Long Noncoding RNA *ANRIL* Regulates Proliferation of Non-small Cell Lung Cancer and Cervical Cancer Cells

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Abstract. *Background: Long noncoding RNA ANRIL (antisense non-coding RNA in the INK4 locus) represses p15 and p16, which induce cell-cycle arrest at G_1 phase, leading to enhanced cell proliferation of normal fibroblasts. Herein we report that ANRIL is also involved in the regulation of cancer-cell proliferation. Materials and Methods: HeLa and H1299 cells were transfected with ANRIL siRNAs. At 72 h post-transfection, cells were subjected to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and cell-cycle analysis. Results: qRT-PCR showed that ANRIL is highly expressed in these cancer cells compared to normal fibroblasts. Depletion of ANRIL increased p15 expression, with no impact on p16 or ARF (alternative reading frame) expression, and caused cell-cycle arrest at the G_2/M phase, leading to inhibition of proliferation of H1299 and HeLa cells. Conclusion: ANRIL positively regulates the proliferation of cancer cells, such as H1299 and HeLa cells, via regulating p15 and other genes related to G_2/M phase control.*

Antisense non-coding RNA in the *INK4* (inhibitors of cyclin dependent kinase 4) locus (*ANRIL*) is a long noncoding RNA (lncRNA) located in the human chromosome 9p21 region. The *INK4* locus encodes three tumor-suppressor genes: CDK (cyclin dependent kinase) inhibitors (CKIs) *p15* and *p16*, and *ARF* (alternative reading frame), which is a positive regulator of p53. This region is frequently mutated or its expression silenced in human cancer (1-4). CKIs bind to and inhibit the activity of specific cyclin–CDK complexes, preventing G_1 to S transition of the cell-cycle (5, 6). Among these CKIs, p15 and p16 are induced by a variety of oncogenes, such as activating *RAS* (rat sarcoma) mutants, causing stable cell-cycle arrest through inhibiting cyclin D-dependent CDK4/6 activity (7-9). *ARF* is also induced by various oncogenes, such as *c-MYC* (cellular myelocytomatosis oncogene), and antagonizes the activity of MDM2 (transformed mouse 3T3 cell double minute 2) ubiquitin ligase, thereby stabilizing p53 tumour suppressor protein and causing cell-cycle arrest (10-13). The activation of the *INK4* locus is therefore important to protect cells from hyper-proliferative stimulation induced by oncogenic insults.

The *ANRIL* promoter is located between *p15* and *ARF*, and *ANRIL* is transcribed in the antisense direction with these genes (14). Yap *et al*. (15) and our group (16) reported that *ANRIL* is involved in the transcriptional repression of the *INK4* locus (15, 16). Inhibition of *ANRIL* in human normal fibroblasts increases the expression of p15 and p16, leading to a decrease in cell proliferation. Yap *et al*. showed that *ANRIL* associates with CBX7 (chromobox 7), a component of polycomb repression complex (PRC)-1 (15). We also showed that *ANRIL* associates with SUZ12 (suppressor of zeste 12 homolog), a component of PRC-2 (16). Inhibition of *ANRIL* disrupts the binding of PRC-1 and -2 on the *INK4* locus, indicating that *ANRIL* recruits PRC-1 and -2 on the INK4 locus, leading to the repression of *p15* and *p16* transcription (15, 16). *ANRIL* is also involved in the occupancy of PRC-1 and -2 on other genes located on different chromosomes, indicating that it functions on different chromosomes to regulate target genes in trans (17). The *trans*-regulation by *ANRIL* is dependent on its Alu motif (17). Several studies showed that depletion or overexpression of *ANRIL* causes changes in the expression levels of many genes involved in cell proliferation, cell adhesion, gene expression, and apoptosis, suggesting that *ANRIL* is involved in multiple cellular functions (17-19). In the present study, we investigated the role of *ANRIL* in the proliferation of cancer cells, namely human non-small cell lung cancer H1299 cells and HeLa cervical cancer cells.

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Materials and Methods

Cell culture. All cell lines used in this study were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (GIBCO, Grand Island, NY, USA) and incubated at 37˚C in an atmosphere containing 5% CO_2.

RNA interference. siRNA oligonucleotides against ANRIL (SIGMA-ALDRICH, Tokyo, Japan) were transfected into HeLa and H1299 cells using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. HeLa and H1299 cells were incubated 72 h after transfection before analyses. The nucleotide sequence of *ANRIL* siRNA was 5'-GGUCAUCUCAUUGCUCUAU-3' with 3' dTdT overlaps.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR). Total RNA was isolated by the RNeasy Plus kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The isolated total RNA was reversed transcribed into cDNA using SuperScript Reverse Transcriptase II (Invitrogen). For qualitative PCR, the produced cDNA was amplified by the specific primer sets: *ANRIL*, 5'-TGCTCTATCCGCC AATCAGG-3' and 5'-GGGCCTC AGTGGCACATACC-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GCAAATTCCATGGCACCGT-3' and 5'-TCGCCC CACTTGATT TTGG-3'; ribosomal protein L32 (*RPL32*), 5'-GGCGGAAACCCAGAGGCATTGA-3' and 5'-CCTGGCATTGGG GTTGGTGACTCT-3'. For qRT-PCR, the produced cDNA was added to SYBR Green PCR master mix (Qiagen) and amplify by the specific primer sets: *p16*, 5'-CGGTCGGAGGCCGATCCAG-3' and 5'-GCGC CGTGGAGCAGCAGCAGCT-3'; *p15*, 5'-AAGCTGAGCCCAGGT CTCCTA-3' and 5'-CCACCGTTGGCCGTAAACT-3'; *ARF*, 5'-CCCTCGTGCTGATGCTACTG-3' and 5'-ACCTGGTCTTCTAG GAAGCGG-3'; *GAPDH*, 5'-GCAAATTCCATGGCAC CGT-3' and 5'-TCGCCCCACTTGATTTTGG-3'. Assays were performed in triplicate on a Mx3000P Real-Time Q-PCR System (Agilent Technologies, Santa Clara, CA, USA).

Cell-cycle analysis. HeLa cells were fixed overnight in 70% ethanol at −20˚C. The DNA of fixed cells was stained by Muse™ Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Cells were analyzed by Muse™ Cell Analyzer and analysis software (Merck Millipore).

Results

We first examined the expression of *ANRIL* in various human cell lines. RT-PCR assay showed that *ANRIL* was highly expressed in several cancer cell types, including ABC-1, H1299 (human non-small cell lung cancer), HeLa (cervical cancer) and Saos-2 cells (osteosarcoma) compared with WI38 and TIG-3 cells (normal diploid foetal lung fibroblasts) (Figure 1).

We previously reported that *ANRIL* positively regulates the proliferation of human normal diploid foetal lung fibroblasts (16). Therefore, we next determined whether *ANRIL* is also involved in the regulation of cancer-cell proliferation. We knocked-down *ANRIL* using specific siRNA oligonucleotides

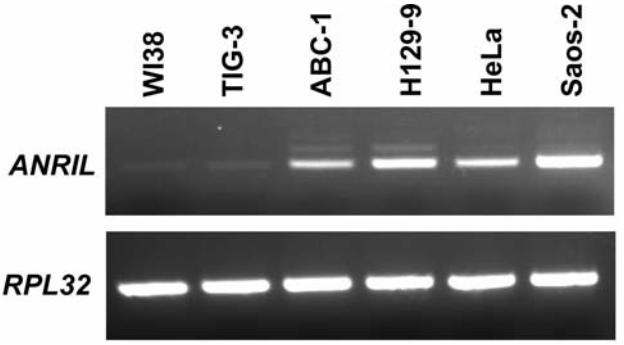


Figure 1. *Expression levels of ANRIL (antisense non-coding RNA in the INK4 locus) in various human cells. The levels of ANRIL expression were determined by RT-PCR. RPL32 (ribosomal protein L32) was used as an internal control.*

in cancer cells with high expression of *ANRIL*, namely HeLa and H1299 cells. RT-PCR assay confirmed that these siRNAs reduced ANRIL expression to nearly undetectable levels in HeLa cells (Figure 2A). The depletion of *ANRIL* resulted in repression of HeLa cell proliferation (Figure 2B and C). In H1299 cells with knocked-down *ANRIL*, we also observed a substantial decrease of H1299 cell proliferation (Figure 2D-F). These results suggest that *ANRIL* positively regulates the proliferation of HeLa and H1299 cells.

Yap *et al*. (15) and our group (16) previously reported that *ANRIL* is involved in the repression of *p15* and *p16* transcription. We, therefore, examined the effect of ANRIL silencing on the expression of genes of the *INK4* locus including *p15, p16*, and *ARF* in HeLa and H1299 cells. qRT-PCR showed that silencing *ANRIL* in HeLa cells resulted in an about two-fold increase in *p15* mRNA, with no changes in *p16* or *ARF* mRNA levels (Figure 3A). Silencing *ANRIL* in H1299 cells also resulted in a more than fourfold increase in p15 mRNA, but no changes were observed for *ARF* mRNA (*p16* was not detected) (Figure 3B). These results suggest that *ANRIL* is involved in *p15* repression in cancer cells, such as HeLa and H1299 cells.

Given that silencing *ANRIL* increases the mRNA level of *p15*, which causes G_1 phase arrest, we next examined the effect of silencing *ANRIL* on the cell cycle using Muse™ Cell Analyzer. Contrary to our expectation, silencing of *ANRIL* in HeLa cells induced a pronounced G_2/M phase accumulation compared with control cells (Figure 4A and B), suggesting that *ANRIL* regulates the G_2/M phase of the cell cycle in HeLa cells.

Discussion

Recent studies revealed that several lncRNAs have pivotal roles in critical cellular processes, including proliferation, differentiation, apoptosis, and senescence (20-23). *ANRIL* is involved in the regulation of cellular senescence of normal

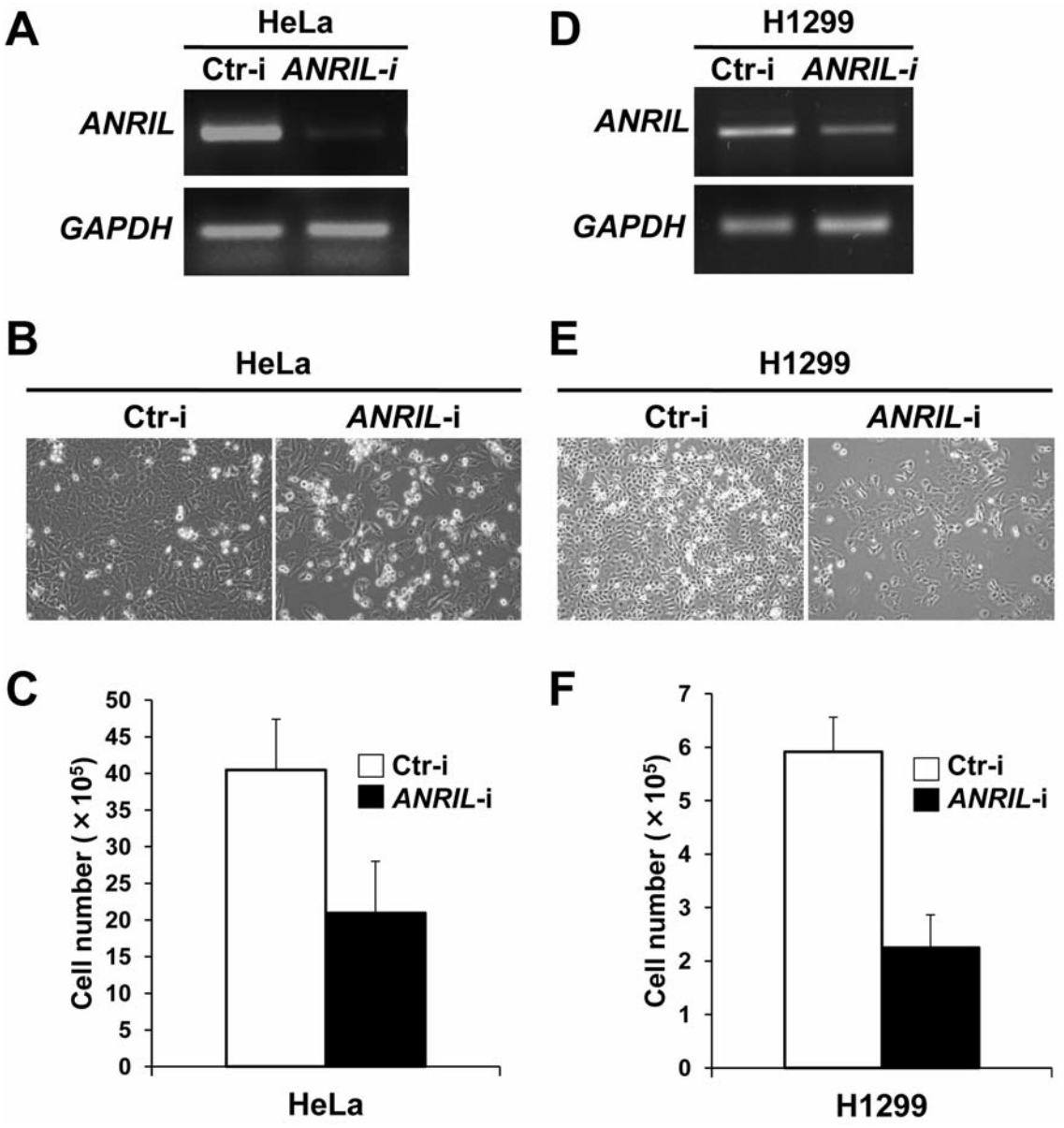


Figure 2. *Silencing of ANRIL (antisense non-coding RNA in the INK4 locus) represses the proliferation of HeLa and H1299 cells. A: HeLa cells were transfected with control (Ctr-i) or ANRIL siRNA oligonucleotides. At 72 h after transfection, cells were harvested and subjected to RT-PCR (reverse transcription-polymerase chain reaction) to determine the level of ANRIL. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. B: HeLa cells transfected with control or ANRIL siRNA oligonucleotides were observed by phase-contrast microscopy at 72 h after transfection. C: After initial seeding of 5×10^5 cells, HeLa cells were incubated overnight, and then transfected with control or ANRIL siRNA oligonucleotides. At 72 h after transfection, viable HeLa cells were counted by Trypan Blue staining. D: H1299 cells were transfected with control (Ctr) or ANRIL siRNA oligonucleotides. At 72 h after transfection, cells were harvested and subjected to RT-PCR as in (A). E: H1299 cells transfected with control or ANRIL siRNA oligonucleotides were observed as in (B). F: After initial seeding of 1×10^5 cells, H1299 cells were incubated overnight, and then transfected with control or ANRIL siRNA oligonucleotides. Viable H1299 cells were counted as in (C).*

human diploid foetal lung fibroblasts through the regulation of *p15* and *p16* transcription (15, 16). In this study, we showed that *ANRIL* is also involved in the regulation of proliferation of cancer cells, such as the human H1299 non-small cell lung cancer cells and HeLa cervical cancer cells. Silencing *ANRIL* increased *p15* expression, but not *p16* and *ARF*, suggesting that *ANRIL* represses mainly p15 transcription in these cancer cells. p15 inhibits the activity of cyclin D–CDK4/6, causing cell-cycle arrest at the G_1 phase. Interestingly, silencing *ANRIL* caused cell-cycle arrest

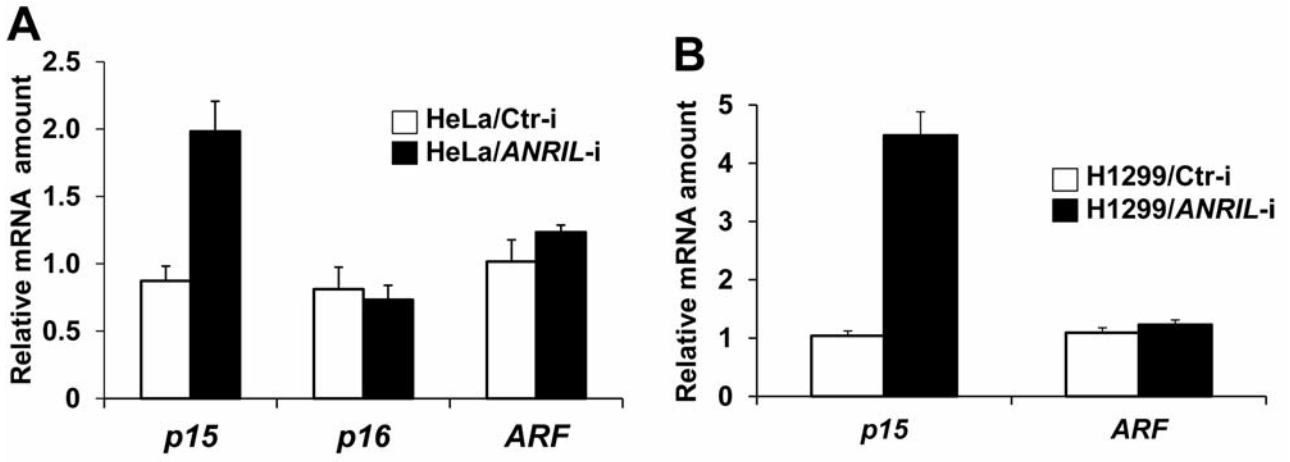
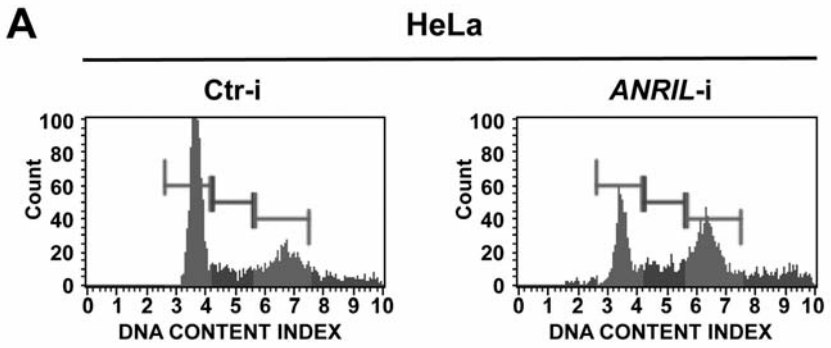


Figure 3. *Silencing of ANRIL (antisense non-coding RNA in the INK4 locus) increases p15 mRNA expression. A: HeLa cells were transfected with control (Ctr-i) or ANRIL siRNA oligonucleotides. At 72 h after transfection, cells were harvested. The effects of ANRIL silencing on the expression of p15, p16 and ARF (alternative reading frame) were determined by qRT-PCR. The results are expressed relative to the corresponding values for control cells. The mean values and standard deviations were calculated from the data of three independent experiments. B: The effects of ANRIL silencing on the expression of p15 and ARF in H1299 cells were determined by qRT-PCR as in (A).*



B

	G_0/G_1	S	G_2/M
Ctr-i	48.6 %	12.1 %	27.5 %
ANRIL-i	28.2 %	15.9 %	38.1 %

Figure 4. *Silencing of ANRIL (antisense non-coding RNA in the INK4 locus) causes cell-cycle arrest at the G_2/M phase. A: HeLa cells transfected with control (Ctr-i) or ANRIL siRNA oligonucleotides were analyzed by Muse™ Cell Analyzer at 72 h after transfection. B: The percentage of total cells present in the G_0/G_1, S and G_2/M phases of the cell cycle are shown.*

at G_2/M phase in these cancer cells, not G_1 phase. Recent reports demonstrated that *ANRIL* recruits PRC-1 and -2 to target genes located on different chromosomes to regulate gene expression (17). In support of this notion, silencing *ANRIL* impacts the expression of a large number of genes (18, 19). Thus, *ANRIL* may be involved in the regulation of genes that control G_2/M phase in a trans-acting manner.

We also showed that *ANRIL* is highly expressed in different types of human cancer cell, such as non-small cell lung cancer (ABC-1 and H1299), cervical cancer (HeLa) and osteosarcoma (Saos-2). High levels of *ANRIL* have been previously observed in human cancer, including prostate (16) and gastric (24). The molecular mechanism underlying aberrant expression of *ANRIL* in cancer remains to be

elucidated. It was reported that transcription factor E2F1 induced by ATM (ataxia telangiectasia mutated) binds to the *ANRIL* promoter and activates its transcription in response to DNA damage (18, 25). Increased expression of E2F1 has been observed in many types of human cancer (26), suggesting that deregulation of E2F1 might lead to aberrant expression of *ANRIL* in cancer.

p15 and *p16* are activated by oncogenes, including an oncogenic form of small GTPase RAS (called oncogenic RAS), causing stable cell-cycle arrest in order to protect cells from hyper-proliferation (7-9). Recently, we reported that oncogenic RAS reduces the expression of *ANRIL* (15, 27). The decrease of *ANRIL* by oncogenic RAS might be required for p15- and p16-dependent cell-cycle arrest. We, therefore, postulate that aberrant expression of *ANRIL* might disrupt protection by p15 and p16, leading to oncogenic transformation.

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