Abstract

This study aimed to determine lncRNA SNHG14 and miR-206 in Thyroid Cancer (TC) and to explore the related mechanisms. Sixty-four samples of thyroid tissue were collected from patients with TC. TC cell lines and a normal human thyroid cell line (HTori-3) were bought. lncRNA SNHG14-siRNA (si-lncRNA SNHG14), lncRNA SNHG14-shRNA (sh-lncRNA SNHG14), blank plasmid (siRNA-NC), miR-206-inhibitor, miR-206-mimics were transfected into BHT101 and Ocut-2C cells. qRT-PCR quantified the expression of lncRNA SNHG14 and miR-206, and the expression of vimentin, Snail, N-cadherin, Slug, E-cadherin and ZO-1 proteins were identified via WB. MTT assay, flow cytometry, and Transwell were employed to determine cellular proliferation, apoptosis, and invasion, separately. The high expression of lncRNA SNHG14 and low expression of miR-206 were exhibited in patients with TC. lncRNA SNHG14 and miR-206 were related to lymph node metastases, TNM staging, as well as differentiation of TC. Silencing lncRNA SNHG14 and over-expressing miR-206 inhibited the expression of Akt, p-ERK1/2, p-p38, p-4EBP1, p-Akt, PI3K, vimentin, Snail, N-cadherin, and Slugn, as well as up-regulated the expression of E-cadherin and ZO-1. Rescue experiment showed that after BHT101 and Ocut-2C cells were transfected with either sh-lncRNA SNHG14+miR-206-mimics or si-lncRNA SNHG14+miR-206-inhibitor, the cellular proliferative, invasive, and apoptotic activities weren't different from those transfected with siRNA-NC. Suppression of lncRNA SNHG14 up-regulates miR-206 and affects EMT, as well as proliferative, invasive, and apoptotic activities of cells, which may become an underlying treatment target for TC.

Keywords: lncRNA-SNHG14, PI3K-Akt/MAPK-ERK, Thyroid cancer, Biological mechanism

1. Introduction

Thyroid cancer (TC) stands as a global endocrine ailment, emerging as the most prevalent thyroid malignancy worldwide [1,2]. Despite the relentless strides in targeted therapy and molecular diagnostics, the survival rates and prognoses for individuals in the late stages of TC remain notably unsatisfactory [3,4]. The scarcity of robust biological markers for the prevention, early diagnosis, metastasis monitoring, and prognostic evaluation of TC underscores the critical need for a more comprehensive understanding of the underlying molecular mechanisms. Such insights are imperative for formulating effective treatment plans that can address the complexity of this prevalent malignancy [5-7].

MicroRNAs (miRNAs), known for their regulatory roles in cellular functions, have gained attention for their potential to influence tumor behaviors. In the context of endocrine tumors, research on functional miRNAs has been limited. However, studies have confirmed suppressed expression of miR-206 in TC, making it a focal point in the exploration of molecular pathways and potential therapeu tic interventions [8-10].

The progression of TC is intricately linked to the dysregulation of long non-coding RNAs (lncRNAs), which exert substantial influence over the Epithelial-Mesenchymal Transition (EMT) and overall biological functions of TC cells. The burgeoning field of targeted diagnosis and treatment in TC, particularly involving lncRNAs, holds promise for revolutionizing therapeutic approaches [11,12].

LncRNAs, acting as versatile modulators, play pivotal roles as either oncogenes or tumor suppressors, exerting profound effects on the cell cycle and apoptosis. Their dynamic alterations significantly impact tumor progression, influencing the prevention and treatment of related diseases [13,14]. Among these, LncRNA SNHG14 has been implicated in various tumors, demonstrating a carcinogenic role. However, the specific effects of LncRNA SNHG14, its correlation with TC diagnosis and prognosis, and the underlying molecular mechanisms remain largely elusive.

Current research suggests that lncRNAs serve as key mediators in extracellular communication within the tu-
mor microenvironment, with lncRNA SNHG14 specifically identified as a facilitator of TC cell proliferation and metastasis through its role as a miRNA sponge [5].

In light of these considerations, our study aims to unravel the intricate molecular mechanisms by investigating the expressions of lncRNA SNHG14 and miR-206. By deciphering how LncRNA SNHG14 influences TC progression through the regulation of miR-206 expression, we aspire to identify robust cancer biomarkers and potential therapeutic targets that could significantly enhance the accuracy of TC diagnoses and prognoses. This research endeavor contributes to the evolving landscape of TC understanding and opens avenues for the development of targeted medicines tailored to the specific molecular intricacies of this prevalent malignancy.

2. Materials and Methods

2.1. General data

Herein, an overall 64 sufferers (34 males and 30 females, aged 54.8±3.52 years average) with TC were enrolled. Inclusive criteria: Every sufferer was diagnosed with papillary adenocarcinoma via pathological, cytological and image-forming methods (15); sufferers received no pre-operation chemical therapy, immune therapy, radiation therapy or other anticancer therapies. Exclusive criteria: sufferers with incomplete general clinical data; patients complicated with coagulation dysfunction or liver cirrhosis; patients who did not cooperate with or failed to follow-up; patients who were expected to survive for less than 1 month. The Ethical Board of our hospital accepted our research and the entire subjects enrolled signed informed consent forms in advance.

2.2. Main instruments and reagents

B-CPAP, BHT101, KTC-1, Ocut-2C, HTh-7 (human TC cell line) and HTori-3 (normal human thyroid cell line) (BNCC100390, BNCC100391, BNCC100400, BNCC100401, BNCC100396, BNCC338687, Be Na Culture Collection Company); ABI Stepone Plus quantitative real-time polymerase chain reaction (qRT-PCR) equipment, Lipofectamine™2000 transfection kit, RNA abstraction tool (TRIzol), Annexin V/propidium iodide (PI) apoptotic tool (Invitrogen, America); SYBR Green PCR Master Mix (ABI, America); β-catenin polyclonal goat IgG, cyclin D1 polyclonal goat IgG, c-myc polyclonal goat IgG, cyclin D1 polyclonal goat IgG, c-myc polyclonal goat IgG, β-actin, HRP conjugated goat antimouse second antisubstance (R&D Systems, America). FACSCanto flow cytometry machine (BD, America); DR5000 ultraviolet-visible spectrophotometry apparatus (BioRad, America); synthesis of primer sequences (Shanghai Sangon Bioengineering Company, PRC).

Table 1. Primer sequences of miR-206, lncRNA SNHG14, and their internal references.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>miR-206</td>
<td>5'-ATCCGAGGATGAATCTCAC-3’</td>
<td>5'-AGCCGAGGAGAAGACGTTG-3’</td>
</tr>
<tr>
<td>U6</td>
<td>5’-CTCGCTTCGCGACACA-3’</td>
<td>5’-AAGGCTTCAAGATTTCGCT-3’</td>
</tr>
<tr>
<td>lncRNA SNHG14</td>
<td>5’-TGCCACGACGACAGCAGCA-3’</td>
<td>5’-GTCGAGGTCGAGGACT-3’</td>
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<tr>
<td>GAPDH</td>
<td>5’-CAAAGGTGGATCATGAGAAG-3’</td>
<td>5’-GGTGAGCATATCAACCGAG-3’</td>
</tr>
</tbody>
</table>

2.3. Detection Methods

2.3.1. Cell culture and transfection

Transfection was performed in DMEM added with 10% FBS and PNC-koowamycin liquor, and TC lineage cells was cultivated at 5% CO₂, 37°C, and saturated humidity. lncRNA SNHG14-siRNA (si-lncRNA SNHG14), lncRNA SNHG14-shRNA (sh-lncRNA SNHG14), and blank plasmid (siRNA-NC) were introduced into the cells through transfection with the greatest diversity in lncRNA SNHG14 expression with a Lipofectamine™2000 transfection kit. After 6 h, the cells were grown in medium supplemented with 10% FBS. qRT-PCR determined transfection efficiency.

2.3.2. qRT-PCR

qRT-PCR was deployed to identify the expression of mRNAs in tissular samples and cells. Total RNAs extracted with TRIzol were dissolved in diethyl pyrocarbonate (DEPC)-treated water (20 μL), reverse-transcribed with a reverse transcription kit, then cultivated under 38°C for 1 h. Each sample was tested in 3 repeating wells for 3 repeated tests. U6 and GAPDH were inner references of miR-206 and lncRNA SNHG14, separately. PCR amplification and melting curves were plotted after the reaction, and the results of the relatively quantitative assay of targeted genes were studied via 2-∆∆CT. (Table 1).

2.3.3. Western Blot (WB)

After being transferred to a centriufuge tube, the lysed cells were subjected to centrifugation at 12000×g for 600 s under 4°C. The protein level in the supernate was identified via BCA. The excess liquid was dried, and development was completed in a dark room by the electrochemiluminescence (ECL) reagent. The protein bands were scanned to obtain the gray values with Quantity One (Molecular Devices Corp, America).

2.3.4. MTT assay

Cellular activities were measured via the MTT assay. Cells collected 24 h posterior to transfection (5×10³ cells/well) were seeded into 96-well dishes under 37°C for 24, 48, and 72 h, separately, and the MTT liquor (20 μL, 5 μmg/mL) was supplemented at every temporal point. Posterior to the inoculation under 37°C for 4 h, 200 μL DMSO was put into all wells. The value of OD was read at 570 nm wavelength by a spectrophotometry instrument.

2.3.5. Transwell

Transwell insert was coated with Matrigel and allowed to stand under 37°C for 0.5 h. Cells were re-suspended with no-serum MEM. Cellular suspension (200 μL, 4×10⁴ cells/mL) and DMEM (800 μL, 10% FBS) were added to the apical and basolateral chambers, respectively. After 24-48 h of culture, the insert was taken out. Cells were immobilised via 4% PFA and stained in 0.1% gentian
violet. Cells in the apical chamber were removed. Cellular invasive abilities were evaluated with a light microscope under 10 randomly selected high-power fields.

2.3.6. Cell apoptosis assay

Cells transfected for 48 h were trypsinized (0.25%), cleaned twice with PBS, re-suspended with AnnexinV binding buffering solution (100 μL), then prepared to a suspension with the density of 1×10⁶ cells/mL. The suspension was supplemented with Annexin-V/FITC (5 μL), and cultivated under 4°C for 15 min. Afterwards, PI dyeing liquor (5 μL) was applied and the suspension was cultivated under 4°C for 300 s. Cellular apoptotic activities were determined using flow cell technique. The assay was finished in triplicate to acquire the average.

2.4. Statistical analysis

Data processing was carried out with Statistic Package for Social Science (SPSS) 20.0 (IBM, Armonk, NY, USA). Data in Gaussian distribution were presented by average ± SD (X±SD), and comparison of measurement data between groups adopted independent samples t-test. The comparison among data at several temporal points was conducted repeatedly by measurement ANOVA, and post hoc test adopted by Bonferroni. One-way ANOVA was used for the contrast of means amongst several groups, and post hoc test adopted Fisher’s least significant difference-t (LSD-t). The diagnosis significance was evaluated via ROC curve. Pearson test was utilized for correlative analysis. P<0.05 had significance on statistics.

3. Results

3.1. Expression of LncRNA SNHG14 and miR-206 in TC

qRT-PCR demonstrated that LncRNA SNHG14 was regulated upward and miR-206 was regulated downward in TC tissular samples. ROC found that the AUC of LncRNA SNHG14 was greater than 0.08. The sufferers were allocated into a high-expression group and a low-expression group by the mid-value of LncRNA SNHG14 expression. The expression of LncRNA SNHG14 was related to lymph node metastases, TNM staging, as well as TC differentiation activities. In contrast to normal mankind thyroid cell HTori-3, LncRNA SNHG14 in TC cells was remarkably increased while miR-206 was remarkably decreased. Correlative analysis revealed the negative association between LncRNA SNHG14 and miR-206 in TC. In addition, LncRNA SNHG14 and miR-206 had the most significant changes in BHT101 and Ocut-2C cells, so these two cells were selected as research objects (Table 2, and Figure 1).

3.2. Expression of LncRNA SNHG14 and miR-206 and their impacts on cellular biofunction

qRT-PCR was employed to identify the expressing of LncRNA SNHG14 in lineage cells. Compared with normal mankind thyroid cell HTori-3, the expression of LncRNA SNHG14 in B-CPAP, BHT101, KTC-1, Ocut-2C, and HTh-7 cells increased markedly (P<0.05). BHT101 and Ocut-2C cells in which LncRNA SNHG14 expression showed the greatest difference were enrolled for transfecion. The expressing of LncRNA SNHG14 in siRNA-lncRNA SNHG14 group was remarkably lower than in contrast to the siRNA-NC group (P<0.01), and shRNA-lncRNA SNHG14 group was significantly greater in contrast to the siRNA-NC group (P<0.01). MTT test demonstrated that the proliferative activities in the siRNA-lncRNA SNHG14 group were significantly hindered in contrast to the siRNA-NC group (P<0.05), and that in the shRNA-lncRNA SNHG14 group was enhanced (P<0.05). Flow cytometry showed that compared with siRNA NC group, the apop-

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Table 2. Association between LncRNA SNHG14 and pathological data.

<table>
<thead>
<tr>
<th>Factor</th>
<th>LncRNA SNHG14 expression</th>
<th>( \chi^2 )</th>
<th>P</th>
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<tr>
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<tr>
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<td>18(56.25)</td>
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<tr>
<td>Female (n=30)</td>
<td>14(43.75)</td>
<td>16(50.00)</td>
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<td></td>
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<tr>
<td>≥40 years old (n=42)</td>
<td>22(68.75)</td>
<td>20(62.50)</td>
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<tr>
<td>&lt; 40 years old (n=22)</td>
<td>10(31.25)</td>
<td>12(37.50)</td>
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<td>T staging</td>
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</tr>
<tr>
<td>T1+T2(n=28)</td>
<td>19(59.38)</td>
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<td>Differentiation</td>
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<td>7.752</td>
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<td>Poorly differentiated (n=27)</td>
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<td>19(59.38)</td>
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tosis of siRNA-lncRNA-SNHG14 group increased (P < 0.001), and the apoptosis of shRNA-lncRNA-SNHG14 group was inhibited (P < 0.001). Transwell revealed that the cellular aggression in the siRNA-lncRNA SNHG14 group was dramatically hindered in contrast to the siRNA-NC group (P<0.001), and that in the shRNA-lncRNA SNHG14 group was significantly enhanced (P<0.001) (Figure 2).

qRT-PCR was employed to quantify the expressing of miR-206 in lineage cells. Compared with normal mammalian thyroid cell HTori-3, miR-206 in BHT101 and Ocut-2C cells was remarkably regulated downward (P<0.05). BHT101 and Ocut-2C cells in which miR-206 expression showed the greatest difference were enrolled for transfection. The expressing of miR-206 in the miR-206-suppressor group was remarkably suppressed in contrast to the miR-NC group (P<0.01), and the expression in the miR-206-mimetic substance group was remarkably elevated (P<0.01). CCK-8 revealed that the proliferative activities in the miR-206-mimetic substance group were remarkably hindered in contrast to that in the miR-206-suppressor group with low differentiation is remarkably greater in contrast to that in those with moderate and high differentiation, and the expressing of miR-206 in sufferers with low differentiation is remarkably lower in contrast to that in those with moderate and high differentiation. a denotes P<0.001.

3.3. Effects of lncRNA SNHG14 and miR-206 on EMT
WB demonstrated that after cells were subjected to transfection with sh-lncRNA SNHG14 and miR-206-suppression, separately, the expressing of vimentin, Snail, N-cadherin and Slug proteins was remarkably up-regulated, and that of E-cadherin and ZO-1 was remarkably down-regulated. Nevertheless, contrary outcomes were revealed in cells transfected with si-lncRNA SNHG14 and miR-206-mimics, separately (P<0.001) (Figure 4).

3.4. Determination of targeted genes of lncRNA SNHG14
Targeted binding loci between miR-206 and lncRNA SNHG14 were found by predicting target genes downstream of lncRNA SNHG14 through Targetscan6.2. Therefore, DLR test was completed and found that pmirGLO-lncRNA SNHG14-3'UTR Wt fluorescein enzyme activity was remarkably reduced after over-expressing miR-206 (P<0.001), but no changes showed in pmirGLO-lncRNA SNHG14-3'UTR Mut fluorescein enzyme activity (P>0.05). PCR demonstrated that the expressing of miR-206 in BHT101 and Ocut-2C cells was regulated downward remarkably posterior to the transfection of sh-lncRNA SNHG14, and up-regulated posterior to the transfection of si-lncRNA SNHG14 (P<0.001) (Figure 5).
3.5. SNGH14 reverses the EMT transformation of Thyroid cancer by miR-206

Sh-lncRNA SNHG14+miR-206-mimics and Si-lncRNA SNHG14+miR-206-suppressor were transfected into BHT101 and Ocut-2C cells independently to evaluate cell biological function. After transfection, the growth, aggression, and metastasis in either Sh-lncRNA SNHG14+miR-206-mimics group or Si-lncRNA SNHG14+miR-206-suppressor group had no difference in contrast to those in the siRNA-NC group, and no differences existed between the two groups (P>0.05). Nevertheless, Sh-lncRNA SNHG14+miR-206-mimics and Si-lncRNA SNHG14+miR-206-suppressor groups showed remarkably enhanced proliferation, invasion, and migration than Sh-lncRNA SNHG14 group (P<0.05), which were remarkably weakened than Si-lncRNA SNHG14 group (P<0.05). The expression of EMT-related proteins in Sh-lncRNA SNHG14+miR-206-mimics and Si-lncRNA SNHG14+miR-206-inhibitor groups had no difference compared with that in NC group (P>0.05). In contrast to Sh-lncRNA SNHG14 group, the expression of E-cadherin and ZO-1 proteins was remarkably suppressed, and the expression of vimentin, Snail, N-cadherin and Slug protein was remarkably elevated. However, compared with Si-lncRNA SNHG14 group, reversed results were obtained (P<0.05) (Figure 6).

4. Discussion

Recently, the regulatory effect of lncRNA-miRNA has aroused much interest in clinical research for the design of novel molecule-targeted therapies [16]. LncRNAs and miRNAs participate in protein-coding in human life acti-
lncRNA SNHG14 and Kirsten rat sarcoma viral oncogene homolog (KRAS) was regulated upward while miR-944 was regulated downward in CRC. In addition, studies have shown that lncRNA SNHG14 knock-down hinders tumor growth, and lncRNA SNHG14 reduces proliferation and accelerates apoptosis of CRC cells via suppressing the PI3K/AKT signal path [23]. Ye et al. [24] also stated that lncRNA SNHG14 affects biological function of oncocytes via targeting miR-32-5p, which further suggests the influence of lncRNA SNHG14 changes on TC. However, how lncRNA SNHG14 influences the biofunction and EMT of TC cells remains largely unknown.

lncRNA SNHG14 induces EMT in the process of cancer cell proliferation, thus accelerating disease progression [21]. Herein, our team monitored EMT-associated protein after suppressing or over-expressing lncRNA SNHG14 and miR-206 in TC cells. Vimentin, Snail, N-cadherin and Slug proteins in cells with low lncRNA SNHG14 expressing or high miR-206 expressing were regulated downward significantly, and E-cadherin and ZO-1 were remarkably increased, while the results in cells with high miR-206 expression or low lncRNA SNHG14 expression were opposite. Therefore, lncRNA SNHG14 inhibits EMT of cells via regulating miR-206. There are also studies on EMT of TC cells that E-cadherin, ZO-1 and other connexins are down-regulated and cell adhesion is decreased; up-regulation of N-cadherin, Vimentin and zinc finger protein superfamily promotes the dissociation of tumor cells, resulting in increased invasiveness [25,26].

The rescue experiment found that transfecting either miR-206-mimics+sh-lncRNA SNHG14 or miR-206-inhibitor+si-lncRNA SNHG14 did not change the biological function of BHT101 and Ocut-2C, while compared with si-lncRNA SNHG14 group, the growth, aggression, and metastasis were enhanced, and the results were reversed in sh-lncRNA SNHG14 group, suggesting that lncRNA SNHG14 regulates miR-206 targeted. We further verified the relationship between miR-206 and lncRNA SNHG14 through DLR assay. The fluorescein enzyme activity of lncRNA SNHG14-3'UTR Wt increased significantly after over-expressing miR-206, but no changes were revealed in sh-lncRNA SNHG14 group, suggesting that lncRNA SNHG14 regulates miR-206 targeted. The progression of tumors was associated with EMT, and the presence of EMT promoted the biofunction variations of oncocytes. Therefore, miR-206 can serve as an available target for TC treatment, moreover, the suppression of lncRNA SNHG14 can repress the growth, invasion, and EMT of TC cells. The expression of lncRNA SNHG14 and miR-206 hindered cell proliferation and invasion. The progression of tumors was associated with EMT, and the presence of EMT promoted the biofunction variations of oncocytes. Therefore, miR-206 can serve as an available target for TC treatment, moreover, the suppression of lncRNA SNHG14 can repress the growth, invasion, and EMT of TC cells. The expression of lncRNA SNHG14 and miR-206 hindered cell proliferation and invasion.

lncRNA SNHG14 and miR-206 thyroid cancer

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5. Conclusions
To sum up, lncRNA SNHG14 modulates the growth and apoptosis of TC cells via regulating miR-206, and may become an underlying clinical treatment target for TC. Moreover, miR-206 and lncRNA SNHG14 may be available markers for diagnoses and prognoses of TC.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the ethics committee of The Second Hospital of Nanjing.

Informed Consent
Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Yiming Sang and Rui Min designed the study and performed the experiments, Tao Huang collected the data, Jizong Zhang analyzed the data, Yiming Sang and Rui Min prepared the manuscript. All authors read and approved the final manuscript.

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This study did not receive any funding in any form.

Abbreviations
Thyroid cancer (TC); microRNAs (miRNAs); Dulbecco's Modified Eagle Medium (DMEM); Western Blot (WB); small nucleolar RNA host gene 14 (lncRNA SNHG14); propidium iodide (PI); Methyl thiazolyl tetrazolium (MTT); horseradish peroxidase (HRP); diethyl pyrocarbonate (DEPC); electrochemiluminescence (ECL); quantitative real-time polymerase chain reaction (qRT-PCR); dimethyl sulfoxide (DMSO); standard deviation (SD); receiver operating characteristic (ROC); tumor–node–metastasis (TNM); colorectal cancer (CRC); epithelial-mesenchymal transition (EMT); The Cancer Genome Atlas (TCGA); Kirsten rat sarcoma viral oncogene homolog (KRAS); long non-coding RNAs (lncRNAs); fetal bovine tissue (FBS); glyceraldehyde-3-phosphate-dehydrogenase (GAPDH); dual-luciferase reporter (DLR).

References
landscape identifies a role for ARLNC1 in prostate cancer progression. Nat Genet 50:814-824. doi: 10.1038/s41588-018-0120-1


