Long Noncoding RNA, *ANRIL*, Regulates the Proliferation of Head and Neck Squamous Cell Carcinoma

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Abstract. *Background/Aim:* *ANRIL* is a long noncoding RNA located on *INK4* locus, which encodes *p15* and *p16* that cause *G*₁ phase arrest in the cell cycle. *ANRIL* positively regulates proliferation of several kinds of cancer cells such as lung and gastric cancers. This study, examined the effect of *ANRIL* in head and neck squamous cell carcinoma cells. *Materials and Methods:* Cells were transfected with siRNA oligonucleotides targeting *ANRIL*. Transfected cells were subjected to cell-cycle and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. *Results:* Depletion of *ANRIL* increased *p15* mRNA in FaDu cells, and *p15* and *p16* mRNA in CAL27 cells and inhibited proliferation of these cells. Cell cycle analysis showed that depletion of *ANRIL* caused arrest at the *G*₁ phase of the cell cycle. *Conclusion:* *ANRIL* promotes *G*₁ phase progression by repressing *p15* and *p16*, and thus promotes FaDu and CAL27 cell proliferation.

INK4 locus (inhibitor of cyclin dependent kinase 4 locus) is located in the human chromosome 9p21 region. This locus encodes *p15*, *p16*, and *ARF* genes, products of which function as tumor suppressors. Both *p15* and *p16* are CDK inhibitors that cause *G*₁ phase arrest in the cell cycle via inhibition of the CDK4 and CDK6 kinase activities (1). *ARF* functions to stabilize tumor suppressor p53 at the protein level by inhibiting the ubiquitin ligase activity of MDM2 (2, 3). This locus is frequently either mutated, deleted, or its transcription is repressed in many types of human cancers

(4). The transcriptional activation of this locus is therefore thought to be important in tumor suppression.

Long noncoding RNAs (lncRNAs) are more than 200 nucleotides long and lack a functional open reading frame. Recent studies have revealed that many lncRNAs participate in the regulation of differentiation, growth, senescence, and apoptosis (5). An antisense noncoding RNA transcribed from the *INK4* locus (*ANRIL*) is the lncRNA (6). Along with Yap *et al.*, we revealed that *ANRIL* associates with polycomb repression complex 1 (PRC1) and 2 (PRC2) and recruits them on the *INK4* locus to repress the transcription of *p15* and *p16* genes (7, 8). In the gene silencing model of polycomb protein complexes, PRC2 methylates histone H3 at lysine 27 on the target region, leading to the recruitment of PRC1, which ubiquitinates histone H2A at lysine 119 (9-11). These histone modifications establish and maintain the epigenetic repression of target genes. Silencing *ANRIL* results in the induction of *p15* and *p16* transcription, thus inhibiting cell proliferation (7, 8). Other than *p15* and *p16*, *ANRIL* is involved in the transcriptional regulation of many genes related to cell adhesion, gene expression and apoptosis (12-14), indicating that *ANRIL* participates in the regulation of diverse cellular functions.

Expression analysis showed that *ANRIL* is expressed at higher levels in several human cancer cells, including human cervical cancer, osteosarcoma and non-small cell lung cancer than normal lung fibroblasts (15). *ANRIL* regulates cancer cell proliferation in an *INK4* locus-dependent (15) and – independent manner (16). Many studies on clinical cancer specimens have shown that the expression of *ANRIL* is up-regulated in various human cancers such as lung, gastric, ovarian and cervical cancers (17). It has also been reported very recently that *ANRIL* expression is up-regulated in oral cancer and that *ANRIL* participates in the proliferation, migration and invasion of oral squamous cell carcinoma cells (18). However, the role of *ANRIL* in head and neck squamous cell carcinoma is largely unknown. In this study, we examined the function and mechanism of action of *ANRIL* in head and neck squamous cell carcinoma cells.

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Materials and Methods

Cell culture and RNA interference. A human hypopharyngeal cancer cell line, FaDu and a human oral cancer cell line, CAL27 (American Type Culture Collection, Frederick, MD, USA), were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in an atmosphere containing 5% CO₂. For *ANRIL* silencing, siRNA oligonucleotides targeting *ANRIL* were transfected by using Lipofectamine RNAiMAX according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The nucleotide sequence of the *ANRIL* siRNA oligonucleotide was 5'-GGUCAUCUCAUUGCUCUAU-3' with 3' dTdT overhangs.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). Seventy-two h after transfection of siRNA oligonucleotides, cells were subjected to qRT-PCR. qRT-PCR was performed as previously described (19). Briefly, the isolation of total RNA was performed using RNeasy Plus kit (Qiagen, Hilden, Germany). cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and amplified using QuantiTect SYBR Green PCR kit (Qiagen). The specific primer set for *ANRIL* was 5'-CTATCCGCCAATCAGGAGGC-3' and 5'-GCGGCAGCGGTTTAGTTTAAT-3'. The specific primer sets for *p15*, *p16*, *ARF* and *GAPDH* have been previously described (15).

Cell cycle analysis. Cell cycle analysis was performed as previously described (20). Briefly, cells were fixed for at least 3 h at -20°C using 70% ethanol about 72 h after transfecting siRNA oligonucleotides. The fixed cells were processed with Muse™ Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) according to the instructions of the manufacturer and subjected to cell cycle analysis using Muse Cell Analyzer (Merck Millipore).

Statistical analysis. Data are presented as means and standard deviations from triplicate samples. A two-tailed *t*-test was performed to determine significant differences. A *p*-values of <0.05 was considered to be statistically significant.

Results

We had previously reported that *ANRIL* is involved in the repression of cellular senescence in normal lung fibroblasts (8). Recently, several other research groups, including ours, reported that *ANRIL* functions to promote the proliferation of cancer cells, such as colorectal cancer, lung cancer, cervical cancer, gastric cancer, hepatocellular carcinoma and bladder cancer cells (17). Accordingly, we first examined the involvement of *ANRIL* in head and neck squamous cell carcinoma cell proliferation. The expression of *ANRIL* was downregulated using siRNA oligonucleotides that target *ANRIL* in a human hypopharyngeal cancer cell line, FaDu, and a human oral cancer cell line, CAL27. Depletion of *ANRIL* significantly repressed the proliferation of both FaDu (Figure 1A and B) and CAL27 (Figure 1C and D) cells, suggesting that *ANRIL* functions to promote the proliferation of both FaDu and CAL27 cells.

We (8), along with Yap *et al.* (7) have previously shown that *ANRIL* inhibits *p15* and *p16* expression, thus resulting in the promotion of cell proliferation. Accordingly, we examined the involvement of *ANRIL* in *p15* and *p16* expression in FaDu and CAL27 cells. Data obtained from qRT-PCR demonstrated that transfection with siRNA oligonucleotides significantly reduced *ANRIL* expression (Figure 2A and B) in both FaDu and CAL27 cells. Depletion of *ANRIL* resulted in an increase in *p15* mRNA expression in FaDu cells (*p16* was not detected) (Figure 2A). Interestingly, silencing *ANRIL* also increased the expression levels of *ARF* mRNA that is transcribed from the *INK4* locus and functions to stabilize the p53 tumor suppressor protein. Depletion of *ANRIL* resulted in an increase in *p15*, *p16* and *ARF* mRNA expression levels in CAL27 cells (Figure 2B). These results suggest that *ANRIL* is also involved in the repression of *p15* and *ARF* transcription in FaDu cells, and *p15*, *p16* and *ARF* in CAL27 cells.

Given that depletion of *ANRIL* increased the mRNA levels of *p15* and *p16*, both of which cause arrest at the G₁ phase of the cell cycle, we next examined whether depletion of *ANRIL* affects the cell cycle in FaDu and CAL27 cells. Cell cycle analysis showed that depletion of *ANRIL* significantly reduced the number of cells in S and G₂/M phase and increased the number of cells in the G₁ phase in both FaDu (Figure 3A and B) and CAL27 (Figure 3C and D) cells. This result suggests that *ANRIL* is involved in the regulation of G₁ phase progression in FaDu and CAL27 cells.

Discussion

Recent studies have shown that *ANRIL* induces proliferation of several cancer cells by regulating gene expression (15-17). In this study, we also showed that silencing *ANRIL* inhibits the proliferation of head and neck squamous cell carcinoma cells such as the human hypopharyngeal cancer cell line, FaDu and the human oral cancer cell line, CAL27, indicating that *ANRIL* functions as a positive regulator of proliferation of these cells. Furthermore, silencing *ANRIL* increased the expression of CDK inhibitors *p15* and *p16* on the *INK4* locus, which lead to G₁ phase arrest in the cell cycle in FaDu and CAL27 cells, suggesting that *ANRIL* functions to promote G₁-S transition via repression of *p15* and *p16*. *ANRIL* is known to repress *p15* and *p16* transcription through the recruitment of polycomb complexes PRC1 and PRC2 on *INK4* locus (7, 8). *ANRIL* also participates in the regulation of cell adhesion, apoptosis and metabolic activity via regulation of the transcription of many genes, in a *trans*-acting manner (14). The roles of *ANRIL* in processes other than cell cycle regulation and in target genes other than those of the *INK4* locus are yet to be determined in head and neck squamous cell carcinoma and are important topics that warrant further study.

Many studies have shown that *ANRIL* expression is up-regulated in various human cancers such as lung, gastric,

Figure 1. Depletion of *ANRIL* inhibits proliferation of FaDu and CAL27 cells. A: FaDu cells were seeded at 5×10^5 cells/100mm dish. One day later, the cells were transfected with either control siRNA (Ctrl-i) or siRNA silencing *ANRIL* (ANRIL-i) and 72 h later, cells were photographed. B: The number of viable cells was measured by trypan blue staining. $**p < 0.01$. C: CAL27 cells were transfected with siRNA and observed as in (A). D: Viable CAL27 cells were counted as in (B). $***p < 0.001$.

ovarian and cervical cancers (17). It has also been reported very recently that *ANRIL* is highly expressed in head and neck squamous cell carcinoma and correlates with tumor progression (21). Accordingly, we propose that *ANRIL* up-regulation causes aberrant progression of the cell cycle *via* repressing *p15* and *p16* transcription, resulting in head and neck squamous cell carcinoma progression.

Conflicts of Interest

The Authors have no conflicts of interest directly relevant to the contents of this article.

Authors' Contributions

Conceptualization and design, N.M., T.W., R.Y., and Y.K.; Supervision, Y.K.; Materials, N.M., T.W., R.Y., and Y.K.; Data collection, N.M., and Y.K.; Analysis, N.M., and Y.K.; Writer, Y.K.

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Figure 2. Depletion of ANRIL increases the expression of INK4 locus genes. A: FaDu cells were seeded at 5×10^5 cells/100mm dish and cultured for one day, transfected with either control siRNA (Ctr-i) or siRNA silencing ANRIL (ANRIL-i) and 72 hours later, were harvested to determine the mRNA levels of ANRIL, p15 and ARF by qRT-PCR. The results were expressed relative to the corresponding values for FaDu cells/Ctr-i. ** $p < 0.01$. B: qRT-PCR using CAL27 cells transfected with siRNA was performed and the data was analyzed as in (A). * $p < 0.05$, ** $p < 0.01$.

Figure 3. Depletion of ANRIL causes arrest in the G₁ phase of the cell cycle in both FaDu and CAL27 cells. A: At 72 h after transfection with either control siRNA (Ctr-i) or siRNA silencing ANRIL (ANRIL-i), FaDu cells were subjected to cell-cycle analysis by flow cytometry. The x-axis of histogram indicates propidium iodide staining intensity which is reflective of DNA content. The y-axis indicates cell count. B: The graph indicates percentages of FaDu cells in G₁, S and G₂/M phases. *** $p < 0.001$. C: CAL27 cells transfected with siRNA were subjected to cell-cycle analysis as in (A). D: The graph indicates percentages of CAL27 cells in G₁, S and G₂/M phases. ** $p < 0.01$, *** $p < 0.001$.

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