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Experimental Research

Evaluating the effect of Alantolactone on the expression of N-cadherin and Vimentin genes effective in epithelial-mesenchymal transition (EMT) in breast cancer cell line (MDA-MB-231)

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ABSTRACT

Objective: Breast cancer is the second leading cause of death and the most common cancer among women. 10 to 20 percent of breast cancer samples have a negative triple phenotype that is more metastatic and more difficult to diagnose. Tumor invasion to other tissues and the formation of a secondary tumor depend on the epithelial to mesenchymal transition process, and the STAT3 pathway, which is associated with tumor proliferation and invasion and is the target gene for the drug, alantolactone. In this study, the EMT process is evaluated in negative triple-breasted cancer cells treated with alantolactone.

Methods: We used MDA-MB-231 cell line for assessing the survival rate of triple negative breast cancer cells and MTT test for determining alantolactone dose. We used three doses of 0.01 0.1, and 1 μM of alantolactone for evaluating the cell behavior in cancer invasion pathway. Real-time PCR was used to evaluate the expression of Vimentin, and N-cadherin genes. All of the tests were repeated thrice and the data were analyzed using Prism version 7.0.

Results: The expression of Vimentin and N-cadherin decreased significantly at 1 μM alantolactone compared to the control group, $p < 0.05$.

Conclusion: Alantolactone affects the expression of Vimentin and N-cadherin through STAT3 signaling pathway and suppresses EMT process, metastasis and cancer invasion. This component may be used for treatment of patients with breast cancer.

1. Introduction

Cancer, as a common non-infectious disease from an epidemiological point of view, is considered as a multifactorial disease. One of the most common cancers reported in women is breast cancer. According to WHO reports, 1.7 million people are diagnosed with breast cancer each year, accounting for 25% of all cancer patients, which is the second leading cause of death from cancer after lung cancer [1]. One of the subtypes of breast cancer is triple-negative cancer (TNBC) where cancer cells lack estrogen and progesterone receptors and HER2 receptor expression [2–4]. It is clinically and pathologically considered as an invasive cancer, and has a high growth and proliferation rate and occurs in women at a young age [5]. Increased production of IL-6 by these cells through the STAT3 signaling pathway contributes chiefly in its invasion and metastasis [6,7]. Recent studies show that the epithelial-mesenchymal transition (EMT) process plays an important role in the pathophysiology of fibrosis in some organs as well as in cancer metastasis [8]. EMT is the process by which epithelial cells assimilate the migrating and invading characteristics of mesenchymal cells [9]. During this process, the epithelial cells lose their connections and their apical-basal polarity. On the other hand, the cytoskeleton is rearranged. These events are the result of changes in the signaling pathway of cells that increase cell motility and migration [10]. Several cellular signaling pathways play a key role in the EMT process such as Wnt, Notch, TGF-B, Hedgehog and STAT3 [10]. Among all members of the STAT family, STAT3 is often known to be associated with tumorigenesis and is considered an oncogene. Evidence shows that in most malignancies, the amount of this protein increases abnormally, and recent studies show that 80% of

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triple-negative breast cancer cells have high levels of STAT3 activity. This protein acts as an important mediator in oncogenic signaling pathway by reducing the expression of apoptosis inhibitor genes, regulating cell cycle and inducing angiogenesis, metastasis and cell invasion [6]. Studies show a list of key transcription factors that play a key role in the EMT process. These factors are controlled by STAT3 and play an important role in the rapid change of cell phenotype from epithelial to motile mesenchymal cells [11].

STAT3 can alter epithelial cell markers and convert them to mesenchymal cell markers by acting on the expression of snail1, Zeb1 and Twist transcription factors. STAT3, through the these transcription factors, reduces the expression of E-cadherin and increases the expression of Vimentin and metalloproteases 2 and 9, and induces the EMT process, ultimately promoting cell invasion [12–14]. One of the changes that occurs in the EMT process is the loss of cellular connections. In this process, cadherins play a key role. During the EMT process, the expression of E-cadherin decreases whereas that of N-cadherin increases. Unlike E-cadherins, N-cadherins bind loosely to each other, thereby causing the cells to move [10,15]. On the other hand, in the EMT process, changes occur in the cytoskeleton and protein complex that affects cell polarity. In this process, the composition of the intermediate filaments changes by decreasing the expression of cytokeratin and increasing the amount of Vimentin. Changes in the expression of intermediate filaments cause cell motility, which is likely due to the interaction of Vimentin with the complexation of cellular polarity proteins [10]. Because cells lack surface markers for estrogen, progesterone, and HER2, hormone or anti-triple-negative breast cancer HER2 therapies cannot be used. For this reason, chemotherapy has become the only treatment option for this cancer [4,16]. Conventional and traditional chemotherapeutic drugs, which cause toxicity in the body due to their low specificity. This reason prompted researchers to explore new therapeutic compounds with high specificity against cancer cells and non-toxicity in normal cells [17]. Sesquiterpene lactone terpenes are a large group of natural compounds, made up of 15 carbons and consisting of two isoprene [18]. These compounds have attracted the attention of many researchers due to their biological activities, especially their anti-cancer activity in in-vitro [18]. Alantolactone is a natural compound in the Sesquia family of lactone terpenes derived from the roots of the helenium plant. Recent studies have suggested their anti-inflammatory, antibacterial and antifungal effects. On the other hand, this natural compound has recently been reported to have inhibitory effects on cell growth and proliferation in many cancer cell lines [19]. Recent studies have shown that sesquiterpene lactone alantolactone exerts a variety of effects on cancer cells through different mechanisms such as inhibition of STAT3. This compound selectively inhibits STAT3 and exerts its anti-cancer effects [20]. Since STAT3 expression is greatly increased in most metastatic cells, in this study the effect of alantolactone as a STAT3 inhibitor on EMT process markers, N-cadherin and Vimentin, the two key markers in the transformation of normal cells into cancerous cells is evaluated.

2. Method

2.1. Materials & Equipment

DMEM culture medium, penicillin, and streptomycin, fetal bovine serum (FBS), Trypsin-EDTA prepared from Biowest Company (Nuaille, France), diphenyl tetrazolium bromide *(MTT)*, trypan blue, and phosphate-buffered saline tablets (PBS) from Sigma Company; Dimethyl sulfoxide (DMSO) from Santa Cruz Company; Alantolactone (Cat. No: SML0415), from Sigma-Aldrich (St Louis, MO; USA); Master Mix Real Time-PCR and PCR from Yekta Tajhiz Azma, (Iran); primer was synthesized by Pishgam Company; RNA extraction kit and cDNA synthesis from Yekta Tajhiz Azma (Iran). The instruments include: Reverse Phase Microscope, EcoLab, (Serial n. 2016000003218, Echo AB IMB600); Co2 incubator cell culture chamber, memmert (Germany), Cell RECOLAB laminar Air Flow LF550 Iran; Real Time-PCR Mic qPCR (Bio Molecular Systems) Australia; Measurement of light absorption for MTT by BioTek Plate Reader.

2.2. Cell culture

MDA-MB-231 cell line (NCBI code: IBRC C10684) was obtained from Pasteur Institute, which is the National Cell Bank of Iran, Tehran (NCBI) and cultured in DMEM medium supplemented with 10% FBS, 100U/ml Penicillin and 100 μg/ml streptomycin and kept in a humidified atmosphere in a 37 °C incubator with 5% CO2 with humid 95% air. Cell media was replaced done twice a week until confluency of 80–90% was achieved. Following this, the cells were trypsinized and sub-cultured for passage and storage of the cells. All cells were used at passage 3–6 in culture.

3. MTT assay

The MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide) assay was performed to evaluate the effect of ALT on cell survival. Briefly,5000 cells/well seeded in each well of the 96 well plates. After 24 h, the cell culture medium was removed and cells treated with various concentrations of ALT (0.01, 0.1, 1, 5, 10, 25, 50, 75, 100, 150 μM) for 24, 48, 72 h. Control cells were also treated with DMSO. Following incubation for 24, 48, and 72 h, the culture medium was removed and the culture medium containing MTT (Final concentration 0.5 mg/mL) was added to each well and incubated at 37 °C for 4 h. After the incubation, 100 μl of DMSO was added to each well and shaken for 5 min. Finally, Absorbance was measured at 570 nm via a microplate reader and IC50 values were assessed. Experiments were performed in triplicates.

3.1. Quantitative real-time PCR

Real-time PCR was used to evaluate gene expression. $5*10^5$ cells/well were cultured in each well of the 6-well plate. Subsequently, the cells were treated with safe concentrations (no significant difference to control) 0.01, 0.1, 1 μM of ALT for 72 h. Total RNA was extracted using a total RNA extraction kit, according to the manufacturers' protocol. In order to evaluate the purity of the extracted RNA, absorbance was measured at 260 and 280 nm. 2 μg of total RNA was used for the First-strand cDNA synthesis. For first-strand cDNA synthesis, RNA reverse transcription kit was used according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using SYBR® Green real-time PCR master mix on Mic *qPCR* Cycler (*Bio Molecular Systems*, *Australia*). Each reaction consists of 2 μM cDNA, 0.5 μL forward primer, 0.5 μM reversers primer (Primer sequences were listed in Table 1), 7 μL PCR master mix and 4 μL water. The reactions were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20s, 61 °C for 30s, and 72 °C for 30s. Experiments were performed in triplicates and the $2^{-\Delta\Delta Ct}$ (Livak) method was used to analyze the results.

Table 1
The sequences of primers for real time PCR.

Target Gene		Primer Sequence	Size of product (bp)
GAPDH	Forward	5′- CTGGAACGGTGAAGGTGACA-3′	161
	Reverse	5′-TGGGGTGGCTTTTAGGATGG-3′	
STAT3	Forward	5′-GTTCTCAAGGCACAGGTCTC-3′	194
	Reverse	5′- GCATACTGGTGGAATTTGGTC-3′	
N-cadherin	Forward	5′- GGGAAATGGAAACTTGATGGCA -3′	210
	Reverse	5′- TGGAAAGCTTCTCACGGCAT -3′	
Vimentin	Forward	5′- CAGGACTCGGTGGACTTCTC -3′	110
	Reverse	5′- TAGTTGGCGAAGCGGTCATT -3′	

3.2. Statistical analysis

The experimental results were statistically analyzed using Graph Pad Prism 7 (USA) and expressed as the mean ± SD of at least three independent experiments. Statistically significant differences were calculated with one-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. $P < 0.05$ was considered statistically significant.

The methods have been stated in accordance with STROCSS 2021 guidelines [21].

unique identifying number (UIN) of your study. researchregistry7375.

4. Results

4.1. Morphology of MDA MB 231 cells

Triple-negative breast cancer cells were grown in culture medium at a double-time rate of 38 h, and the cells were used in the logarithmic phase and between passages 3 to 6 (Fig. 1).

4.2. Effect of alantolactone on the viability of MDA-MB-231 cells

The results of MTT assay showed that alantolactone has a concentration- and time-dependent inhibition, i.e. with increasing dose and time, the inhibitory effect of alantolactone on the viability of MDA-MB-231 cells increases. Our results demonstrated that the amount of calculated IC50 was 17.54 μmol in 24 h, 8.1 μmol in 48 h and 6.068 μmol in 72 h. Since the aim of this study was to investigate the molecular mechanism affecting cell metastasis, therefore, concentrations that did not cause significant changes in cell survival compared to control were selected to continue the study (of 0.01, 0.1 and 1 μM) within 72 h.

The effect of Alantolactone on EMT markers (N-cadherin and Vimentin) **in MDA-MB-231**.

The expression level of N-cadherin gene in the groups treated with alantolactone compared to the control group is shown in Fig. 2.

The results indicated that N-cadherin was downregulated at 72 h at concentrations of 0.1, 0.01, and 1 μM, which was not statistically significant ($P < 0.05$) (Fig. 2).

The expression level of Vimentin gene in the groups treated with alantolactone compared to the control group is shown in Fig. 3. The results indicated that the expression level of Vimentin at 72 h at concentrations of 0.01, 0.1 and 1 μmol were significantly reduced in comparison to control group ($P < 0.05$) (Fig. 3).

5. Discussion

In this study, the effect of alantolactone as a STAT3 inhibitor on EMT process markers, N-cadherin and Vimentin, which are the two key markers in the transformation of normal cells into cancer was evaluated (Fig. 4). According to the results of this study, alantolactone has an anti-metastatic effect even at low doses. The expression level of N-cadherin and Vimentin genes in breast cancer cells decreases after treatment with alantolactone.

Reduced expression of N-cadherin and Vimentin genes inhibits cell proliferation and ultimately cell migration and invasion [22]. Also,

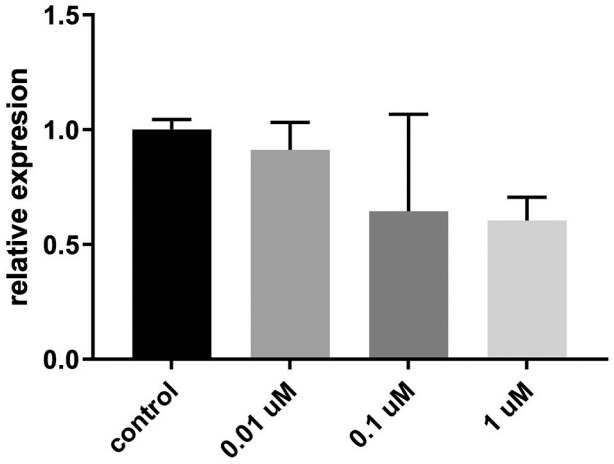

Fig. 2. Real-time PCR results of N-cadherin gene expression in alantolactone treatment on MDA-MB-231 cells. The 72 h' treatment at concentrations of 0.01, 0.1 and 1 μM of alantolactone. The findings were assessed by GAPDH internal control. The vertical axis indicates the fold changes in the standard deviation of at least three repetitions at each tested concentration. According to statistical analysis, the level of this gene decreased after drug treatment, which is only one uM dose compared to the control group. (A star symbol means that p-value is less than 0.05).

according to the results of the study conducted by Chun et al. [12], decreased expression of STAT3 gene in breast cancer inhibits metastasis. The study by Cui et al. [23] also showed that decreased expression of STAT3 gene induces apoptosis and inhibits metastasis in breast cancer. This flavonoid also reduces the expression level of N-cadherin and Vimentin as mesenchymal cell factors and increases the expression level of E-cadherin, thereby inhibiting metastasis and cell invasion. Therefore, the use of compounds that reduce the expression of STAT3, N-cadherin and Vimentin genes can inhibit metastasis in various cancers. So far, the effects of limited compounds on the expression of N-cadherin and Vimentin genes have been investigated and the combination of curcumin and nimostine hydrochloride are rare compounds whose effects on the expression of these two genes have been studied [22].

Alantolactone is one of the compounds that has been used to investigate the inhibitory effect on the expression of related genes in metastasis, including STAT3 in various cancers such as breast, lung and liver cancers. A study conducted by Chun et al. [12] on MDA-MB231 breast cancer cell line concluded that alantolactone is effective against triple-negative breast cancer by inhibiting overexpression of STAT3. The results of our study are completely consistent with the findings of these studies, showing that alantolactone can lead to reduction in STAT3 gene expression. Given that in the present study, only the effect of alantolactone on gene expression is investigated, it is recommended that in the future more studies on breast cancer focus on other mechanisms such as the production of reactive oxygen radicals, the effect on mitochondrial membranes and increased glutathione production, and their effect on the expression of different genes. In cervical cancer and colorectal cancers researches, Ala significantly inhibit the EMT genes (N-cadherin

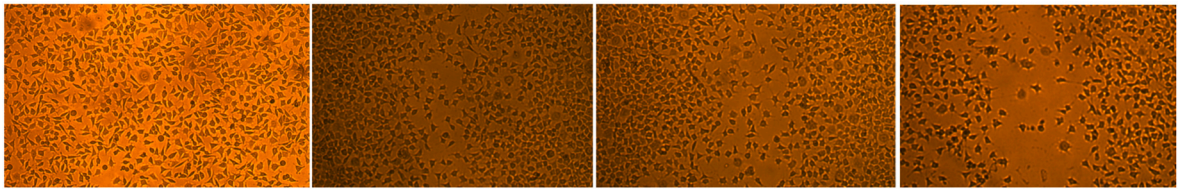

Fig. 1. The MDA-MB-231 cells with epithelial-like morphology, growth in monolayer attached form and look phenotypically as spindle shaped cells (40× magnification was consumed). Respectively from left to right; control group, 0.01, 0.1 and 1 μM concentration of ALT which cells were treated them.

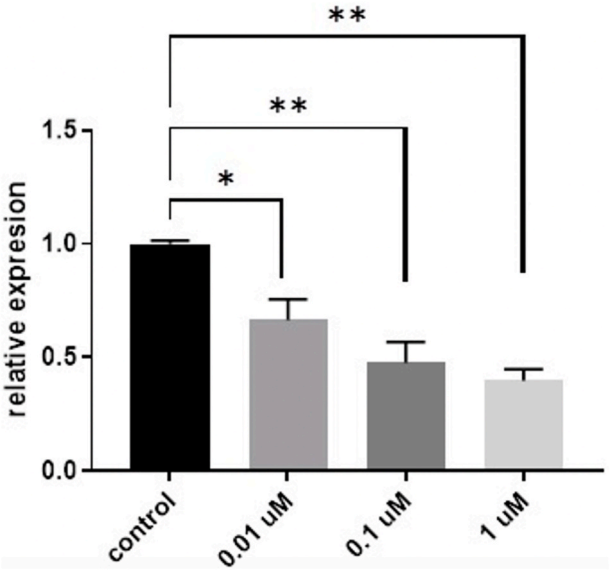

Fig. 3. Real-time PCR results of Vimentin gene expression level in alantolactone treatment on MDA-MB-231 cells. 72 h' treatment at concentrations of 0.01, 0.1 and 1 μM of alantolactone. The findings were assessed by GAPDH internal control. The vertical axis represents the ± Fold changes of standard deviation from at least three repetitions at each tested concentration. According to statistical analysis, the level of this gene decreased after drug treatment, which is significant for all three concentrations of the drug compared to the control group. (Two-star icon represents 1 and 0.1 dose with p-value 0.005 and 0.003 respectively and one-star icon stands for 0.01 dose with p-value 0.02).

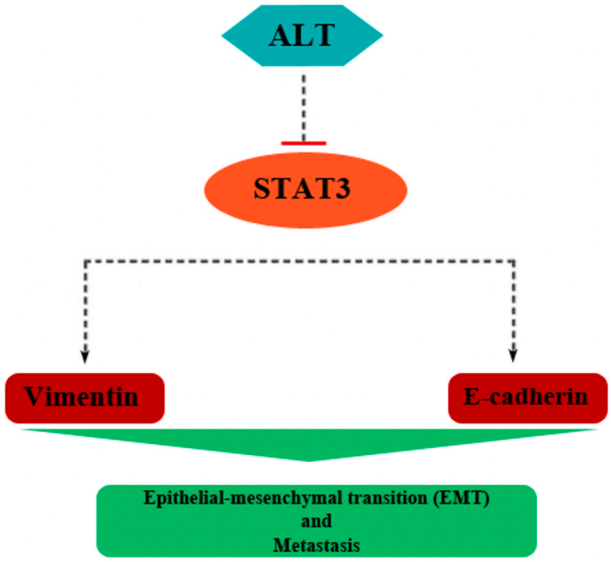

Fig. 4. Effect of Alantolactone on breast cancer cell's metastasis via STAT3 Signaling pathway.

and Vimentin) via STAT3 signaling pathway and NF-kB respectively [24]. Discovering the signaling pathway and critical molecule such as FGF, EGF, and TGF, by which N-cadherin promotes motility in epithelial cells, is important to effective targeted therapy that will decline the invasiveness of tumor cells [25]. As negative prognosis marker, increased Vimentin in breast cancer cells is evident in poor differentiated carcinomas [26] so scientist can use these biomarkers for early prediction and diagnosis [27].

Therefore, further studies should be performed on the effect of alantolactone on the expression of these two genes and its effects on apoptosis and metastasis of various cancers.

According to the findings of this study, in breast cancer cells, the levels of both, Vimentin and N-cadherin genes, have been increased to establish the metastasis process, and in general, STAT3, the main target of alantolactone, is the first gene in this pathway for cancer cell proliferation and survival. Treatment of alantolactone causes reduction in the production of STAT3, followed by inhibition of metastasis as a result of decrease in the expression of Vimentin and N-cadherin genes. Therefore, it can be concluded that the alantolactone reduces the expression of STAT3, N-cadherin and Vimentin genes and thus ultimately leads to the induction of apoptosis, reduction of cell invasion and inhibition of metastasis in MDA-MB-231 breast cancer cell line.

6. Conclusion

According to this study alantolactone reduces the viability and invasion of MDA-MB-231 cells through the inhibition of STAT3 signaling pathway at the initiation of procedure by downregulation of N-cadherin and Vimentin genes. The dosage of both the biomarkers has linear relationship with alantolactone concentration.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent

Not applicable.

Author contribution

Dr. Roya Naderi: conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript.

Dr Shiva Gholizadeh-Ghaleh Aziz: Designed the data collection instruments, collected data, carried out the initial analyses, and reviewed and revised the manuscript.

Dr. Amir Salar Haghigi-Asl: Coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Registration of research studies

1. Name of the registry:
2. Urmia University of Medical Sciences, Iran (Study ID: ir.umsu.rec.1397.06-32-1589).

Hyperlink to the registration (must be publicly accessible):

Guarantor

Dr. Shiva Gholizadeh-Ghaleh Aziz.

Declaration of competing interest

The authors deny any conflict of interest in any terms or by any means during the study. All the fees provided by research center fund and deployed accordingly.

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