Indian Journal of Gynecologic Oncology (2020) 18:41
https://doi.org/10.1007/s40944-020-00384-w

ORIGINAL ARTICLE

Degradation of HOX Transcript Antisense RNA Provoked Apoptosis and Necrosis in Human Ovarian Cancer Cells

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Received: 8 February 2020 / Accepted: 28 March 2020 / Published online: 9 April 2020

Abstract

Purpose Ovarian cancer (OC) is one of the common malignancies with poor survival rates in women. Long noncoding RNAs (lncRNAs) are a class of regulatory RNA via gene expression regulation and dysregulation results in several disorders such as cancer. LncRNA HOX transcript antisense RNA (HOTAIR) is reported to be upregulated in OC. It plays a major role in cell proliferation and metastasis. The aim of this study is to investigate the effect of HOTAIR blockage in OC cells proliferation and death.

Methods We blocked HOTAIR in OC cell line SKOV3 by Antisense LNA GapmeRs. The qPCR and Annexin V/propidium iodide staining assay were performed at three time points after transfection. Moreover, we examined *STAT3* and *MAPK8* expression levels as cancer proliferation-associated agents under the influence of GapmeRs using qPCR at 24, 48 and 72 h after transfection.

Results The rate of apoptotic cells increased in Antisense LNA GapmeRs-transfected group compared with the two other groups at all three time points. The ratio of the necrotic cell was also higher in Antisense LNA GapmeRs-treated group in contrary to the other groups in a time-dependent manner. However, among all selected genes only *MAPK8* was significantly downregulated at 24 h, followed by upregulation at 48 and 72 h. *STAT3* was remarkably increased in all three time points.

Conclusion These data demonstrated that HOTAIR can act as an onco-lncRNA and its inhibition significantly boosted apoptosis and necrosis in the SKOV3 cell line. Our findings can pave the way for antisense-based therapy as a potentially effective approach in the treatment of OC.

Keywords Apoptosis · LncRNA HOTAIR · Long noncoding RNA · Necrosis · Ovarian cancer

Introduction

Ovarian cancer (OC) is the most common malignant gynecologic cancer and remains the leading cause of cancer-related death in the world. It accounts for 5% of female cancer deaths due mainly to low survival rates, largely driven by late-stage diagnoses [1]. The first line of OC treatment is surgery which is curative in more than 90% of patients. However, in most patients, the tumor has spread beyond the ovaries by the time it is diagnosed. Thus, combined treatment of surgery and chemotherapy appears to be more effective [2]. Despite advances in surgery and chemotherapy, the overall survival of epithelial ovarian cancer (EOC) patients remains unsatisfactory, with a five-year survival rate of only 30% [3]. One of the main reasons for the poor prognosis of OC in patients is associated with the occurrence of metastasis and recurrence [4]. Moreover, it was shown the key role of acquired resistance for failure in cancer chemotherapy. As suggested by Cuello et al. [5], the synergetic induction of apoptosis via biological therapies against molecular targets as well as chemotherapy can overcome this issue. Similar to other types of cancers, OC

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is supposed to arise due to the dysregulation of cellular proliferation and apoptotic pathways. Besides, microarray analysis indicated that several individual genes involved in growth and apoptosis were differentially expressed in OC [6].

Long noncoding RNAs (lncRNAs) are a new class of regulatory molecules in many biological processes that play crucial roles in a wide variety of functions including nuclear organization, epigenetic modification of post-transcriptional regulation and RNA splicing [7]. LncRNAs, $>$ 200 nt in length, are polyadenylated spliced RNAs that mainly transcribed by RNA polymerase II and mostly localized in the nucleus [8]. Accumulating reports displayed that lncRNAs are involved in the development and progression of cancer [7]. HOX transcript antisense RNA (HOTAIR), human ovarian cancer-specific transcript 2 (HOST2) and three lncRNA derives from 5′ end of HOXA cluster are identified lncRNAs that have been dysregulated in OC [4, 9, 10]. HOTAIR, initially known as epigenetic regulatory lncRNA, is involved in promoting metastasis. It is believed to be a prognostic marker in a number of types of cancer, including breast cancer [11], hepatocellular carcinomas [12] and OC [4]. Further, Qiu and his colleagues showed that HOTAIR is upregulated in OC patients which are associated with aggressiveness and poor prognosis. Moreover, they showed the pro-metastasis effect of HOTAIR in OC via regulating the expression of a large number of genes involved in cell metastasis and epithelial–mesenchymal transition (EMT) [4]. According to their report, HOTAIR might be considered as an oncogenic lncRNA and its downregulation might provide a new therapeutic strategy in OC treatment.

Signal transducers and activators of transcription (STATs) are transcription factors that are activated in response to inflammation or growth factor-associated pathways. STAT3 is regarded as an oncogenic agent that might suppress tumor cell apoptosis upon over activating of anti-apoptotic molecules such as bcl2 family. It also induces resistance to conventional therapies in several cancers especially human OC [13].

The mitogen-activated protein kinase (MAPK) cascade is another critical proliferation-associated pathway for human cancer cell survival, dissemination and resistance to drug therapy [14]. MAPK is a serine/threonine protein kinase that is widely present in eukaryotic cells. There are different MAPKs, namely extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) also termed as MAPK8, p38 MAPK and ERK5. MAPK/ERK pathway is activated by mitogenic stimuli and strongly induces cell proliferation as well as inhibits the apoptosis pathway [15]. On the other hand, JNK and p38 MAPKs pathways are pro-apoptotic pathways which are activated upon oxidative stresses or inflammation. These pathways negatively regulate cell cycle progression and are able to provoke cell death [16].

Considering the key role of HOTAIR in the development of OC, in this study we sought to investigate the effect of HOTAIR suppression on cell proliferation and death in OC. Antisense LNA GapmeRs are a novel class of single-strand antisense oligonucleotides that induce degradation of the target sequence by the RNase H-dependent mechanism. Accordingly, we used Antisense LNA GapmeRs to induce HOTAIR degradation in a cell line (SKOV3) and then we examined apoptosis and necrosis along with the expression levels of *STAT3* and *MAPK8* under this condition.

Materials and Methods

Cell Culture

SKOV3 cell line (human) was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were cultured in RPMI 1640 (Gibco, Paisley, UK) medium supplemented with 15% of fetal bovine serum (FBS, Gibco, Paisley, UK), 100 U/ml of penicillin and 100 mg/ml of streptomycin (Sigma-Aldrich, Saint Louis, MO, USA). The cells were grown at 37 °C in a humidified incubator with 5% CO_2. To maintain the exponential phase, cells were passaged two times per week [17].

Cell Transfection

The lncRNA HOTAIR sequence was obtained from a reputable site: http://www.lncrna.org. Antisense LNA GapmeRs and antisense LNA GapmeR negative control (ALGNC) oligonucleotides for lncRNA HOTAIR were purchased from the Exiqon (Copenhagen, Denmark). Antisense LNA GapmeRs and ALGNC were labeled at their 5′ end with a fluorescent dye, 6-FAM (6-carboxyfluorescein). SKOV3 cell transfection was performed using the PolyFect transfection reagent kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 5×10^5 cells, in the exponential growth phase, were seeded in six-well culture plates (Nunc, Roskiled, Denmark) containing 1.8 ml RPMI 1640 per well without antibiotic and FBS. Six picomoles of Antisense LNA GapmeRs were mixed with 12 µl of PolyFect in 300 µl of Opti-DMEM I Medium (Gibco, Paisley, UK) and subsequently incubated at room temperature for 10 min. Then, the complex was added to the cells and rotated cautiously to ensure even distribution over the entire plate surface. After 6 h of incubation, FBS and antibiotics were added to the cells and then the cells were incubated for 24, 48 and 72 h. Untreated cells and cells transfected with ALGNC

were cultured in parallel to Antisense LNA GapmeRs-transfected cells. Antisense LNA GapmeRs were conjugated with 6-FAM fluorescein, and SKOV3 cells transfected with Antisense LNA GapmeRs were seen by a fluorescence microscope [17, 18].

Reverse Transcriptase Real-Time PCR

Reverse transcriptase (RT) real-time PCR was performed to determine the efficiency of lncRNA HOTAIR inhibition as well as differential expression alteration of *STAT3*, *MAPK8* and *AKT* under the influence of antisense LNA GapmeRs. Briefly, total RNA was extracted with miRCURY RNA Isolation Kit (Exiqon, Copenhagen, Denmark) at 24, 48 and 72 h after transfection. RNA concentration and purity were measured at an OD of 260 to 280 nm with spectrophotometer (BioTek Instruments, Winooski, VT, USA). Then, the isolated total RNA was reverse transcribed to complementary DNA (cDNA) using the Universal cDNA Synthesis Kit (Exiqon, Copenhagen, Denmark). Real-time PCR was performed using the SYBR Green Master Mix kit (Exiqon, Copenhagen, Denmark) and specific primers for lncRNA HOTAIR and selected genes (all consumables in this section were from Exiqon, Copenhagen, Denmark) (Table 1). The real-time polymerase chain reaction was performed using BioFACT^{TM} 2X Real-Time PCR Master Mix kit (BIOFACT, Korea) and the StepOne Plus^{TM} quantitative real-time PCR detection system (Applied Biosystems). PCR reactions were carried out in a total volume of 10 µl. The PCR amplification conditions consisted of 15 min at 95 °C followed by 45 cycles of denaturation step at 95 °C for 15 s and annealing and extension for 1 min at 60 °C. The expression level of each target gene was calculated by the $2^{-\Delta\Delta CT}$ method, and *GAPDH* gene (glyceraldehydes-3-phosphate dehydrogenase) was used to normalize gene expression. All experiments were carried out in triplicate and independently repeated at least three times.

Apoptosis and necrosis assay

The Annexin-V-FLUOS Staining kit (Roche, Manheim, Germany) was used for detection of apoptosis and necrosis in SKOV3 cells. For the detection of phosphatidylserine, an apoptosis marker on the outer leaflet of the apoptosis cell member, Annexin V was used. To distinguish necrotic cells, propidium iodide (PI) staining was performed. The procedure was performed at 24, 48 and 72 h after transfection according to the manufacture's instruction. (Untreated cells were used for controls.) Concisely, 24, 48 and 72 h after transfection, the SKOV3 cells which were seeded to six-well plates at a density of 5×10^5 cells per well were transferred to flow cytometry tubes and centrifuged for 5 min at 1500 rpm. The supernatant was removed away, and cells were washed with 1 ml of cold phosphate-buffered saline (Gibco, Paisley, UK). 100 ml of the prepared solution (based on kit instruction) was added to each tube and incubated at room temperature in the dark for 15 min. After incubation, 300 µl of AVBB solution was added to each tube and analyzed by FACS Calibur flow cytometer (BD, California, USA) with 488 nm excitation, 518 nm band-pass filter for fluorescein-conjugated Annexin V detection and a filter > 600 nm for PI detection [18].

Statistical Analysis

All tests were performed in triplicate, and the results were analyzed using SPSS version 22 (IBM, New York, NY, USA) software. Two-way ANOVA (two-way analysis of variance) and also post hoc test to examine were considered. Data were presented as mean ± SEM. Statistical significance was defined as $P < 0.05$.

Results

For inhibition of lncRNA HOTAIR, the Antisense LNA GapmeRs were transfected to SKOV3 cell line with PolyFect transfection reagent kit. As the transfected

Table 1 Primer sequences for qPCR

Primer name	Sequence	Length	Accession number
HOTAIR	F: 5′TCTGGAGCTTGATCCGAAAG3′	97	NR_003716.3
	R: 5′GGTGTTGGTCTGTGGAACT3′		
MAPK8	F: 5′CCAGGACTGCAGGAACGAGT3′	102	NM_001323328.1
	R: 5′CCACGTTTTCCTTGTAGCCC3′		
STAT3	F: 5′GGCTTTTGTCAGCGATGG3′	151	NM_001369520.1
	R: 5′GATTCTGCTAATGACGTTATCC3′		
GAPDH	F: AAGCTCATTTCCTGGTATG	125	NM_002046.7
	R: CTTCCTCTTGTGCTCTTG		

HOTAIR, HOX transcript antisense RNA; MAPK8, mitogen-activated protein kinase 8; STAT3, Signal transducer and activator of transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

oligonucleotides were fluorochrome-conjugated, transfection efficiencies were evaluated by fluorescence microscopy. The expression level of lncRNA HOTAIR was measured by reverse transcriptase lncRNA real-time PCR in SKOV3 cells transfected with the antisense LNA GapmeRs, antisense LNA GapmeR negative control (ALGNC) and untreated SKOV3 cells at 24, 48 and 72 h after transfection. The expression level of lncRNA HOTAIR in ALGNC group was significantly lower than that of the untreated group at all three time points ($P < 0.001$). In Antisense LNA GapmeRs groups, the expression level of lncRNA HOTAIR was remarkably declined compared to both ALGNC and untreated groups in 24, 48 and 72 h ($P < 0.001$). Moreover, our qPCR results showed the lowest expression level of lncRNA HOTAIR was at 24 h after transfection in SKOV3 cells and then raised slightly at 48 and 72 h after transfection (Fig. 1).

To evaluate the effect of lncRNA HOTAIR inhibition on apoptosis and necrosis, cells were stained with Annexin V and PI at 24, 48 and 72 h after transfection. Apoptotic cells were almost undetectable in untreated cells. However, the rates of apoptotic cells were slightly increased at 48 and 72 h after ALGNC transfection contrary to untreated cells (Fig. 2). At all three time points, the apoptotic assay analysis displayed a significant increase in the apoptotic cells after transfection with Antisense LNA GapmeRs compared with both ALGCN-transfected cells and controls ($P < 0.001$). The highest amount of apoptosis was observed 24 h after transfection, whereas this ratio was gradually decreased at 48 and 72 h post-transfection. Collectively, the results exhibited that blockage of HOTAIR by transfection of Antisense LNA GapmeRs noticeably boosted the apoptosis of SKOV3 cells (Fig. 2). Consistent with apoptosis assay results, necrosis was

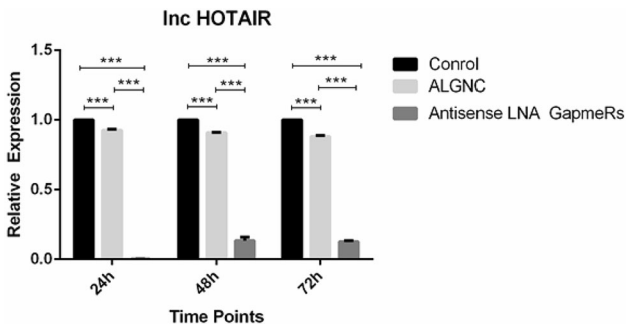

Fig. 1 Antisense LNA GapmeRs suppressed the expression of lncRNA HOTAIR at 24, 48 and 72 h after transfection. The expression level of HOTAIR was significantly decreased in both ALGNC and Antisense LNA GapmeRs group compared with untreated controls. The minimum rate of the expression level of HOTAIR was at 24 h post-transfection. Relative mRNA expression was normalized to GAPDH. The values are expressed as means (± SEM); ***$P < 0.001$ indicates significant differences between groups

undetectable in untreated cells, while the ratio of the necrotic cells slightly raised at 48 and 72 h after ALGNC transfection in comparison with the untreated cell group (Fig. 3). The necrosis ratio was also significantly increased in the Antisense LNA GapmeRs-transfected cells compared with the other groups in all three time points. The lowest amount of necrosis was seen at 24 h post-transfection whereas, and this ratio was gradually increased at 48 and 72 h post-transfection. Taken together, these results demonstrated that inhibition of HOTAIR by Antisense LNA GapmeRs is able to induce the necrosis in SKOV3 cells ($P < 0.001$) (Fig. 3).

The expression level of *STAT3* and *MAPK8* was measured by real-time qPCR. Based on our result, *STAT3* is noticeably increased in the Antisense LNA GapmeRs group compared with the other groups in all three time points ($P < 0.001$) (Fig. 4a). However, the qPCR analysis showed that the expression level of *MAPK8* was significantly downregulated in SKOV3 cells at 24 h after transfection of Antisense LNA GapmeRs in comparison with ALGCN group and untreated control ($P < 0.001$ for Antisense LNA GapmeRs group vs. untreated control and $P < 0.01$ for Antisense LNA GapmeRs vs. ALGCN group) (Fig. 4b).

Discussion

In this study, we investigated the inhibitory effect of lncRNA HOTAIR on ovarian cell lines SKOV3. In order to reach this goal, we used Antisense LNA GapmeRs to degrade lncRNA HOTAIR in SKOV3 cells. We confirmed the downregulation of lncRNA HOTAIR after LNA GapmeRs transfection using real-time qPCR. The transfection of Antisense LNA GapmeRs significantly raised the rate of SKOV3 cell death. Besides, degradation and blockage of HOTAIR noticeably induced apoptosis and necrosis in SKOV3. Moreover, necrosis induction in Antisense LNA GapmeRs-transfected cells occurred in a time-dependent manner. These results highlighted the notion that degradation of lncRNA HOTAIR can augment the apoptosis and necrosis of SKOV3 cells. These data are consistent with the previous report that considered HOTAIR as an oncogenic lncRNA [19]. HOTAIR is highly correlated with lymph node metastasis as well as overall survival and disease-free survival reduction in OC patients. The pro-metastatic impact of HOTAIR is mainly mediated by the regulation of MMP/EMT-related genes [4]. Furthermore, a recent report suggested that HOTAIR overexpression might play a crucial role in cellular senescence and platinum sensitivity in OC through regulating NF-κB activation [20]. Zhang and his colleagues claimed that knockdown of HOTAIR reduced cell viability and promoted cell apoptosis in OC

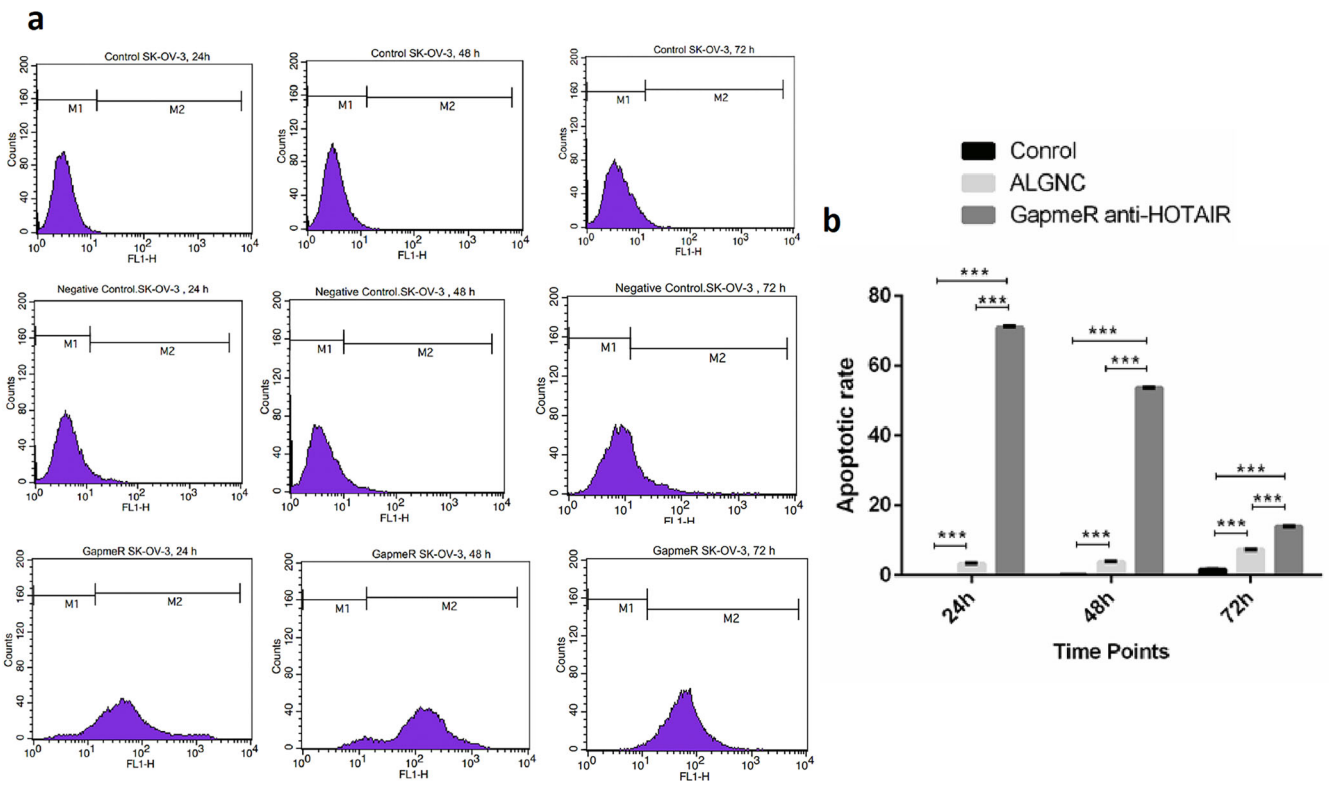

Fig. 2 Antisense LNA GapmeRs transfection induced apoptosis at 24, 48 and 72 h in SKOV3 cell lines. **a** Representative cytofluorometric graphs are shown the assessment of apoptosis by Annexin V–propidium iodide staining performed at 24, 48 and 72 h after transfection. **b** Annexin V–PI staining indicated that Antisense LNA GapmeRs significantly increased apoptosis at all three time points. The value is expressed as means (± SEM); *** $P < 0.001$ indicates significant differences between groups

cell line HEYC2. They also displayed the positive regulation between HOTAIR and Rab22a, in which Rab22a gene is involved in the EMT process during OC progression, via miR-373 sponging [21]. These reports are consistent with our results that the suppression of lncRNA HOTAIR induced apoptosis as well as necrosis in SKOV3 cells.

Based on qPCR results, transfection of Antisense LNA GapmeRs provoked the expression level of *STAT3* but decreased the expression level of *MAPK8* at 24 h, followed by a significant upregulation at 48 and 72 h post-transfection. Burke and colleagues demonstrated that *STAT3* is overexpressed in OC and the inhibition of *STAT3* predominantly blocks the OC and also breast cancer progression. Their results introduced *STAT3* as one of the potential therapeutic targets for treating OC [22]. Besides, a previous study disclosed that active MAPK/ERK pathway is supposed to be considered as a good prognostic marker in patients with high-grade OC [23]. Furthermore, it is reported that phenethyl isothiocyanate, as a cytotoxicity factor, prevented the proliferation of OS cell line (OVCAR-3) via suppression of *ERK1/2*, *AKT* and *c-myc* as well as simultaneously activating pro-apoptotic *p38* and *JNK1/2* [24]. JNK (MAPK8) signaling cascades are activated upon stress signals such as UV- and γ-irradiation, heat shock as well as chemotherapeutic drugs, and supposed to be involved in protective responses, stress-dependent apoptosis and inflammatory responses [25, 26]. Cisplatin is a chemotherapeutic drug that suppresses OC cell growth by provoking JNK/p38 pathway [27]. Thus, a comprehensive understanding of the complex interaction network of genes and their regulators needs to be achieved to unravel the molecular mechanism underlying lncRNA HOTAIR-mediated tumor development.

Due to high metastatic and drug resistance features of OC, it is dispensable to develop novel therapeutic targets to effectively address these challenges. The blockage of lncRNA HOTAIR using Antisense LNA GapmeRs triggered apoptosis in SKOV3 cells. These findings confirmed that HOTAIR could be considered as an onco-lncRNA in OC. Since uncontrolled cell growth is the main issue of cancer progression, the increased rate of apoptosis/necrosis

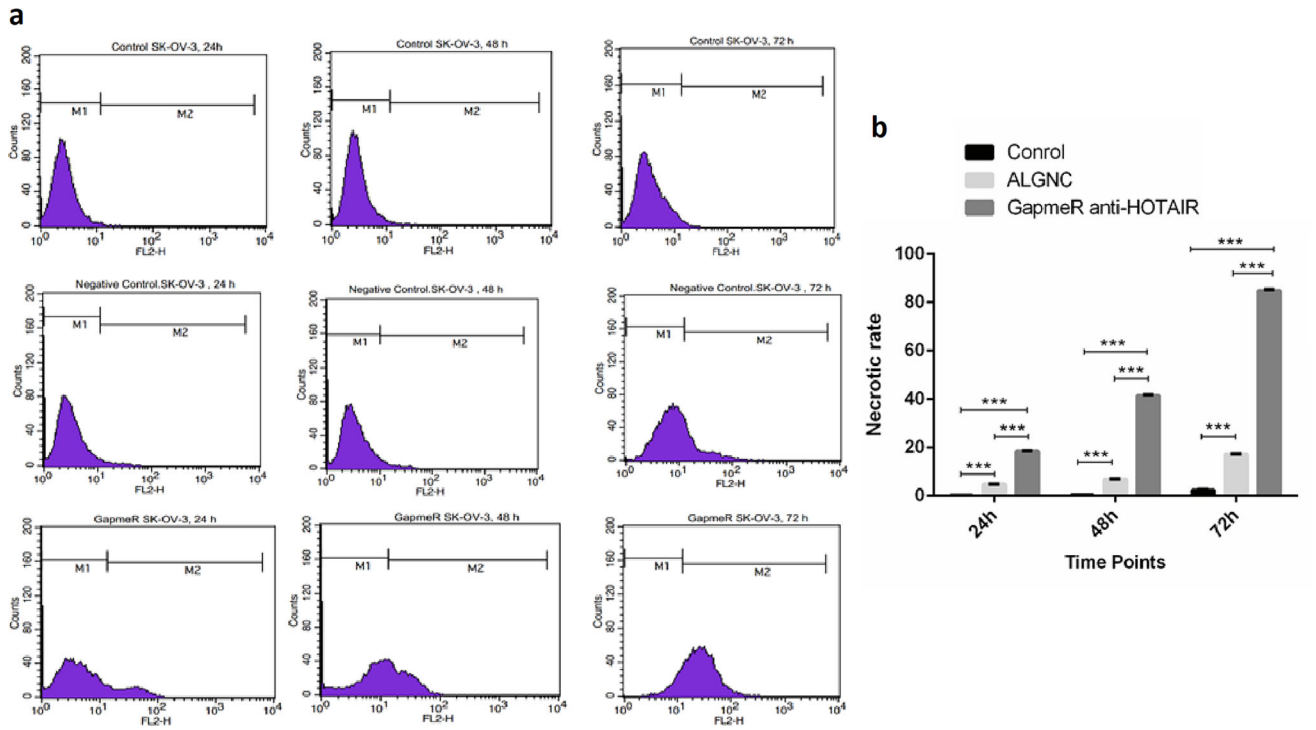

Fig. 3 Antisense LNA GapmeRs transfection induced necrosis at 24, 48 and 72 h in SKOV3 cell lines. **a** Representative cytofluorometric graphs are shown the evaluation of necrosis by Annexin V–propidium iodide staining performed 24, 48 and 72 h after transfection. **b** Annexin V–PI staining displayed that Antisense LNA GapmeRs gradually increased the necrotic rate in SKOV3 cells at 24, 48 and 72 h. The value is expressed as means (± SEM); $***P < 0.001$ indicates significant differences between groups

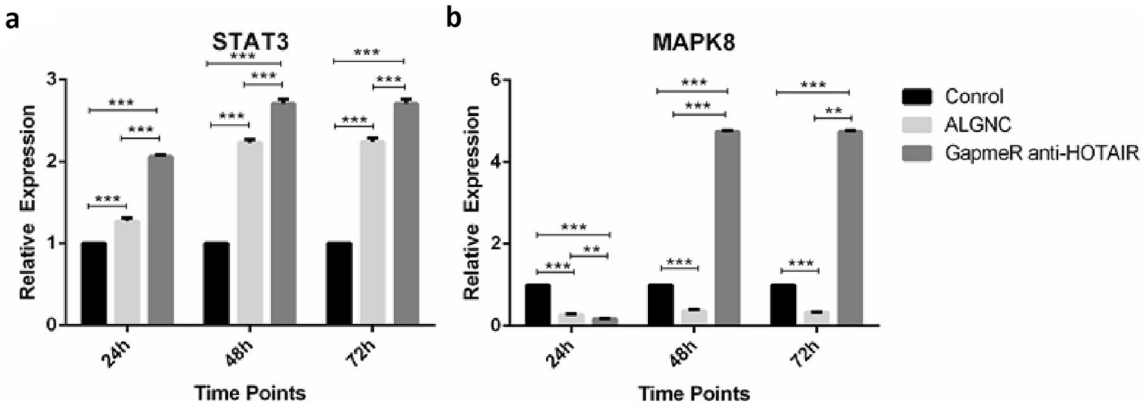

Fig. 4 The expression level of *STAT3* and *MAPK8* was evaluated under the influence of Antisense LNA GapmeRs and ALGNC by qPCR. **a** *STAT3* was significantly upregulated in both Antisense LNA GapmeRs and ALGNC group compared to untreated control in all three time points. **b** The expression level of *MAPK8* in ALGNC group was significantly lower than that in untreated control; however, Antisense LNA GapmeRs statistically declined the expression level of *MAPK8* at 24 h with significantly increased at 48 and 72 h. Relative mRNA expression was normalized to *GAPDH*. The value is expressed as means (± SEM); $***P < 0.001$ and $**P < 0.01$ indicate significant differences between groups

in proliferating cells is regarded as an efficient strategy toward cancer treatment. Accordingly, inhibition of onco-lncRNA HOTAIR by Antisense LNA GapmeRs method might be a potentially effective approach in the treatment of OC. Taken together, our study indicated antisense-based therapy can act as one of the potential options in OC treatment. However, well-tailored in vivo investigations are highly needed to validate the feasibility of this strategy in clinical settings.

Acknowledgements This study was conducted with the support of Isfahan University of Medical Science Bushehr Branch, Islamic Azad University (Iran).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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