing the mechanism and preventive medical efforts.

Acknowledgments

This work was supported by Scientific Research Projects Coordination Unit of Istanbul University-Cerrahpasa. Project number is #25732.

This study was presented as oral presentation at VII International Congress on Molecular Biology and Biotechnology in Konya/Turkey (2018).

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Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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