

Original Research Article

Tectorigenin functions as a potential drug for melanoma treatment via inhibition of cell growth, migration and aerobic glycolysis

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Abstract

Purpose: To investigate the role of tectorigenin (TG) in melanoma cells.

Methods: Viability of A375 and A2058 melanoma cells was determined by cell counting kit (CCK-8) assay. Cell cycle progression was analyzed by 5-ethynyl-2'-deoxyuridine (EdU) staining. Migration and invasion of melanoma cells were determined by Transwell assay. The epithelial-mesenchymal transition (EMT) of melanoma cells was investigated by determining expression of E-Cadherin and Snail. Expression of glucose transporter 1 (GLUT1) and lactose dehydrogenase A (LDHA) was assessed via western blotting. Glucose and lactate production was evaluated by corresponding assay kit. The NOX4 and FOXM1 expressions were determined using western blotting.

Results: Tectorigenin inhibited proliferation, migration and invasion of A375 and A2058 melanoma cells. Tectorigenin regulated cell cycle progression by decreasing the number of cells in S-phase and significantly inhibited snail expression and increased expression of E-Cadherin ($p < 0.05$), thus inhibiting the EMT of A375 and A2058 cells. Tectorigenin inhibited expression of GLUT1 and LDHA, thereby leading to reduction in glucose consumption and lactate production. It also significantly inhibited expressions of NOX4 and FOXM1 ($p < 0.05$), indicating an inhibitory effect on the activity of NOX4/FOXM1 pathway.

Conclusion: Tectorigenin inhibits aerobic glycolysis, growth and migration in melanoma cells by suppressing the activity of NOX4/FOXM1 pathway, suggesting its potential in melanoma treatment.

Keywords: Thyroid hormone receptor interactor 13, Gastric cancer, Proliferation, Migration, Stemness, Cisplatin resistance

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INTRODUCTION

Melanoma is a type of skin cancer that develops in melanocytes responsible for producing skin pigment [1]. Melanoma is a serious skin cancer that accounts for only 1 % of skin cancers, but causes over 80 % of all skin cancer deaths [2].

Treatments for melanoma has been improved with approval of new targeted drugs and immune check points, especially PD-1 inhibitors [3]. However, results from clinical trials of PD-1 inhibitors revealed that nearly 60 % advanced melanoma patients are non-responsive to PD-1 inhibitors [4]. Among patients responsive to PD-1

inhibitors, nearly 40 % of them have developed resistance [4]. Thus, development of new drugs for single treatment or in combination with PD-1 inhibitors is urgently needed.

Tectorigenin (TG) is an O-methylated isoflavone with various biological functions, including anti-inflammatory, anti-apoptosis, anti-microbial and anticancer effects [5,6]. Tectorigenin is used in treatment of many cancers, including breast, prostate, ovarian cancer and glioblastoma [7,8]. In glioblastoma and breast cancer, tectorigenin treatment induced cell cycle arrest at G0/G1 phase and cell apoptosis, and also inhibited tumor cell migration and invasion [9,10]. In ovarian cancer, tectorigenin promoted sensitivity of ovarian cancer cells to paclitaxel through downregulation of Akt and NF- κ B pathways [8]. In prostate cancer, tectorigenin inhibited proliferation through downregulation of PDEF, PSA, hTERT and IGF-1R expression [7]. However, there is currently no report on tectorigenin in melanoma, and underline mechanism of tectorigenin in melanoma treatment is unclear.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is a member of NADPH oxidase family, which is highly expressed in melanoma cells, as well as in other cancers [11-13]. The NOX4/FOXM1 pathway has been shown to promote aerobic glycolysis in glioblastoma cells, thereby promoting proliferation of tumor cells [13]. Down-regulation of FOXM1 expression inhibits melanoma cell growth and survival [14]. In addition, down-regulation of FOXM1 expression also suppresses melanoma tumor metastasis [15]. Therefore, NOX4/FOXM1 pathway plays an important role in melanoma.

In this study, the inhibitory effect of tectorigenin on proliferation, migration, invasion and epithelial-mesenchymal transition of melanoma cells as well as the effect of tectorigenin on aerobic glycolysis and NOX4/FOXM1 pathway was investigated.

EXPERIMENTAL

Cell Counting Kit-8 cell proliferation assay

The A375 or A2058 melanoma cells were seeded into 96-well plates at a cell number of 5,000 cells per well. The cells were treated with 0, 50, 100 or 200 μ M TG respectively. Cell viability was measured at 24, 48, 72, 96 h by Cell Counting Kit-8 (Beyotime, code no. C0038) according to manufacturer's instructions.

S-phase cell cycle progression

Cell cycle progression was analyzed by 5-ethynyl-2'-deoxyuridine (EdU) staining (Invitrogen, code no. C10340) according to manufacturer's instructions. The A375 or A2058 melanoma cells were seeded into 96-well plates at a cell number of 5,000 cells per well, treated with 0, 50, 100 or 200 μ M TG respectively, and incubated with EdU. Cells were fixed and permeabilized, and then stained with 4'-diamidino-2-phenylindole (DAPI). Images were captured and analyzed. Five fields of view were taken in total and calculated based on average number of cells.

Cell migration and invasion assay

Matrigel[®] (Corning, code no. 354230) was thawed on ice, and 350 μ L of Matrigel[®] was added to upper chamber of 24-well transwell plate, and solidified for 30 min. Thereafter, 100 μ L of A375 or A2058 cell suspension (100,000 cells) was added into the upper chamber, and 600 μ L of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) was added to the bottom chamber. After 24 h incubation, insert was removed from the plate, and cells remaining on upper chamber were carefully removed and inserted in 4 % paraformaldehyde (PFA) for 30 min to allow for cell fixation. Cells were then stained with 0.2 % crystal violet for 20 min, and washed with phosphate buffered saline (PBS). Images were captured and analyzed with Image analysis software. Five fields of view were taken in total and calculated based on average number of cells.

Western blotting assay

Total proteins were extracted from A375 and A2058 cell pellets with radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, code no. P0013B). The RIPA lysis buffer contained multiple inhibitors which inhibit protein degradation efficiently during sample lysis. Total protein samples were separated using 8 % sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gels electrophoresis. Western blotting was performed according to standard procedures. Primary antibodies and corresponding dilution ratio used are listed as follows: β -actin (Abcam, code no. ab6276, 1:5000), E-Cadherin (Abcam, code no. ab231303, 1:1000), Snail (Abcam, code no. ab216347, 1:1000), GLUT1 (Abcam, code no. ab115730, 1:5000), LDHA (Invitrogen, code no. 702747, 1:500), NOX4 (Abcam, code no. ab154244, 1:1000), FOXM1 (Abcam, code no. ab207298, 1:1000). Semi-quantitative analysis

was performed using image analysis software to evaluate relative expression levels of E-Cadherin, Snail, GLUT1, LDHA, NOX4 and FOXM1.

Glucose consumption and lactate content determination

The A375 or A2058 melanoma cells were seeded into 6-well plates and treated with 0, 50, 100 or 200 μM TG for 48 h. Glucose content in culture medium was measured by glucose content assay kit (Solarbio, code no. BC2505) and glucose consumption calculated. Lactate production was measured by lactic acid (LA) content assay kit (Solarbio, code no. BC2235). Detailed protocols were performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM), and analyzed by t-test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Tectorigenin inhibited proliferation and cell cycle progression of melanoma cells

Inhibitory effect of tectorigenin on proliferation and cell cycle progression of melanoma cells were investigated with CCK-8 and EdU assays. A375 and A2058 melanoma cells were used as representative melanoma cells in this study. After treatment with 50, 100 or 200 μM tectorigenin, proliferation of A375 or A2058 cells was significantly inhibited ($p < 0.05$) compared to control in a dose dependent manner (Figure 1 A). Furthermore, percentage of EdU positive cells decreased in a dose dependent manner after tectorigenin treatment (Figure 1 B). This result indicated that tectorigenin treatment decreased number of cells in S-phase. Thus, tectorigenin inhibited DNA replication and proliferation of A375 and A2058 melanoma cells.

Tectorigenin inhibited migration and epithelial-mesenchymal transition (EMT) of melanoma cells

Transwell assay was performed to evaluate inhibitory effect of tectorigenin on migration and invasion ability of A375 and A2058 melanoma cells. Number of migrated A375 or A2058 cells was significantly decreased ($p < 0.05$) by tectorigenin treatment, and this inhibitory effect increased with increasing tectorigenin concentration (Figure 2 A). A similar result was

observed in Figure 2 B, suggesting that tectorigenin inhibited invasion ability of A375 and A2058 cells.

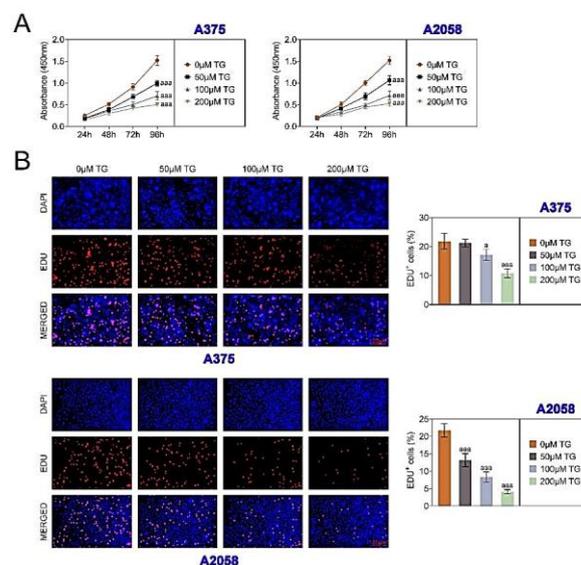


Figure 1: Tectorigenin inhibited the proliferation and cell cycle progression of melanoma cells. (A) The proliferation of A375 or A2058 melanoma cells were assayed by CCK-8. Cells were treated with 0, 50, 100 or 200 μM TG; (B) Cell cycle progression of A375 or A2058 melanoma cells was analyzed by EdU. Five fields of view were taken in total and calculated based on average number of cells

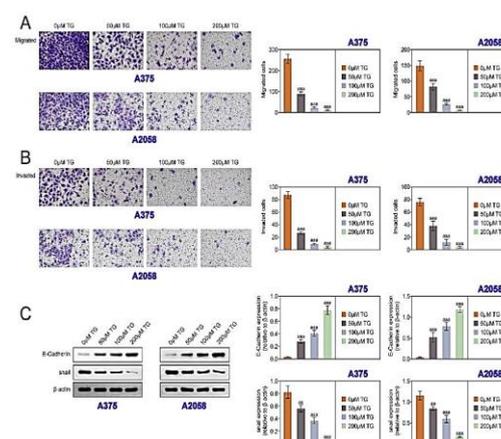


Figure 2: Tectorigenin inhibited migration and epithelial-mesenchymal transition of A375 and A2058 melanoma cells. (A) Migration ability of A375 and A2058 cells was determined after tectorigenin treatment. Migrated cells were counted and represented using a histogram. (B) Invasion ability of A375 and A2058 cells determined after tectorigenin treatment. The invading cells were counted and represented using a histogram. (C) Expression of E-Cadherin and Snail in A375 or A2058 cells with or without tectorigenin treatment. Semi-quantitative analysis was performed through image analysis software to evaluate relative level of E-Cadherin and Snail. ^{aa} $P < 0.01$, ^{aaa} $p < 0.001$ vs 0 μM TG

Epithelial-mesenchymal transition of A375 or A2058 cells was further investigated by determining the expression of EMT markers E-Cadherin and Snail. The results in Figure 2 C revealed that Snail was highly expressed in A375 and A2058 cells, which further suppressed expression of E-Cadherin, leading to a loss of cell adhesion and acquisition of mesenchymal properties. Tectorigenin treatment significantly regulated expression of Snail and E-Cadherin ($p < 0.05$), which inhibited EMT of A375 and A2058 cells. Inhibitory effect of tectorigenin on EMT of melanoma cells increased with tectorigenin concentration.

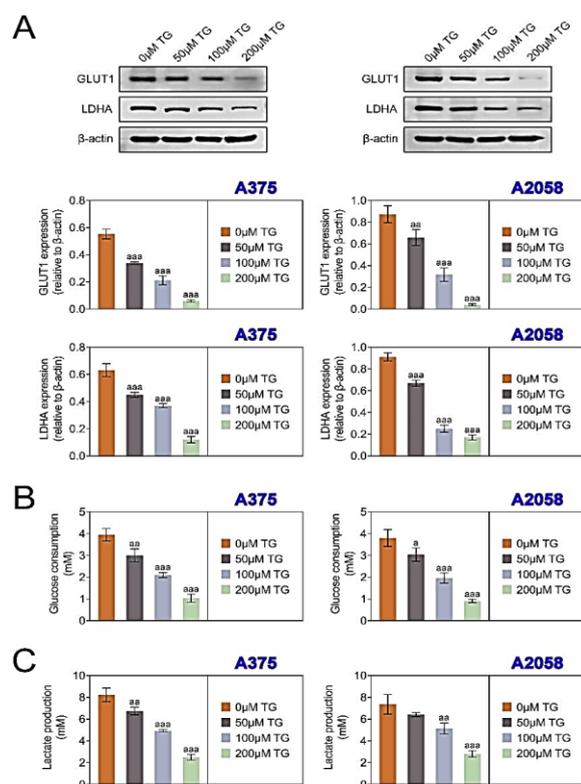


Figure 3: Tectorigenin inhibited aerobic glycolysis in melanoma cells. (A) Expression of GLUT1 and LDHA in A375 or A2058 cells with or without tectorigenin treatment. Semi-quantitative analysis was performed using image analysis software to evaluate relative level of GLUT1 and LDHA, (B) Glucose consumption in A375 or A2058 cells with or without tectorigenin treatment, (C) Lactate production in A375 or A2058 cells with or without tectorigenin treatment. $^aP < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ vs 0 μM TG

Tectorigenin inhibited activation of NOX4/FOXM1 pathway

The A375 and A2058 melanoma cells were treated with different concentrations of tectorigenin, and expression levels of NOX4 and FOXM1 were determined by western blotting. Expression of NOX4 and FOXM1 was relatively high in A375 and A2058 cells, which was

consistent with previously reported results (Figure 4). With tectorigenin treatment, NOX4 expression significantly decreased in a dose dependent manner. In addition, a reduction in FOXM1 expression in A375 and A2058 cells was observed with tectorigenin treatment. This down-regulation in FOXM1 expression is due to reduction in ROS production as a result of decrease in NOX4 expression. These results demonstrated that NOX4/FOXM1 pathway was highly activated in melanoma cells, and tectorigenin inhibited activation of NOX4/FOXM1 pathway.

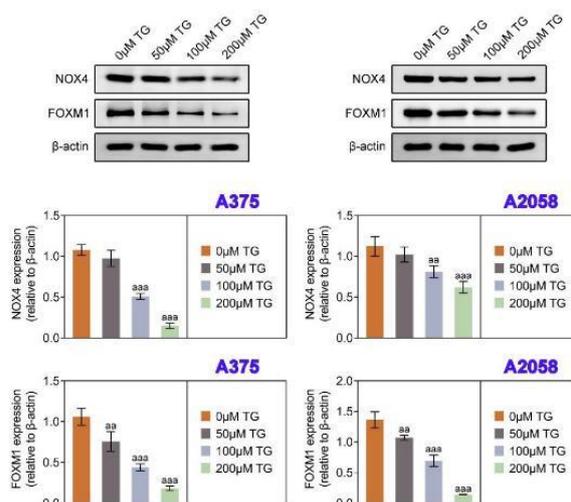


Figure 4: Tectorigenin inhibited activity of NOX4/FOXM1 pathway. Expression of NOX4 and FOXM1 in A375 or A2058 cells with or without tectorigenin treatment. Semi-quantitative analysis was performed through image analysis software to evaluate relative level of NOX4 and FOXM1. $^{aa}P < 0.01$, $^{aaa}p < 0.001$ vs 0 μM TG

DISCUSSION

Tectorigenin has demonstrated anti-tumor activity in several cancers, including breast, prostate, ovarian cancer and glioblastoma [7-10]. However, this was the first study to report the function and mechanism of tectorigenin in melanoma. Mechanisms through which tectorigenin regulated melanoma cell proliferation, migration, invasion and epithelial-mesenchymal transition was also investigated in this study.

Tectorigenin inhibited melanoma cell proliferation by regulating cell cycle progression. S-phase is a critical phase during cell proliferation where DNA replication occurs. The results illustrated that tectorigenin treatment decreased number of cells in S-phase, thereby inhibiting cell proliferation. Previous studies found that epithelial-mesenchymal transition (EMT) was associated

with tumor progression and metastasis, especially in epithelial cancers [16]. Loss of E-cadherin is a hallmark of EMT, and low E-cadherin expression was determined in metastatic melanoma [17]. Results in this study revealed that tectorigenin treatment could significantly regulate expression levels of Snail and E-Cadherin, thus inhibiting EMT of melanoma cells. As a result of this inhibition on EMT, migratory and invasive properties of the melanoma cells were weakened. These results indicated that tectorigenin inhibited proliferation, migration, invasion and EMT of melanoma cells, thus contributing to the inhibition of tumor metastasis, including local invasion, intravasation, circulation, extravasation, and colonization of secondary sites.

Expression levels of glucose transporter 1 (GLUT1) and lactose dehydrogenase A (LDHA) have been reported to be up-regulated in cancer cells, and GLUT1 was highly expressed in melanoma [18]. High expression of GLUT1 is closely related to metastasis of malignant melanoma cells [18]. Furthermore, LDHA is also reported to be upregulated in melanoma, which enables melanoma cells to meet the high energetic demands associated with their rapid growth and proliferation [19]. In this study, tectorigenin inhibited the expression of GLUT1 and LDHA, which further reduced glucose consumption and lactate production. All these findings demonstrated that tectorigenin modulates aerobic glycolysis and disrupts the energy supply of cancer cells. Detailed research in this study showed that tectorigenin inhibited activation of the NOX4/FOXO1 pathway, which was related to aerobic glycolysis. However, the specific mechanism by which the NOX4/FOXO1 pathway regulates aerobic glycolysis is not fully understood and further research is required.

CONCLUSION

Tectorigenin inhibits aerobic glycolysis in melanoma cells by suppressing activation of NOX4/FOXO1 pathway. Reduction in aerobic glycolysis further inhibits proliferation, migration, invasion, and epithelial-mesenchymal transition of melanoma cells. Therefore, tectorigenin is a potential agent for melanoma treatment by inhibiting NOX4/FOXO1 pathway, and consequently melanoma development.

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. Qiao Yan and Fei Wang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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