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Research Article

SOX2 Overlapping Transcript (*SOX2-OT*) Enhances the Lung Cancer Malignancy Through Interaction with *miR-194-5p/*SOX5 Axis

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Background: Lung cancer is one of the most common types of cancer and a leading cause of cancer-related deaths worldwide. Therefore, it is useful to know the biomarkers involved in the malignancy of lung cancer.

Objectives: This study aimed to show that *SOX2-OT* as a long non-coding RNA (IncRNA) regulates gene expression via the *SOX2-OT/miR-194-5p/*SOX5 axis molecular pathway in lung cancer.

Materials and Methods: A549 cells transfected with siRNA-*SOX2-OT* and the expression of *SOX2-OT* and *miR-194-5p* genes were analyzed by real-time PCR before and after transfection. In addition, the expression of the B-catenin, MMP9, phosphorylated and activated STAT3 (p-STAT3), SOX5, and VEGF proteins before and after transfection was investigated by Western blotting.

Results: After using siRNA-*SOX2-OT*, an increase in the expression of *miR-194-5p* and a decrease in the expression of B-catenin, SOX5, p-STAT3 activated STAT3, VEGF, and MMP9 proteins was observed.

Conclusions: According to the results of the present study, an increase in *SOX2-OT* in lung cancer seems to stimulate the expression of beta-catenin, SOX5, MMP9, and VEGF thus support the malignancy of lung cancer cells.

Keywords: β-catenin, *miRNA-194-5P*, MMP9, p-STAT3, SOX5, *SOX2-OT*, VEGF

1. Background

Lung adenocarcinoma (LAC) has the highest mortality rate in the world (1, 2). Despite advances in targeted therapy, most LAC patients die due to recurrence and drug resistance. In fact, the diagnosis is delayed due to a lack of understanding of the pathogenesis of lung cancer. Therefore, it is important to identify reliable predictive biomarkers and study the disease at an early stage. Therefore, the lack of a better molecular biomarker to predict prognosis accounts for the poor outcomes. LAC requires the identification of reliable prognostic predictors that can improve diagnosis and serve as therapeutic targets (3). It is known that large parts of the genome are transcribed as non-coding RNA (4) that have been classified into short and long ncRNAs (lncRNAs). Short ncRNAs are composed of small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), transfer RNA (tRNA), microRNAs (miRNAs), ribosomal RNA (rRNA), and other RNAs. lncRNAs are usually longer than 200 nucleotides (5). lncRNAs are increasingly being considered for cancer early detection and prognosis to be introduced as biomarkers. For example, high expression of *SOX2-OT* lncRNA (*Sox2-overlapping transcript*) is associated with poor survival in lung adenocarcinoma patients (6).

SOX2-OT is a IncRNA located in a sequence that overlaps with the *SOX2* gene (7). *SOX2-OT* is highly conserved and plays an important role in conserved ontogenetic processes (8). Despite its increased ex-pression in lung cancer (9-11), the details of the *SOX2-OT* mechanism in lung tumors need further investigation. Several

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lncRNAs have been discovered that act as competitors for endogenous RNAs (ceRNAs). In the ceRNA hypothesis, lncRNA can sponge microRNA (miRNA) and control the expression of downstream genes (12). miRNAs, short non-coding RNAs, affect gene expression through mechanisms including repressing messenger RNA translation or mediating mRNA degradation (13). Indeed, lncRNAs can associate with miRNA molecules to form a broad regulatory network and protect their target RNAs from inhibition. (14).

Wei *et al*. (2018) reported that *SOX2-OT* targets *miR-194-5p* in gastric cancer (GC). In addition, they reported that *SOX2-OT* knockdown suppresses MMP2 and MMP9 gene expression in gastric cancer (15) and likely has the same effect in lung cancer. It is also reported that *SOX2-OT* acted as a ceRNA for *miR-194-5p* in colorectal cancer and upregulated SOX5 by sponsoring *miR-194-5p*. This downregulated *SOX2-OT* increased *miR-194-5p* expression and decreased SOX5 protein levels, which suppresses colorectal cancer tumorigenesis (16). There is no report on the interaction between SOX2-OT and SOX5 in lung cancer, probably the same function is predicted for *SOX2-OT* who studied this process in lung cancer. Recently, in 2021, this topic that *SOX2-OT* sponging *miR-194-5p* was reported by Ni *et al*. (17). Angiogenesis is an important signal of poor prognosis required for the process of cancer metastasis and cell proliferation. Pathways of tumor angiogenesis in lung adenocarcinoma are therapeutic targets, while the precise mechanisms underlying angiogenesis in lung adenocarcinoma are still unknown. It is important to identify key molecules in the regulator of angiogenesis as a poor prognostic signal (18, 19). According to previous articles, SOX5 induces angiogenesis of lung adenocarcinoma by inducing VEGF expression through phosphorylation and stat3 activation (20).

According to Song *et al*. (2019), *SOX2-OT* inactivated the Wnt/β-catenin signaling pathway by modulating the *miR-452-5p*/*HMGB3* axis in prostate cancer. This means that in prostate cancer, *SOX2-OT* increases the expression of the HMGB3 protein by inhibiting *miR-452-5p* and decreases beta-catenin protein levels (21). Therefore, it is hypothesized that inhibition of *SOX2-OT* is likely to lead to an increase in beta-catenin expression, growth, and proliferation in lung cancer, and beta-catenin levels were measured after *SOX2-OT* knockdown.

2. Objectives

This study focused on the investigation of the relationship between *SOX2-OT* and the *miR-194-5p/* SOX5/P-STAT3 axis in lung cancer, and the effects of *SOX2-OT* knockdown on MMP9 (metastatic marker), VEGF expression (angiogenesis marker) and beta-catenin expression at the protein level.

3. Materials and Methods

3.1. Culture Media and Cell Lines

The A549 cell line was obtained from Pasture Institute (Tehran, Iran). It was cultivated in a humidified atmosphere with 5% CO_2. The A549 cells were seeded into 6-well tissue culture plates and incubated overnight at 37 °C. After about 24 hours, the medium was changed to DMEM with 5% FBS. After about 3 hours, the cells were transfected with 50 or 100 nM *SOX2-OT* siRNA. Transfection was performed using the Lipofectamine 2000 reagent according to the manufacturer's protocol. Then, after about 6.5 hours, the transfected medium was discarded and replaced with DMEM with 10% FBS. The transfected cells were incubated for 48 h before being harvested for further analysis. After the extraction, the quality of the RNA extraction product was checked using 1% agarose gel electrophoresis. Sequences of siRNAs for human *SOX2-OT* are siRNA1: GGAUAGGCCUCACUUACAA & siRNA2: GGAGAUUGUGACCUGGCUU. **Figure S1** shows the position of siRNAs on the *SOX2-OT* gene (**Fig. S1**).

3.2. RNA Isolation, cDNA Synthesis, and Quantitative RT-PCR

Two days after cell transfection with siRNAs (siRNA 1 or siRNA 2 or siRNA 1 + siRNA 2) for samples and no transfection for control, total RNA was extracted from A549 cells using the Tripura reagent according to the manufacturer's protocol. The Add bio cDNA synthesis kit and two micrograms of total RNA treated with DNase I (Termo Fisher Scientific, Inc.) were used for the reverse transcription experiment. The primer sequences are shown in **Table 1**. Results were analyzed using Excel software. The relative mRNA levels of the target genes were adjusted for beta-actin for *SOX2-OT* and RNU48 for *miRNA194-5p* and then determined as 2^-ddCq.

Table1. Sequences of the primers (5′ to 3′ direction) used in real-time PCR

SOX2OT F	GCTCGTGGCTTAGGAGATTG
SOX2OT R	CTGGCAAAGCATGAGGAACT
β- actin F	AGACGCAGGATGGCATGGG
β-actin R	GAGACCTTCAACACCCCAGCC
Sox2 F	TACAGCATGTCCTACTCGCAG
Sox2 R	GAGGAAGAGGTAACCACAGGG
miR194-5p F	AACGCAGTGTAACAGCAACTC
miR194-5p RTPrimer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCACA
universal miRNA Primer	GTCGTATCCAGTGCAGGGT
RNU48 RTPrimer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTCAG
RNU48 F	CTCTGAGTGTGTCGCTGATGCC

3.3. Western Blotting

The transfected and control cells were prepared and total proteins were extracted as recommended in the protocol of Sara Company in Tabriz, Iran. Western blotting was determined by Sara Company in Iran.

4. Results

4.1. The Cell Culture of A549

To compare the expression of the studied genes, A549 cells were cultured in two 3 cm plates (**Fig. 1**) and the treatment groups were transfected with siRNA1 or siRNA2 or both siRNAs. Two RNA-related bands show the high quality of the RNA extraction products (data not shown). After DNase treatment, cDNA synthesis was performed.

A549 before transfection | A549 + siRNA 1 & 2 after 48 h

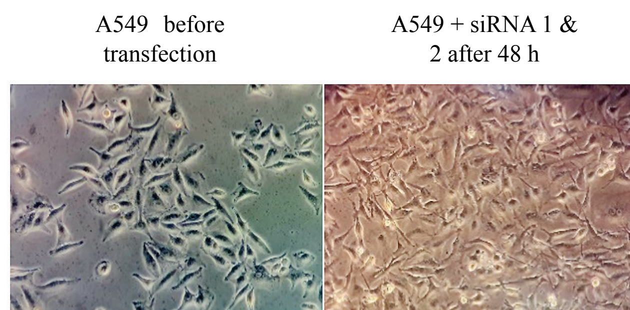

Figure 1. The A549 cells before and after si-*SOX2-OT* transfection. magnification, 20X.

4.2. Verification of SOX2-OT Knockdown with SiRNA

After transfection, the expression level of *SOX2-OT* in the transfected cells and control cells were checked and compared after 48 hours using real-time PCR. At this stage, the beta-actin gene was used as a reference gene. After setting the Mic system software to LinReg, the Cq data was extracted into Excel, and the expression was calculated using the 2^-ddCq method. Statistical significance was checked by a t-test in Excel software. As a result, *SOX2-OT* expression decreased significantly (P-value < 0.05) compared to the control group (**Fig. 2**). SiRNA 1 does not significantly reduce expression of the *SOX2-OT* gene (P-value > 0.05). However, siRNA 2 (50nM) caused a significant reduction in *SOX2-OT* at the 0.05 level. The use of siRNA 2 (100nM) caused a significant decrease in *SOX2-OT* at the 0.01 level. According to bioinformatic analysis, siRNA 1 decreased the expression of short variants and siRNA 2 decreased all variants.

4.3. SOX2-OT Knockdown, Decreases SOX2 Expression and Increases miR-194-5p Expression in A549 Cell Line.

To examine the relationship between the *SOX2* and *SOX2-OT* genes, their expression was measured by real-time PCR after reducing *SOX2-OT* gene expression, and t-tests were performed with Excel to determine significance. β-actin was the reference gene when measuring gene expression. As shown in **Figure 3**, *SOX2* gene expression decreased significantly in line with the decrease in *SOX2-OT* gene expression.

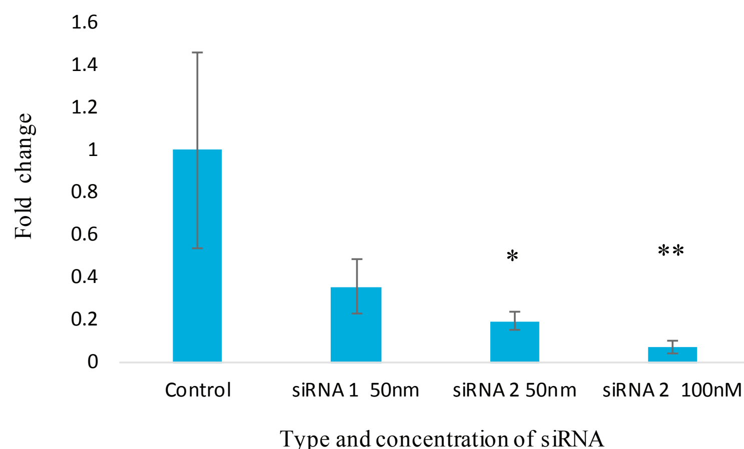

Figure 2. The Expression assessment of *SOX2-OT* in A549 cells before and after si-*SOX2-OT* transfection. The control sample is the untreated A549 cells; siRNA 1 50nM are A549 cells treated with siRNA 1, 50 nM only, and the third group are A549 cells transfected with siRNA 2, 50 nM. The final column shows A549 cells transfected with 100 nM of siRNA2. The A549 siRNA 1 (50nM) group was not significantly different from the A549 control group ($P > 0.05$). *SOX2-OT* expression exhibited a dramatic decrease (to about 0.196) in the A549 siRNA 2 (50nM) group (* $P < 0.05$). The cells with siRNA2 (100nM) transfection suppressed *SOX2-OT* expression significantly (to about 0.073) 2 days after transfection (** $P < 0.01$). The error bar indicates the mean±SE. B-actin gene expression was used as the housekeeping gene control to normalize Cq values.

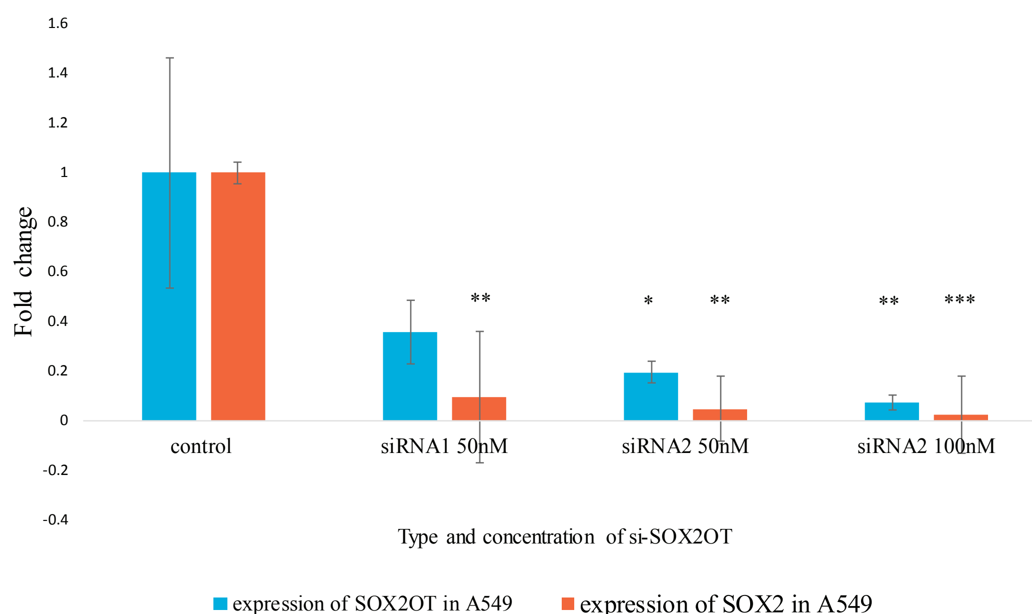

Figure 3. The Expression assessment of *SOX2* in A549 cells before and after si-*SOX2-OT* transfection. The expression of the *SOX2* gene is reduced with the decrease in the *SOX2-OT* gene expression compared to the control group. *SOX2* expression is 0.097 (** $P < 0.01$), 0.050 (** $P < 0.01$), and 0.025 (*** $P < 0.001$) for A549 treated with siRNA1 50 nM, siRNA2 50 nM, and siRNA2 100 nM, respectively. The error bar indicates the mean±SE. B-actin gene expression was used as the housekeeping gene control to normalize Cq values

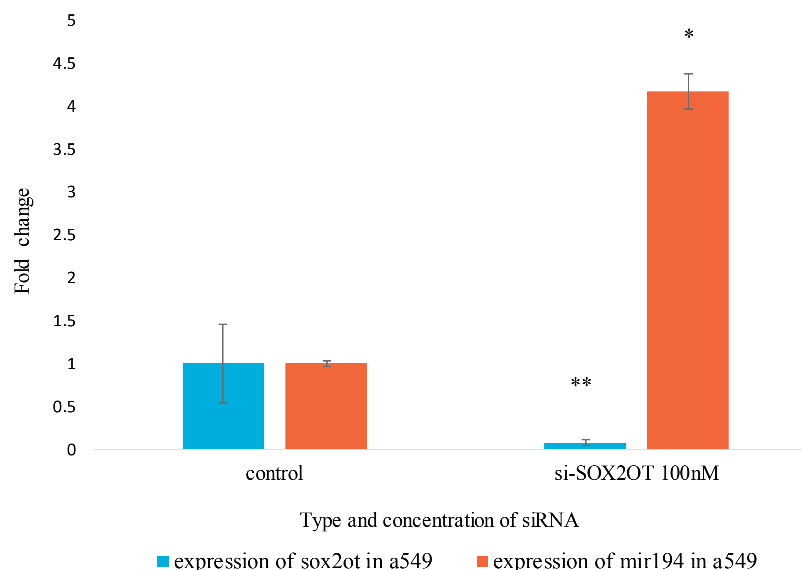

Figure 4. The Expression assessment of *miR-194-5p* in A549 cells before and after si-SOX2-OT transfection. The *miR-194-5p* gene expression significantly increased (4.17 fold) (* $P < 0.05$) after the decrease in *SOX2-OT* gene expression (** $P < 0.01$) compared to the control groups. The error bar indicates the mean±SE. The RNU48 gene expression was used as the housekeeping gene control to normalize Cq values.

To investigate the relationship between *SOX2-OT* and *miR-194-5p* after reducing *SOX2-OT* gene expression, the expression of both genes was measured in real-time PCR. A graph was drawn with Excel and a t-test was performed to check for significance. The RNU48 gene was used as a reference gene to measure *miR-194-5p* gene expression. As shown in **Figure 4**, *miR-194-5p* gene expression increased significantly and concomitantly with the decrease in *SOX2-OT* gene expression.

4.4. SOX2-OT Knockdown Decreases β-catenin, SOX5, P-STAT3, VEGF, and MMP9 Proteins

To study the effects of *SOX2-OT* on poor prognostic factors such as metastasis and angiogenesis, after reducing *SOX2-OT* expression, changes in the expression of key proteins in this signaling pathway were measured, including MMP9 (effective in metastasis) and VEGF (effective in angiogenesis). According to the articles, STAT3 plays an important role in the progression of various cancers, including proliferation, invasion, angiogenesis, and evasion from immune surveillance (23). Therefore, after reducing *SOX2-OT* expression, changes in phosphorylated and activated STAT3 were measured. In addition, *SOX2-OT* promotes epithelial-mesenchymal transition by *miR-194-5p* sponging, induces some cancer cells to grow (15), and also affects the *miR-194-5p*/SOX5 axis and leads to malignant cancer (16). After *SOX2-OT* knockdown and an increase in *miR-194-5p* gene expression, SOX5 protein expression was examined using Western blotting. Also, altering B-catenin expression as one of the key proteins of the Wnt/β-catenin signaling pathway that causes cell growth was investigated. Changes in beta-actin protein expression were examined as a reference gene. As shown in **Figure 5**, β-actin housekeeping gene expression did not change significantly after reducing *SOX2-OT* expression. On the other hand, the expression of MMP9, β-catenin, VEGF, and SOX5 proteins, as well as phospho-STAT3 (activated STAT3) decreased. Quantification of Western data using the image j program showed tthat the β-catenin, SOX5, P-STAT3, VEGF, and MMP9 proteins decreased to about 58.77%, 32.88%, 66.11%, 20.70%, and 31.41% respectively after *SOX2-OT* knockdown. The results showed that the expression of these genes changed concordantly with *SOX2-OT* gene expression.

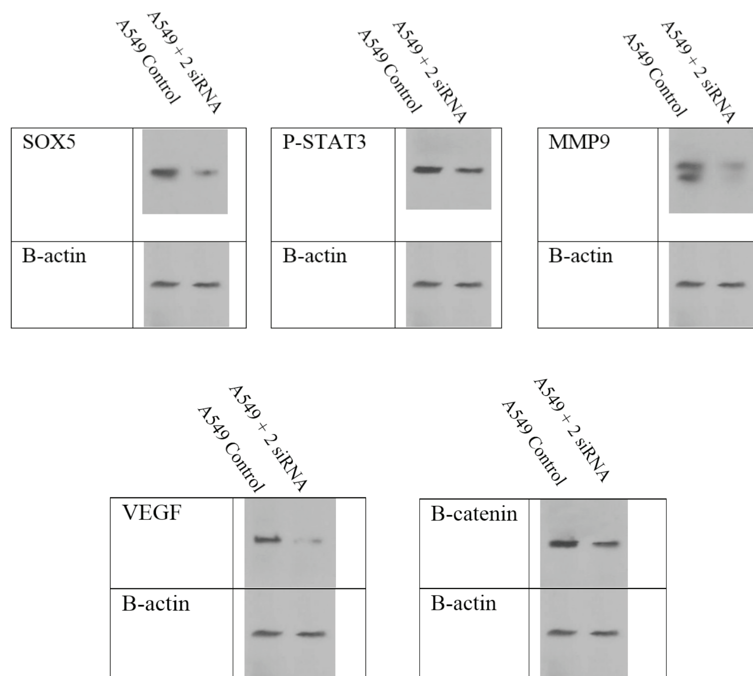

Figure 5. Western blot of β-actin, β-catenin, SOX5, p-STAT3, VEGF, and MMP9 proteins before and after si-*SOX2-OT* transfection. Western blotting showed a decrease in the intensity of the band and, as a result, a decrease in the expression of all the studied proteins after si-*SOX2-OT* transfection (50nM from each siRNAs). β-actin was used as a reference protein.

5. Discussion

SOX2-OT is expressed in several diseases, including human lung cancer (24), esophageal squamous cell carcinoma (25), breast cancer (26), and prostate cancer (27). However, the specific action and molecular mechanism of *SOX2-OT* in cancer are not yet fully understood. In this study, the molecular pathway of *SOX2-OT* function in lung cancer was investigated. The expression level of *SOX2-OT* was twice that of non-tumor samples adjacent to the tumor in 53.01% of people with primary lung cancer. In addition, high *SOX2-OT* expression is associated with poor survival in lung cancer patients (9). Studies showed that expression of *SOX2-OT* was significantly higher in non-small cell lung cancer (NSCLC) tissues and serum samples than in normal controls (11, 28, 29). Wang *et al*. reported that *SOX2-OT* knockdown reduces the ability of cells to form colonies and induces G2/M cell cycle arrest (29). Teng *et al*. reported that *SOX2-OT* increased in patients with lung squamous cell carcinoma compared to negative controls (30). Wei *et al*. showed that *SOX2-OT* with *miR-194-5p* sponge promotes the transition from epithelial to mesenchymal and causes the growth of gastric cancer cells (15). Feng *et al*. reported that *SOX2-OT* leads to cancer malignancy progression in colorectal cancer by affecting the *miR-194-5p*/SOX5 axis (16). In this work, it is shown that *SOX2-OT* knockdown leads to a 4-fold increase in *miR-194-5p* expression in the A549 lung cancer cell line. In fact, it is possible that by reducing *SOX2-OT* expression using siRNA, the number of spongy *miR-194-5p* decreases and expression increases. This finding agrees with those of Nei *et al*. They studied the effect of *SOX2-OT* on *miR-194-5p* in lung cancer and reported that *SOX2-OT* promotes bone metastasis in lung cancer by affecting the *miR-194-5p*/RAC1 axis in osteoclasts. Furthermore, they demonstrated the association between *SOX2-OT* and *miR-194-5p* using the MS2 RIP assay in the A549 cell line (17).

The Wnt/β-catenin signaling pathway is activated in many types of cancer (31). By examining the expression of proteins associated with the Wnt/β-catenin signaling

pathway in prostate cancer using a Western blot method, Song *et al*. found that accumulation of *SOX2-OT* increased protein levels of b-catenin and c-myc, suggesting that an increase in *SOX2-OT* expression activates the Wnt/β-catenin signaling pathway (21). Liu *et al*. investigated glioblastoma drug resistance to temozolomide as one of the main reasons for glioblastoma recurrence and poor prognosis. They reported that *SOX2-OT* regulates this drug sensitivity by increasing the expression of *SOX2* and further activating the Wnt5a/β-catenin signaling pathway *in vitro* and *in vivo*. Their results showed that *SOX2-OT* lncRNA inhibited cell apoptosis, and increased cell proliferation and drug resistance by increasing *SOX2* expression, which activates the Wnt5a/β-catenin signaling pathway (32).

Based on data in articles, we hypothesized that increased expression of *SOX2-OT* in lung cancer alters *SOX2* and beta-catenin expression and results in Wnt/β-catenin signaling pathway progression. Therefore, after reducing the expression of *SOX2-OT*, we measured the expression of *SOX2* and the key protein of the Wnt/β-catenin signaling pathway, beta-catenin. The results showed that reducing *SOX2-OT* expression leads to a reduction in the *SOX2* and β-catenin expression. Therefore, it is possible that *SOX2-OT* increases cell growth in lung cancer by stimulating *SOX2* and β-catenin expression, compatible with Song's (21) results. Our results are also reported by Hou *et al*. who showed that *SOX2-OT* knockdown inhibits cell proliferation by reducing the number of cellsin the S phase and triggering G2/M arrest (9).

Studies have shown that *miR-194-5p* regulates SOX5 gene expression in age-related osteoarthritis (33). Based on this, *SOX2-OT* is predicted to increase SOX5 gene expression by reducing *miR-194-5p* in lung cancer. Hence the SOX5 expression decreased after *SOX2-OT* reduction. Our results showed that reducing *SOX2-OT* expression led to a reduction in SOX5 protein expression. The present result is consistent with Feng's reports on the colorectal cancer, which found that *SOX2-OT* causes malignancy through the *miR-194-5p*/SOX5 axis (16). The results suggest that the SOX5 protein metastasizes through the epithelial-mesenchymal transition and predicts a poor prognosis in lung adenocarcinoma (3). According to reports and studies, SOX5 activates STAT3 in A549 cells (20). Phosphorylation of STAT3 at tyrosine 705 activates STAT3 through signaling from upstream regulators (34). STAT3 plays an important role in the development of various cancers, including proliferation, invasion, angiogenesis, escape from immune surveillance, etc. (23).

Therefore, since our study showed that *SOX2-OT* reduction decreased the expression of SOX5, it was investigated whether it also activates STAT3. Therefore, after *SOX2-OT* knockdown, phosphorylated and activated STAT3 was measured by Western blotting and a decrease in activated STAT3 (p-STAT3) was observed. This finding is consistent with that of Chens in lung adenocarcinoma who reported that SOX5 causes increased activation of STAT3 in A549 cells (20). Therefore, we propose that in lung cancer, the increase in *SOX2-OT* expression causes *miR-194-5p* sponging and thereby increases SOX5, leading to the activation of STAT3. In the study by Chens (2018), SOX5 and VEGF expression showed a positive correlation in patients with lung adenocarcinoma. VEGF is probably the most commonly involved proangiogenic factor. Some studies have shown that VEGF expression is always regulated by the transcription factor STAT3 and the extracellular signal-regulated kinase (ERK) or protein kinase B (AKT) signaling pathway. The Janus kinase 2/STAT3/VEGF pathway has been reported to induce tumor angiogenesis in non-small cell lung cancer (NSCLC). Studies have shown that STAT3 increases VEGF expression and stimulates angiogenesis in lung cancer (20).

Therefore, it appears that *SOX2-OT* increases the expression of SOX5 through *miR-194-5p* sponging and capturing in lung cancer. On the other hand, SOX5 appears to stimulate VEGF by regulating the activation of STAT3 as a key signaling protein. Chen *et al*. (2018) linked STAT3/VEGF activation in A549 cells to overexpression of SOX5 (20). So we hypothesize that *SOX2-OT* increases VEGF by activating STAT3. Our *in vitro* results showed that reducing *SOX2-OT* expression led to a reduction in SOX5 protein and therefore VEGF protein and activated STAT3 are reduced. Consequently, our results predict the importance of *SOX2-OT* as a biomarker that induces adenocarcinoma angiogenesis by inducing VEGF expression through STAT3 activation in lung cancer.

Wei *et al*. showed that in gastric cancer, *SOX2-OT* increases the expression of matrix metalloproteinases 2 and 9 by inhibiting *miR-194-5p* (15).

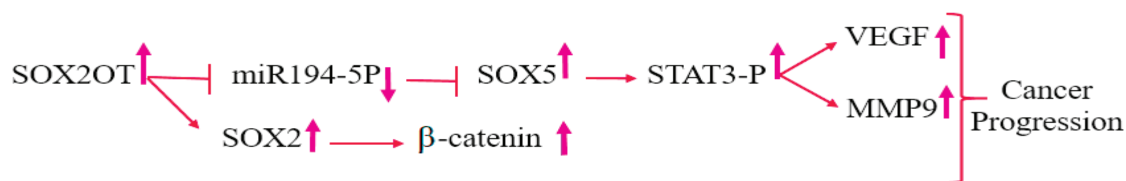

Figure 6. Proposed pathway for the *SOX2-OT* molecule in lung cancer. It seems that *SOX2-OT* increases the expression of SOX5 by inhibiting *miR-194-5p,* and SOX5 increases VEGF and MMP9 by activating STAT3. On the other hand, *SOX2-OT* increases the expression of beta-catenin.

Shi *et al*. reported that the SOX5 transcription factor enhances migration and invasion by regulating MMP-9 expression in a type of rheumatoid arthritis (35). On the other hand, it was shown in ovarian cancer research that activation and phosphorylation of STAT3 in tyrosine 705 caused regulation and production of MMP9 (36). Therefore, the expression of *miR-194-5p*, SOX5, p-STAT3, and MMP9 were measured in lung cancer *in vitro* after a reduction in *SOX2-OT* by siRNA. The results showed that after *SOX2-OT* reduction, *miR-194-5p* expression increased while SOX5, p-STAT3, and MMP9 decreased. It is assumed that increased *SOX2-OT* expression by reducing *miR-194-5p* increases the SOX5 transcription factor, which transcribes MMP9 by activating STAT3. Based on our findings in this research, the molecular signaling pathway of **Figure 6** for the action of *SOX2-OT* in lung cancer can be proposed and traced for the first time.

6. Conclusion

According to the results of the present study, an increase in *SOX2-OT* in lung cancer appears to stimulate the expression of beta-catenin and thus support the growth of lung cancer cells. On the other hand, this suggests that *SOX2-OT* increases the SOX5 transcription factor by scavenging *miRNA-194-5P* and this molecule stimulates the expression of MMP9 and VEGF, the key molecules of metastasis and angiogenesis, by activating STAT3. Therefore, *SOX2-OT* can be introduced as a biomarker for lung cancer and the result is that inhibition of *SOX2-OT* can prevent the upstream expression of important poorly prognostic molecules such as MMP9, VEGF and beta-catenin.

Acknowledgments

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