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Long Non-coding RNA *ANRIL* and *Polycomb* in Human Cancers and Cardiovascular Disease

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Abstract

The long non-coding RNA *CDKN2B-AS1*, commonly referred to as the *Antisense Non-coding RNA in the INK4 Locus* (*ANRIL*), is a 3.8-kb-long RNA transcribed from the short arm of human chromosome 9 on p21.3 that overlaps a critical region encompassing three major tumor suppressor loci juxtaposed to the *INK4b-ARF-INK4a* gene cluster and the methyl-thioadenosine phosphorylase (*MTAP*) gene. Genome-wide association studies have identified this region with a remarkable and growing number of disease-associated DNA alterations and single nucleotide polymorphisms, which corresponds to increased susceptibility to human disease. Recent attention has been devoted on whether these alterations in the *ANRIL* sequence affect its expression levels and/or its splicing transcript variation, and in consequence, global cellular homeostasis. Moreover, recent evidence postulates that *ANRIL* not only can regulate their immediate genomic neighbors in *cis*, but also has the capacity to regulate additional loci in *trans*. This action would further increase the complexity for mechanisms imposed through *ANRIL* and furthering the scope of this lncRNA in disease pathogenesis. In this chapter, we summarize the most recent findings on the investigation of *ANRIL* and provide a perspective on the biological and clinical significance of *ANRIL* as a putative biomarker, specifically, its potential role in directing cellular fates leading to cancer and cardiovascular disease.

1 The DNA and RNA Landscape Overlapping Chr9p21 Loci

A growing number of genome-wide association studies (GWASs) have identified specific regions of the human genome with a strong non-random correlation to complex human traits with predisposition to disease (de los Campos et al. 2010). Indeed, several single nucleotide polymorphisms (SNPs) have been identified on the *INK4b-ARF-INK4a* locus located on the human chromosome 9p21 that are tightly related with the increase of cardiovascular disease (CVD) (de los Campos et al. 2010; Gschwendtner et al. 2009) ischemic stroke

(Gschwendtner et al. 2009; Matarin et al. 2008), aortic aneurysm (Helgadottir et al. 2008), type II diabetes (Zeggini et al. 2007; Scott et al. 2007), glioma (Shete et al. 2009; Wrensch et al. 2009), and cancer predisposition (Shete et al. 2009; Wrensch et al. 2009; Cunningham et al. 2010; Bishop et al. 2009), among other conditions.

The *INK4b-ARF-INK4a* locus encodes three critical tumor suppressor genes, p14^{ARF} (p19^{ARF} in mice), p15^{INK4b}, and p16^{INK4a}, all of which play a central role in cell-cycle arrest, thus affecting key cellular processes such as senescence, apoptosis, and stem cells self-renewal by triggering the activities of both retinoblastoma (Rb) and p53 pathways (Gil and Peters 2006; Popov and Gil 2010). Specifically, p15^{INK4b} and p16^{INK4a} target cyclin-dependent kinases CDK4 and CDK6, preventing the binding of these proteins to D-type cyclins and, as a consequence, inhibiting CDK4/6-mediated phosphorylation (inactivation) of retinoblastoma (RB1) family members. In contrast, the unrelated p14^{ARF} protein acts primarily by binding to the E3 ubiquitin-protein ligase MDM2, promoting its degradation, and therefore abrogating MDM2 inhibition of the TRP53 activity (Popov and Gil 2010). The locus contains a fourth gene, methylthioadenosine phosphorylase (MTAP), which has annotated exons overlapping the *INK4b-ARF-INK4a* locus (Nobori et al. 1996). MTAP catalyzes the phosphorylation of 5'-methyladenosine (MTA) in the polyamine pathway, and it has also been associated with cancerogenesis (Behrmann et al. 2003; Schmid et al. 1998).

The long non-coding RNA *ANRIL* (*Antisense Non-coding RNA in the INK4 Locus*) was first identified within the 403-kb germ-line deletion of a French family with a history of melanoma and neural system tumors (Pasmant et al. 2007). *ANRIL* is transcribed as a 3,834-bp lncRNA in the opposite direction from the *INK4b-ARF-INK4a* cluster (Yu et al. 2008), and it shares a bidirectional promoter with p14^{ARF}, as the 5' end of the first exon of *ANRIL* is located 300 bp upstream of the transcription start site (TSS) of the p14^{ARF} gene. Hence, the expression of both genes is coordinated, and reporter assays have shown a transcriptional activation of this divergent promoter by E2F1 and the insulator CTCF (Sato et al. 2010; Rodriguez et al. 2010). Specifically, CTCF binding is required to maintain the *INK/ARF* locus in an inducible conformation, which is abrogated upon DNA methylation, having consequences in cancer progression (Rodriguez et al. 2010).

ANRIL transcript contains 20 exons, many of them consisting of LINE, SINE, and *Alu* repetitive elements (Jarinova et al. 2009), that can be alternatively spliced. *ANRIL* transcripts are expressed at very low levels, and the two short forms, both terminating with polyadenylated exon 13, EU741058 (exons 1, 5, 6, 7, 13) and DQ485454 (exons 1–13), and the long form NR_003529 that lacks the exon 13 and terminates with polyadenylated exon 20 (exons 1–20), are the most abundant transcripts. Circular *ANRIL* (*cANRIL*) isoforms have also been described (Burd et al. 2010), which result from exon skipping events occurring during RNA splicing. Thus, *cANRIL* show non-sequential linkages between various *ANRIL* exons, appearing species like exons 4–6 and 14–5, to name some examples. A fusion transcript between the MTAP gene and the 3' end of *ANRIL* has also been identified in cell lines with 9p21 deletion but not in normal cell lines (Burd et al. 2010; Schmid et al. 2000). Many of the *ANRIL* isoforms can coexist in the same cell type although others are tissue-specific (Burd et al. 2010; Folkersen et al. 2009), increasing the complexity of its regulatory mechanism. These alternative splicing events might modify *ANRIL*

structure leading to changes not just in *Polycomb* group (PcG) proteins-mediated *INK4b-ARF-INK4a* locus regulation. In fact, the overexpression of exons 13–19 in HeLa cells resulted in the repression of a wide set of genes involved in chromatin architecture remodeling, being Centrosomal protein 290 kDa (CEP290), E1 A binding protein p300 (EP300), and transcription factor 7-like 1 (TCF7L1) the most repressed proteins (Sato et al. 2010). Interestingly, Ras responsive element binding protein 1 (RREB1) and Zinc finger and BTB domain containing 32 (ZBTB32) were upregulated upon *ANRIL* 13–19 overexpression (Sato et al. 2010).

2 ANRIL and Polycomb Group Proteins

The PcG proteins were originally identified in *Drosophila melanogaster*, as transcriptional repressors of homeotic (Hox) genes, required for the correct spatiotemporal expression of developmental regulators along the body axis (Lewis 1978). In most metazoan species, the PcG proteins form two macromolecular repressive complexes named polycomb repressive complex-1 (PRC1) and polycomb repressive complex-2 (PRC2) (Levine et al. 2002). The PRC2 complex consists of three subunits: embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and enhancer of zeste 2 or 1 (EZH2/1), which catalyze the mono-, di-, and trimethylation of lysine 27 of histone H3 (H3K27me1, H3K27me2, and H3K27me3) (Margueron et al. 2008; Shen et al. 2008). H3K27me3 is a signature for chromobox-domain (CBX) protein recognition and PRC1 recruitment. The PRC1 composition is heterogeneous, depending on the cellular context, and contains several PcG proteins, including one member of the PCGF family (PCGF1-PCGF6) and of the HPH family (HPH1-HPH3), together with chromobox-domain (CBX) protein and RING1a/1b, which catalyze the monoubiquitination of H2a on K119 (H2AK119ub1) for the maintenance of silent chromatin (Cao et al. 2005; Wang et al. 2004).

Several long non-coding RNAs have a direct role in recruiting PcG proteins to specific loci to modify the epigenetic chromatin state and thereby to repress gene expression. Some documented examples include *XIST* RNA (Mak et al. 2002; Zhao et al. 2008), *KCNQTLOT1* (Fitzpatrick et al. 2002; Pandey et al. 2008), *HOTAIR* (Rinn et al. 2007) and *ANRIL* (Kotake et al. 2011; Yap et al. 2010). Indeed, *ANRIL* specifically associates with the chromodomain of chromobox homolog 7 (CBX7), a subunit of the PRC1 complex, and participates in CBX7 recognition of H3K27me3 to silence the *INK4b-ARF-INK4a* cluster (Yap et al. 2010). This interaction is abolished after treatment of cell nuclei with the transcriptional inhibitor α amanitin, indicating that *ANRIL* is stably associated with CBX7 as a nascent transcript generated by the RNA polymerase II. Moreover, knockdown of *ANRIL* decreases H3K27me3 levels and it is associated with increased p16^{INK4a} expression, which coincides with a reduction in CBX7 and EZH2 binding at the p16^{INK4a} TSS (Yap et al. 2010). Overall, this mechanism is important for the *INK4b-ARF-INK4a* locus repression in order to control senescence [reviewed by (Aguilo et al. 2011)] (Fig. 1).

On the other hand, *ANRIL* can also interact with the PRC2 component SUZ12 and influence SUZ12 binding to the p15^{INK4b} locus. Thus, depletion of *ANRIL* increases the expression of p15^{INK4b}, but not p16^{INK4a} or p14^{ARF}, and inhibits cellular proliferation, thereby influencing human disease progression (Aguilo et al. 2011). Recently, RIP sequence (RIP-

seq) experiments performed in MonoMac cells in which two specific exon-combinations of *ANRIL* were overexpressed, showed a binding of *ANRIL* with CBX7 and RING1B from PRC1, a binding with the PRC2 subunits EED, JARID2, RBAP46, and SUZ12, and PRC-associated proteins RYBP and YY1 (Holdt et al. 2013).

3 *ANRIL* and Cardiovascular Disease

CVD covers a wide array of disorders, including diseases of the cardiac muscle and of the vascular system supplying the heart, brain, and other vital organs.

ANRIL locus has been highlighted as the strongest genetic susceptibility locus for CVD, being numerous polymorphisms located in this locus directly associated with increased risk of developing CVD (Cunnington et al. 2010; Folkersen et al. 2009; Holdt et al. 2010; Holdt and Teupser 2012; Liu et al. 2009). In particular, the coronary artery disease (CAD)-associated SNPs are located on chromosome 9p21.3, specifically in a linkage disequilibrium block that does not contain known protein-coding genes, spanning a region of 58-kb named the CAD interval (Guttman et al. 2009). For example, the SNP rs496892-G is linked to atherosclerotic stroke, whereas the rs10757276-G is the lead SNP for CVD risk. These polymorphisms affect the expression of *ANRIL* (Holdt et al. 2010; Congrains et al. 2012), which in turn regulates the expression of downstream genes involved in several atherogenic pathways and/or inflammation response. For example, decreased expression of *ANRIL* transcripts containing exon 13 correlates with decreased expression of adiponectin receptor 1 (ADIPOR1), vesicle-associated membrane protein 3 (VAMP3), and transmembrane protein 258 (C11ORF10) (Bochenek et al. 2013).

Another possibility is that the polymorphism in the CAD interval may affect *ANRIL* splicing and, as a consequence, *ANRIL* structure. Specifically, two SNPs (rs.7341786 and rs7341791) identified in the exon 15, from where most of the *cANRIL* transcripts arise, were shown to be in linkage disequilibrium with the ASVD-associated SNP rs1075728 and were predicted to increase the ability of exon 15 of acting as splice acceptor. Furthermore, individuals harboring the casual variants mentioned above exhibit a derepressed INK4b-ARF-INK4a expression (Burd et al. 2010), indicating that the alteration of *ANRIL* structure may affect the efficiency of *ANRIL* at repressing the INK4b-ARF-INK4a locus.

Additionally, many of the polymorphisms in the 9p21 locus can also disrupt predicted transcription factor binding sites (Harismendy et al. 2011). For instance, rs564398, one of the SNPs most strongly correlated with *ANRIL* expression, disrupts 'Ras Responsive Element Binding Protein 1' (RREB1) binding site, and the SNP (rs10757278) disrupts the binding of the STAT1 (Signal-transducer and activator of transcription) transcription factor, increasing CVD susceptibility (Harismendy et al. 2011).

The presence of multiple enhancers in this region suggests that the expression of the *INK4b-ARF-INK4a* locus is regulated in a temporal and tissue-specific manner. Thus, some enhancers in the CAD interval appear functional in certain cell types and have cell-type-specific effects. An example is given from the transcription factor STAT1. In physiological conditions, activation of the JAK-STAT pathway is triggered when type II interferons (IFN)

bind to their receptor, inducing Janus kinase (JAK) phosphorylation, which in turn phosphorylates STAT family of transcription factors. Upon tyrosine phosphorylation, STAT dimerizes and translocates to the nucleus where it modulates a number of target genes. Although the STATs are generally associated with transcriptional activation, examples of STAT-dependent transcriptional repression have also been reported (Aaronson and Horvath 2002). Thus, studies in lymphoblastoid cell lines (LCL) show that there is a correlation between the CAD risk variants and CDKN2A/B and *ANRIL* expression in lymphocytes (Helgadottir et al. 2008), and that the CAD risk alleles preclude binding of STAT1 at the enhancer ECAD9. STAT1 occupancy on this enhancer correlates with the repression of *ANRIL* expression. On the other hand, IFN γ treatment of endothelial cells (HUVEC) induces STAT1 binding to the same enhancer, which in turn results in increased *ANRIL* expression (Helgadottir et al. 2007).

The Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) cascade is another essential signaling transduction pathway involved in *INK4b-ARF-INK4a* locus regulation (Malumbres et al. 2000). In fact, it has been shown that the pro-oncogenic Ras protein inhibits *ANRIL* expression and activates p15^{INK4b}, suggesting a potential negative regulation of p15^{INK4b} by ANRIL (Kotake et al. 2011). Ras has an important role in atherosclerosis progression, promoting vascular senescence and inducing the expression of pro-inflammatory cytokines. In particular, a constitutive activation of Ras is involved in atherogenesis by inducing vascular smooth muscle cell (VSMC) senescence and expression of proinflammatory cytokines (Minamino et al. 2003). Furthermore, activation of ERK and vascular inflammation is associated with VSMC senescence in human atherosclerosis, which suggests that the Ras/Raf/MEK/ERK signaling cascade plays an important role in regulating VSMC lifespan and function in vivo (Minamino et al. 2003).

Previous mechanistic studies postulated that *ANRIL* serve as a scaffold for the chromatin modifying complexes PRC1 and PRC2, mediating the repression in *cis* of the *INK4b-ARF-INK4a* locus (Kotake et al. 2011; Yap et al. 2010). Nonetheless, a recent study revealed that *ANRIL* association with CVD susceptibility can be related to its capability of regulating gene expression in *trans* (Holdt et al. 2013), leading to decreased apoptosis and increased cell proliferation and cell adhesion, characteristic and essential alterations of atherogenesis (Lusis 2000). In particular, *ANRIL*-regulated genes contain an *Alu* repeat motif in their promoters, and the occupancy of CBX7 and SUZ12 is highly enriched ~150 bp downstream of this *Alu* motif. *Alu* repeats are a family of primate-specific short interspersed repeat elements (SINEs) with more than one million copies in the human genome (Lander et al. 2001; Dewannieux et al. 2003) and have been linked with genetic disease (Burns and Boeke 2012). Interestingly, the *Alu* motif is also present in the *ANRIL* transcript and it is predicted to locate in a central stem-loop-like structure (Holdt et al. 2013), pointing to RNA-chromatin interactions as an effector mechanism (Mercer et al. 2009).

4 ANRIL and Cancer Predisposition

Cancer is a group of more than 100 diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body.

The *INK4b-ARF-INK4a* gene cluster is homozygously deleted or silenced in approximately 40% of human cancers (Iacobucci et al. 2011), being *ANRIL* one of the most frequently altered lncRNAs in cancer development and progression, including ovarian cancers, breast cancer, lymphoblastic leukemia, nasopharyngeal carcinoma, basal cell carcinoma, and gliomas (Shete et al. 2009; Stacey et al. 2009; Turnbull et al. 2010; Pasmant et al. 2011). Moreover, several polymorphisms identified in the *ANRIL* locus show a significant correlation with tumor development (Shete et al. 2009; Wrensch et al. 2009; Bishop et al. 2009). To name a few examples, the SNP rs1063192-C is highly correlated with glioma, the SNP rs1011970-T with melanoma susceptibility (Cunnington et al. 2010), and the SNP Rs564398 increases the risk of lymphoblastic leukemia development (Iacobucci et al. 2011). These polymorphisms alter the expression pattern of *ANRIL* splice variants (Fig. 2), and in consequence, dysregulate the *INK4b-ARF-INK4a* locus expression. A hypothesis for *ANRIL* function is that this ncRNA is composed of several RNA transcript variants such that the accumulation of transcript variants may focalize PRC1 via CBX7 in proximity to the p16^{INK4a} gene promoter to selectively silence p16^{INK4a} (Aguilo et al. 2011). As sequence technology evolves to incorporate higher resolution, we predict that novel isoforms will emerge and specific diseased states will be represented by the presence (or absence) of the transcript variants for *ANRIL* (Fig. 2).

Although the underlying molecular mechanism by which *ANRIL* increases the risk of cancer progression remains ambiguous, it is believed that high expression levels may lead to cancer predisposition. Indeed, it has been reported that *ANRIL* is overexpressed in preneoplastic and neoplastic epithelial tissues (Yap et al. 2010), gastric cancer tissues (Zhang et al. 2014), esophageal squamous cell carcinoma (ESCC) (Chen et al. 2014), and leukemia leukocytes (Cunnington et al. 2010; Yu et al. 2008) compared with the non-tumor tissues. In normal cells, induction of *ANRIL* transcript levels by E2F1 is required for the suppression of p14^{ARF}, p15^{INK4b}, and p16^{INK4a} expression at the late stage of DNA damage response, in order to return to physiological cellular levels after the completion of the DNA repair. However, in cancerous cells, aberrant expression of *ANRIL* would cause a blockage of the control of the DNA damage response mechanism, leading to genomic instability, and therefore, tumor progression (Wan et al. 2013).

ANRIL also influences cell proliferation by regulating target genes in *trans*. Hence, in gastric cancer tissues, *ANRIL* cooperates with microRNAs in the epigenetic level by binding to EZH2. Specifically, *ANRIL* silences miR-99a/miR-449a, therefore up-regulating the miR-99a/miR-449a target genes mTOR and CDK6, and as a consequence, up-regulating the CDK6 target gene E2F1 (Zhang et al. 2014). This positive feedback loop could in part account for *ANRIL*-mediated cell growth regulation. On the other hand, in esophageal squamous cell carcinoma tissues, *ANRIL* influences cell growth by repression of the TGFβ/Smad signaling pathway (Chen et al. 2014), although the exact molecular mechanisms of interaction between *ANRIL* and TGFβ1 remain elusive.

Collectively, *ANRIL* could serve as a candidate biomarker for cancer detection, and novel cancer therapies should consider *ANRIL* depletion to specifically target highly proliferative cells. However, despite growing knowledge about *ANRIL* function in cancer and other disease models, a broader understanding of the molecular mechanism of action, and the

regulatory pathways, hierarchies and networks in which *ANRIL* and other lncRNA operate, is the essential first step for its therapeutic manipulation.

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Fig. 1.
Illustration of how the *ANRIL* transcript may facilitate polycomb repressive complex 1 to compact chromatin structure of the *INK4b-ARF-INK4a* locus

Fig. 2.

Comprehensive transcript map overlapping the human *INK/ARF* locus determines the assembly of transcripts by long read- and strand-specific RNA sequencing by ISO-Seq. Samples taken for ISO-Seq analysis are from a single prostate invasive carcinoma specimen and compared with the paired normal prostate duct epithelium. Highlighted in red are novel transcript isoforms identified in the tumor specimen when compared to the normal duct epithelium of the prostate