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Original Research Article

Anethole suppresses the growth of human skin cancer cells by targeting microRNA498/STAT4 axis

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Abstract

Purpose: *To investigate the effects of anethole on human skin cancer cells.*
Methods: *Cell viability was determined using CCK-8 and colony formation assay, while AO/EB and Annexin V/PI assays were used to assess apoptosis. The mRNA expressions of genes were assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).*
Results: *The growth of skin cancer cells was significantly reduced by anethole ($p < 0.05$). On the other hand, normal human skin cells were marginally affected by anethole. Furthermore, anethole significantly suppressed the colony formation potential of CRL-6475 skin cancer cells in a concentration-dependent manner ($p < 0.05$). Results from AO/EB and annexin V/PI staining assays revealed that anethole induced apoptosis in skin cancer cells, while qRT-PCR results indicate that anethole significantly up-regulated microRNA-498 in skin cancer cells ($p < 0.05$). However, anethole had no effect on the expression levels of miR-7, miR-9, miR-145, miR-31, miR-27, miR-498 miR-1298, miR-1299, 1179, miR-375, miR-508 and miR-23. CCK-8 assay results confirmed that miR-498 up-regulation significantly mimicked the anti-proliferative effects of anethole. Bioinformatics analysis and luciferase reporter assay led to identification of STAT4 as the regulatory target of miR-498. Furthermore, silencing of STAT4 significantly mimicked the effects of anethole administration ($p < 0.05$).*
Conclusion: *These results indicate that anethole inhibits skin cancer cell growth by targeting the miR-498/STAT4 axis, and therefore, has potentials for use in the management of skin cancer.*

Keywords: *Skin cancer, Melanoma, Metastasis, Anethole, Apoptosis, miR-498/STAT4 axis*

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INTRODUCTION

Skin cancer is one of the common human malignancies which incidence has shown a considerable increase in recent years [1]. Melanoma is ranked the most serious type of skin cancer which is reported to account for more than 90 percent of deaths resulting from skin cancer [2]. Ultraviolet (UV) exposure and sun tan have been identified as major causative factors that induce the onset and progression of human melanoma [3]. On the average, more than 150,000 people were diagnosed with melanoma annually during the past few years throughout the world [4]. A study has revealed that in approximately one-third of the affected

patients, melanoma has been reported to exhibit invasion and metastasis [5]. In view of this, there is a need to study the pathogenesis of skin cancer. In particular, melanoma needs to be studied with a view to identifying the possible prognostic and therapeutic agents.

Flavonoids are plant-based secondary metabolites responsible for imparting characteristic colors to plant parts such as flowers, fruits and seeds [6]. Moreover, flavonoids possess beneficial pharmacological properties such as antioxidant, antimicrobial, antibacterial, antiviral, anti-inflammatory and anti-allergic effects [7]. Interestingly, a number of flavonoid compounds have been reported to produce anticancer effects through inhibition of the proliferation of different types of human cancer cells via targeting of multiple signaling pathways [8]. Flavonoids act as natural protectors of the skin against photo-damage. Thus, flavonoids are valued for their potential role in prevention and treatment of skin ageing. Flavonoid derivatives like diosmetin and luteolin have been shown to exhibit anti-proliferative effects against human skin cancer cells [9].

Anethole, 1-methoxy-4-propenyl-benzene or isoestragole (Figure 1 A), is a flavonoid derivative (alkoxypropenylbenzene) and one of the key components of essential oils of several plant species such as *Foeniculum vulgare* (fennel), *Pimpinella anisum* (anise), and *Illicium verum* (star anise). This compound is known for its several beneficial effects on health, including its anticarcinogenic potential [10]. In the present study, the antiproliferative effects of anethole against human skin cancer cells was investigated.

EXPERIMENTAL

Culture and transfection of cell lines

Human melanoma cell line, CRL-6475 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), while human epidermal melanocytes (HEMa-LP) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The cells were cultured using DMEM (Thermo Fisher Scientific) supplemented with 10 % FBS (Thermo Fisher Scientific) and 1 % streptomycin and ampicillin (Invitrogen, Carlsbad, CA, USA) in a humidified 5-% CO_2 incubator at 37 ^oC.

CCK-8 and colony formation assays

Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was employed to determine cell proliferation, as per the kit manufacturer's instructions. Absorbance at 570 nm (OD_{570}) was determined for different samples using a plate spectrophotometer. For the determination of colony formation, 5000 cells were seeded in a 6-well plate and cultured for 15 days at 37 ^oC. The medium in each well was replaced with fresh medium on alternate days. At the end of 15 days, the medium in each well was discarded, and the colonies were PBS-washed and stained with 0.1 % crystal violet (Sigma-Aldrich, MO, USA) for 15 min at room temperature. Finally, the colonies were counted.

AO/EB and Annexin V-FITC/PI staining

Dual acridine orange/ethidium bromide (AO/EB) staining was used to determine cell viability. In this procedure, the CRL-6475 cells were seeded in a 12-well plate for 24 h at an initial density of 2 x 10^4 cells per well. Thereafter, the cells were fixed with 70 % ethanol and treated with 200 µL of staining mixture of AO and EB (1:1 volume ratio). Then, the cells were examined under a fluorescent microscope (Olympus, Tokyo, Japan) and photographed.

For Annexin V-FITC/PI staining assay, 10^5 of CRL-6475 cells per well were cultured in a 12-well plate at 37 ^oC for 24 h. Then, the cells were subjected to centrifugation, followed by washing with PBS and ethanol fixation. Thereafter, the cells were incubated with 150 µL of binding buffer for 20 min at room temperature, followed by sequential staining with 12.5 µL of Annexin V-FITC and 3 µL of PI solutions. Finally, the cells were subjected to flow cytometric analysis using FACS Calibur flow cytometer (Becton Dickinson, USA).

Extraction of RNA, and qRT-PCR

Total RNA was extracted from the cells using TRIzol reagent kit (Invitrogen) in line with the kit manufacturers' protocol. Following quantification of the RNA with NanoDrop-2000 (Thermo Fisher Scientific), 2.5 µg RNA was reverse-transcribed to complementary DNA (cDNA) using PrimeScript RT Master Mix kit (Takara, Japan), as per the kit manufacturer's protocol. Then, qRT-PCR was performed using Power SYBR Green PCR master mix (Thermo Fisher Scientific). The relative transcript levels of different microRNAs were quantified with the $2^{-\triangle\triangle ct}$ method. Human β-actin gene was used as internal control.

Cell line transfection

The CRL-6475 cells were transfected with miR-498 mimics, miR-negative control (miR-NC), miR-498 inhibitor, si-STAT4, si-NC (STAT4 silencing control), and pcDNA-STAT4, or co-transfected with miR-498 mimics and pcDNA-STAT4, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. The synthetic transfection constructs were purchased from RiboBio (Guangzhou, China).

MiR target analysis

TargetScan analysis was used to predict the target of miR-498. A dual luciferase reporter assay was carried out to determine the interaction between miR-498 mimics and 3′-UTR of STAT4 mRNA. In this process, the wild-type (WT) or mutant type (MT) 3′-UTR stretch of STAT4 was cloned into a pGL3-luciferase reporter vector (Promega, Madison, WI, USA). The reporter vector (WT or MUT) was co-transfected with miR-498 mimics or miR-NC into the CRL-6475 cells using Lipofectamine 2000 reagent. After 24 h of transfection, a dual-luciferase reporter assay system (Promega) was used to estimate the relative luciferase activities of the transfected cells.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). GraphPad Prism 7.0 (GraphPad Software, San Diego, USA) was used for preparation of graphs, while Student's t-test was used for paired comparisons. A p-value less than 0.05 was considered to be representative of a statistically significant difference between two groups.

RESULTS

Anethole inhibited proliferation and colony formation

The human melanoma cell line (CRL-6475) and epidermal melanocytes (HEMa-LP) were treated with graded concentrations of anethole (up to 100 µM). The results showed that anethole reduced the percentage cell viability of CRL-6475 cancer cells in a concentration-dependent manner, with an estimated IC_{50} of 6 µM (Figure 1 B). However, the viabilities of normal melanocytes were marginally affected by anethole, as was evident in IC_{50} of 80 µM (Figure 1 C). These results are indicative of selective growth inhibitory action of anethole against the skin cancer cells, with minimal effect on normal human cells. Moreover, treatment of CRL-1675 skin cancer cells with varying doses of anethole resulted in significant and concentration-dependent inhibition of colony formation (Figure 2). Colony formation of the treated skin cells was decreased by more than 70 % when treated with 12 µM anethole, relative to untreated cancer cells.

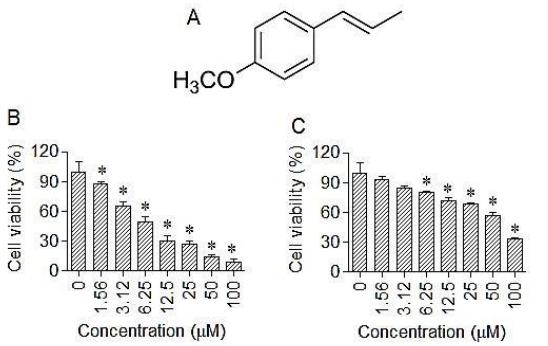

Figure 1: Anethole selectively inhibited skin cancer cell growth. (A) Molecular structure of anethole. (B) Viability of CRL-6475 skin cancer cells exposed to varying concentrations of anethole. (C) Viability of HEMa-LP normal skin cells treated with graded doses of anethole. *P < 0.05

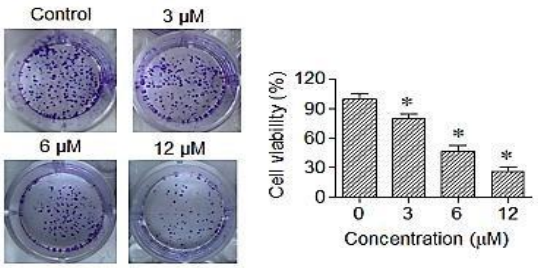

Figure 2: Effect of graded doses of anethole on colony formation on CRL-6475 skin cancer cells. *P < 0.05

Anethole induced apoptosis

In order to determine if anethole induced apoptosis in CRL-6475 cancer cells, the cancer cells treated with graded concentrations of anethole were subjected to AO/EB staining. Fluorescent microscopy indicated that, with increase in anethole concentration, the relative percentage of ethidium bromide-positive cells increased, suggesting induction of cell death (Figure 3 A). For further confirmation, Annexin V-FITC/PI double staining and flow cytometric analysis were carried out. Results from flow cytometric analysis showed that the relative percentage of early and late apoptotic cells were increased with increasing concentrations of anethole (Figure 3 B). These results indicated that anethole inhibited the growth of skin cancer cells *in vitro* through induction of apoptosis.

Anethole targeted miR-498

The microRNA (miR) which was specifically targeted by anethole in skin cancer was identified using qRT-PCR assay. The results showed that among the set of miRs assayed, the expression of miR-498 was significantly up-regulated in CRL-6475 cells by 6 µM anethole (Figure 4 A). To mimic the effects of miR-498, qRT-PCR was carried out after transiently transfecting CRL-6475 cells with miR-498 mimics. The results confirmed the over-expression of miR-498 in cancer cells (Figure 4 A). The CCK-8 assay showed that miR-498 over-expression markedly suppressed the proliferation of host cells (Figure 4 B). On the other hand, transfection of CRL-6475 cancer cells with miR-498 inhibitor resulted in attenuation of the anti-proliferative effects of anethole (Figure 4 C and D). These results revealed that anethole inhibited skin cancer cell growth by up-regulating the expression of miR-498.

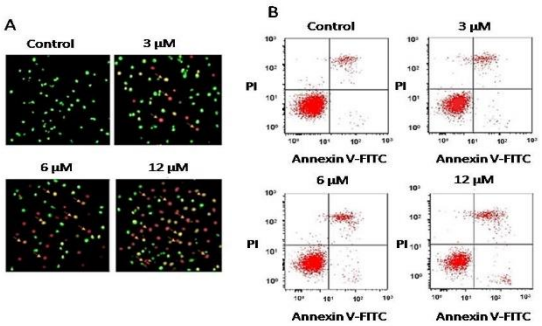

Figure 3: Anethole induced apoptosis of skin cancer cells. (A) Results of AO/EB, and (B) annexin VFITC/PI staining assays for analysis of apoptosis of CRL-6475 skin cancer cells treated with different concentrations of anethole

MiR-498/STAT4 axis modulated the antiproliferative effects of anethole

To further study the mechanism of action of anethole, *in silico* analysis was used to identify the specific target of miR-498. The results showed that signal transducer and activator of transcription 4 (STAT4) acted as the post-transcriptional target of miR-498, and the latter interacted in a sequence-specific manner with 3′-UTR of STAT4 (Figure 5 A). There was significant loss of luciferase activity in CRL-6475 cells co-transfected with miR-498 mimics and luciferase vector bearing STAT4 3′-UTR with wild type miR-498 binding site. This is indicative of interaction of miR-498 with 3′-UTR of STAT4 (Figure 5 B). Interestingly, the silencing of STAT4 in CRL-6475 cancer cells inhibited cell growth in the same manner as anethole, while the over-expression of STAT4 mitigated the anti-proliferative effects of anethole and miR-498 over-expression (Figure 5 C). These results indicate that anethole exerted its *in vitro* anti-proliferative effects on the skin cancer cells by targeting the miR498/STAT4 molecular axis.

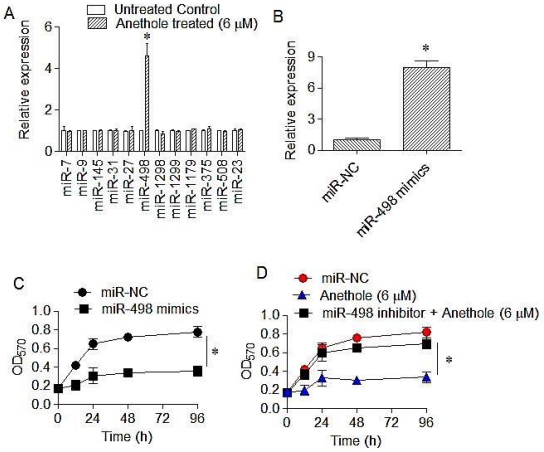

Figure 4: Anethole exerted anti-proliferative effects on the skin cancer cells by up-regulating miR-498. (A) Effect of anethole on the transcript levels of different miRs in skin cancer cells. (B) Confirmation of miR-498 over-expression in miR-498 mimics-transfected CRL-6475 cancer cells, relative to miR-NC-transfected negative control cells. (C) CCK-8 assay of CRL-6475 cancer cells transfected with miR-498 mimics or miR-NC. (D) Data for CCK-8 assay of CRL-6475 cancer cells treated with 6 µM anethole, or transfected with miR-NC, miR498 mimics and treated with anethole. **P* < 0.05

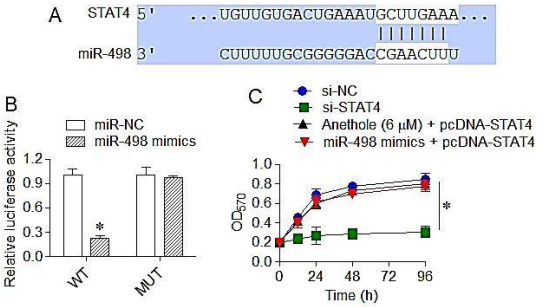

Figure 5: The anticancer effect of anethole on skin cancer cells was modulated via the MiR-498/STAT4 axis. (A) Identification of miR-498 binding site in STAT4 3′-UTR through *in silico* analysis. (B) Results of dual luciferase reporter assay for analysis of interaction of miR-498 with 3′-UTR of STAT4. (C) Results of CCK-8 assay in CRL-6475 cancer cells transfected with si-STAT4, si-NC, miR-498 mimics plus pcDNA-STAT4 or pcDNA-STAT4 prior to anethole treatment. **P* < 0.05

DISCUSSION

Cancer is a serious disease which is currently the second leading cause of human deaths worldwide. The onset and progression of this

disease is yet to be understood owing to its heterogenous nature [11]. Moreover, the prognosis of this malignancy is very poor. However, with advancements in molecular biology approaches in recent years, new insights into cancer progression have come to limelight. It is now known that some transcription products of the human genome which apparently do not code for any proteins play crucial physiological, cellular and metabolic roles [12]. These non-coding RNAs called microRNAs (miRs) have been shown to regulate vital aspects of cellular development, and their dysregulation results in a number of pathological disorders, including cancers [13]. It has been reported that a number of miRs exhibit aberrant expressions in skin cancer, and they affect skin cancer growth and proliferation [14]. In addition, several studies have shown that miRs might emerge as reliable prognostic and therapeutic molecular markers in skin cancer. It has been reported that miR-498 is down-regulated in melanoma, and its re-expression inhibits the growth and metastasis of cancer cells by targeting UBE2T [15]. Thus, induction of miR-498 over-expression might be one of the possible molecular therapeutic strategies for skin cancer.

In the present study, anethole was shown to exhibit selective cytotoxic potential against skin cancer cells. However, it had minimal effect on the viability of normal skin cells. This is consistent with a previous report on the anticancer property of anethole [16]. Similar to the results obtained in the present study, anethole has been reported to exhibit pro-apoptotic potential against human cancer cells [16]. Interestingly, anethole was shown to exert its anti-proliferative effects by up-regulating the expression of miR-498 in skin cancer cells. Signal transducer and activator of transcription 4 (STAT4) was identified as the functional target of miR-498 in skin cancer. The silencing of STAT4 reduced the proliferation of skin cancer cells, thereby mimicking the effect produced by anethole administration. It is known that STAT4 plays important roles in development, proliferation and functioning of human immune system. It acts as an oncogene that regulates tumorigenesis, while its silencing has been shown to inhibit malignant cell growth and proliferation [17]. The targeting of STAT4 by miR-141 was previously shown to inhibit the growth of gastric cancer cells [18]. Overall, the results obtained in the present study have demonstrated that anethole administration inhibited the growth and viability of skin cancer cells through modulation of the miR-498/STAT4 axis.

CONCLUSION

Anethole exerts significant anti-proliferative effects on skin cancer cells, but has minimal toxicity on normal human skin cells. Furthermore, anethole treatment induces apoptosis in skin cancer cells. At the molecular level, the anticancer effects of anethole on skin cancer cells occurs through the modulation of miR-498/STAT4 signal axis. Anethole should be further investigated in animal models of skin cancer for its potentials in the treatment of skin cancer.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The experiments were performed by Jiahang Li and Yingqiu Mao. Wei Li designed the whole study and supervised Jiahang Li and Yingqiu Mao.

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