Biomedicine & Pharmacotherapy 103 (2018) 38–45

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

The role of long non-coding RNA ANRIL in the carcinogenesis of oral cancer by targeting miR-125a

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ARTICLE INFO

Keywords:
lncRNA ANRIL
miR-125a
Oral cancer
Proliferation
Migration
Invasion

ABSTRACT

Recently, increasing evidence has indicated that lncRNAs may play a critical role in the progression of oral cancer (OC). However, whether lncRNA-ANRIL is involved in the tumorigenesis of OC remains undetermined. In the present study, ANRIL showed significantly higher, while miR-125a showed lower, expression in OC tissues and sera than in normal controls. MTT, colony formation, flow cytometry analysis, wound-healing, transwell and mice xenograft model assays were used to detect the proliferation, migration, and invasion of ARNIL-overexpressing HB56 cells and ARNIL-knockdown CAL27 cells. The results showed that cell proliferation, migration, and invasion were significantly increased by ARNIL overexpression and decreased by ARNIL silencing in oral cancer cells. Furthermore, we found a negative correlation between ARNIL and miR-125a, and ARNIL acts as a miRNA-sponge by directly interacting with miR-125a.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) includes various of malignancies occurring in the oral cavity, nasal cavity, pharynx, salivary glands, etc. [1,2]. Among these cancer types, oral cancer (OC) is used as an umbrella term for tumors in the lip, tongue, pyriform sinus, oropharynx, and other sites of the oral cavity [3,4]. OC is considered the fifth most common tumor worldwide, with an estimated 34,780 new cancer cases of OC in men and 13,550 new cancer cases of OC in women in the United States in 2016 [5]. More than 90% of OC is oral squamous cell carcinoma (OSCC), and the most important etiological factors related to the development of OSCC are the consumption of tobacco and alcohol [6,7]. The current treatments for OC primarily include surgery, radiotherapy, and chemotherapy, and these measures have certain curative effects for early stage OC patients with mild side effects [3,4,8]. However, the treatment effects for advanced or metastatic OC patients are limited, with serious systemic side effects [9,10]. Therefore, it is essential to explore the underlying molecular mechanisms of OC tumor initiation and progression, and to identify new effective specific targets for OC patients.

Long non-coding RNAs (lncRNAs), characterized as being more than 200 nucleotides in length, is a critical subgroup of non-coding RNAs (ncRNAs) without protein-coding ability [11–13]. These molecules have been well characterized, and their roles in the formation of various tumors have been established [11,14,15]. The main mechanisms involving lncRNAs in the process of human tumors include cell proliferation, apoptosis, invasion, metastasis, etc. [16,17]. ANRIL (antisense noncoding RNA in the INK4 locus), transcribed by RNA polymerase II, is a lncRNA that has been implicated in the pathogenesis of various tumors, including lung cancer, hepatocellular cancer, and gastric cancer [18–20]. However, whether ANRIL plays a role in the formation of oral cancer remains undetermined.

Recent studies have shown that miRNA regulation plays an important role in health and disease [21]. The abnormal expression of miRNAs may lead to the occurrence and development of tumors [22]. Some studies have indicated that the incidence, development, treatment, prognosis and recurrence of OC are related to abnormal miRNAs [23,24]. Hence, researchers have proposed that miRNAs could be used to determine the pathogenesis, explore the basis for molecular diagnostics, evaluate the prognosis and provide new treatment options for OC [24]. MiR-125a is highly conserved among species, and its expression is different in several human tumors [25,26]. The interaction of lncRNA with miRNA plays an important role in the initiation and development of tumors [27]. Based on this fact, the hypothesis of competing endogenous RNA (ceRNA) was raised and subsequently confirmed by many studies [28,29]. The ceRNA network has been

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https://doi.org/10.1016/j.biopha.2018.01.105
Received 1 October 2017; Received in revised form 15 January 2018; Accepted 24 January 2018

constructed in gastric cancer, hepatocellular cancer, breast cancer and pancreatic cancer [30–32]. However, similar studies are rare in OC.

In the present study, we investigated the expression and function of ANRIL in OC. We also analyzed the effects of ANRIL alternation on the proliferation, cycle, migration and invasion of OC cells by miR-125a. Finally, we constructed an ANRIL/miR-125a ceRNA network in OC.

2. Materials and methods

2.1. Cell lines and tissues

Human oral epithelial cells (HIOEC), OC cell lines (HB56, HB96, TSCC, Tca8113, SCC-9 and CAL27) and HEK-293T cells were all obtained from the American Type Culture Collection (ATCC). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cat#: 11960-044, Invitrogen, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) in a 5% CO_2 at 37 °C. All the procedures of cell functional assays are described in the supplementary materials.

Oral cancer tissues and corresponding normal tissues (n = 130) were obtained from patients diagnosed with OC at the People's Hospital of Yinzhou. Tissues were immersed in liquid nitrogen immediately after removal from patients and stored at −80 °C until further use. Serum samples (n = 114) were respectively extracted from the whole blood of OC patients or healthy controls after centrifugation (3000 g, 10 min) and stored at −80 °C until further processing. Informed consent was provided by all subjects and all experimental protocols were approved by the Ethics Committee of the People's Hospital of Yinzhou.

2.2. LncRNA ANRIL overexpression or knockdown experiments

For ANRIL overexpression, the cDNA of ANRIL was amplified by primers with 5′BamHI and 3′NotI restriction sites, and the PCR products were collected. After digestion with the two restriction endonucleases, BamHI and NotI, the reconstructed ANRIL plasmid was established by inserting the PCR products into the pcDNA 3.1 vector. Next, the reconstructed ANRIL plasmid was transfected into HB56 cells by using Lipofectamine 2000 (Invitrogen, CA) according the manufacturer's instructions. For the ANRIL knockdown, shRNAs for ANRIL or control (shANRIL or shControl) were designed by using BLOCK-iT RNAi Designer software (Invitrogen). The shANRIL was inserted into a lentiviral vector (pSIH-H1), and this reconstructed plasmid was co-transfected into HEK 293T cells with a packing vector by using FuGENE6 Transfection Reagent (Promega). To establish a stable ANRIL knockdown cell model, OC cell lines and CAL27 cells were infected with lentivirus produced by HEK-293T cells.

2.3. RNA extraction, reverse transcription, and quantitative real-time PCR (qRT-PCR)

Total RNA from OC or normal tissues and cell lines was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, RNA samples were reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocols. Quantitative real-time PCR was performed using the FastStart Universal SYBR-Green Master Mix (Roche, Indianapolis, IN, USA). The primer sequences used in the present study are shown in Table 1.

Table 1
The primer sequences in qRT-PCR assay.

Genes	Primers sequences
ANRIL	Forward: 5'-CTTATTTTATTCCTGGCTCCCCT-3'; Reverse: 5'-ATCATTCTCCTCAAATTACAGAG-3'
GAPDH	Forward: 5'-ATG TCG TGG AGT CTA CTG GC-3'; Reverse: 5'-TGA CCT TGC CCA CAG CCT TG -3'
MiR-125a	Forward: 5'-GCGCTCCCTGAGACCCTTTA-3' ; Reverse: 5'-CCAGTGCAGGGTCCGAGGTA-3'
U6	Forward: 5' CTCGCTTCGGCAGCACA-3'; Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

2.4. Cell migration and invasion assay

For cell migration assays, a transwell chamber (8-μm pore size; BD Biosciences, San Jose, CA, USA) without Matrigel coating was used to assess the migration of the treated OC cell lines. Briefly, ANRIL-overexpressed HB56 cells or ANRIL-knockdown CAL27 cells were seeded onto the upper compartment at a concentration of 1×10^5 cells/ml. After incubation for 24 h at 37 °C, the non-migrated cells remaining in the upper chamber were removed, and the cells that migrated to the bottom were fixed with 4% paraformaldehyde for 30 min and then stained with 0.2% crystal violet (Sigma-Aldrich, USA) for at least 10 min. For cell invasion assays, a transwell chamber with Matrigel coating was used to evaluate the invasion of OC cell lines. Briefly, 200 μl of treated OC cells (HB56 or CAL27) were added to the upper chamber at a density of 1×10^5 cells/ml, and 600 μl of complete medium was added to the lower chamber. After incubation at 37 °C for 48 h, the cells on the lower side of the filter were washed three times with PBS, fixed with 4% paraformaldehyde for 30 min, and subsequently stained with crystal violet for 10 min. Images were captured, and the cell number was counted.

2.5. Colony formation assay

ANRIL-treated HB56 and CAL27 cells were seeded onto 6-well plates at a concentration of 1×10^3 cells/well. After incubation for 12 days under an atmosphere of 5% CO_2 at 37 °C, the treated OC cell lines were fixed in 70% ethanol and then stained with 0.2% crystal violet solution for at least 20 min at room temperature. The images were captured, and the visible colonies were counted.

2.6. Cell viability assay (MTT assay)

After transfection with ANRIL or shANRIL plasmid, HB56 or CAL27 cells were seeded onto a 96-well plate at a density of 2×10^3 cells/well and maintained at 37 °C for 24 h. Every day for 5 days, MTT solution (50 μl) was added to each well, and the cells were subsequently incubated at 37 °C for 4 h. Next, the medium was removed and DMSO (200 μl) was added to each well. The absorbance of each well was measured daily by using a microplate reader at 490 nm.

2.7. Cell cycle analysis

Flow cytometry analysis and propidium iodide (PI) staining were performed to explore the cell cycle distribution of treated OC cell lines. Briefly, treated HB56 and CAL27 cells were seeded onto 6-well plates at a concentration of 2×10^5 cell/well and cultured overnight at 37 °C. Next, the cells were trypsinized and washed three times with cold PBS, and then fixed in 80% ethanol. After washing with PBS, the cells were stained with PI working solution for 30 min in darkness at 4 °C. The PI stained cells were subsequently analyzed by using the Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA). Cell cycle distribution was analyzed through MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA). The percentages of HB56 and CAL27 cells in G0/G1, G2M and S phases are shown as histograms.

2.8. Nude mouse xenograft assay

Female nude mice (6 weeks old) with BALB/c background were used in the tumor formation assay. The nude mice were purchased from the National Cancer Institute, Frederick, MD, and maintained according