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Novel Role Expression Between Normal Cell and Long Non-coding RNA

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Abstract

Background and objective: ERICD (E2f1-regulated inhibitor of Cell Death) is a newly found lncRNA located on chromosome 8. ERICD is regulated by E2F1. ARID3A/DRIL1/Bright is a family member of the AT-rich interaction domain (ARID) DNA-binding proteins that are involved in diverse biological processes. ARID3A binds to the E2F transcription factor and activates E2F-dependent transcription. I have found putative binding sites of ARID3A on ERICD. I think that ARID3A and ERICD might be regulated by each other for several biological processes in cancer. The other hypothesis for selecting these two in our study is that both of them have just opposite roles in apoptosis in case of DNA damage indicating a probability of reciprocal interaction between each other. To explore if ARID3A regulates the expression of lncRNA ERICD.

Methods: We took advantage of a human osteosarcoma cell line (U2OS) that each expresses conditionally active ARID3A and ERICD. Overexpression and knockdown experiments were carried out for ARID3A and a knockdown experiment was done for ERICD. Migration assay and colony formation assays were also performed in U2OS. siRNA-mediated knockdown of ARID3A and ERICD inhibited cell migration and reduced the formation of colonies in U2OS cells to a considerable degree. On the other side overexpression of ARID3A confirmed a significant induction in wound closure and increased colony-forming ability of U2OS cells.

Results: The obtained findings in this studyshowed that ARID3A and ERICD interact with each other indirectly via E2F1. Moreover, ARID3A and lncRNA ERICD have oncogenic functions in osteosarcoma. The interaction that has been found between ARID3A and lncRNA ERICD is novel and adds a further milestone regarding lncRNAs targeting DNA-binding proteins.

Keywords: ARID3A, lncRNA ERICD, Knockdown, Overexpression.

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Introduction

Cancer is a genetic disease due to defects in genes that control vital biological processes, especially cell growth and division. Genetic changes that contribute to cancer affect three basic types of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes.[1]Canceris a class of diseases characterized by uncontrolled cell growth. It is an important public health problem all over the world.[2]According to the World Health Organization (WHO), the number of new cancer cases is expected to increase by approximately 70% in the next 20 years. The most common areas of cancer in men are the lung, prostate, colon, rectum, abdomen, and liver, while in women the cancers are breast, colon, rectum, lung, cervix, and stomach.[3]For example, in the USA, the lifetime probability of developing cancer is around 44% in men and 38% in women.[4]

Although "cancer" encompasses a heterogeneous group of diseases, one of the characteristic and unifying features is the creation of abnormal cells that grow beyond their natural boundaries. Since tumorigenesis is a multistage process, normal cells gradually transition to the neoplastic stage and, during this process, acquire special capacities that make them tumorigenic.[5]

The fourth mammalian ARID subfamily is named ARID4 and contains two members, ARID4A and ARID4B. These proteins show 74% similarity in their ARID region and each protein is 40–50% similar along its full length. ARID4A has been characterized as a repressor of E2F transcription recruited by pRb.[6,7]ARID4A is widely expressed. ARID4B expression is highly restricted in normal tissue; It is expressed abundantly only in the testis. However, it was first observed as a frequently expressed tumor marker in human carcinomas.[8]

The fifth ARID subfamily, ARID5, consists of two members, ARID5A and ARID5B. Both proteins were characterized by their ability to bind similar AT-rich sequences in the transcriptional modulator of human cytomegalovirus. Although these proteins are more than 70% similar within their ARID sequence, they are not similar outside the ARID region. ARID sequences are grouped because they are more similar to each other than to other members of the ARID family.[9]

Recent studies have identified various regulatory paradigms for how long ncRNAs function. Programming from the promoter region of the non-coding RNA upstream negatively [10]or positively[11] affects the expression of the downstream gene by suppressing the recruitment of RNA Pol II to that region and chromatin remodeling, respectively.[12] An antisense transcript is capable of hybridizing with the overlapping sense transcript and prevents recognition of fusion sites by the spliceosome. Hence alternative splicing causes.[13] Alternatively, the hybridization of sense and antisense transcripts allows Dicer to produce endogenous siRNA. By binding to specific protein partners, a noncoding transcript may

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alter the activity of the protein[14], serve as a structural component that allows the formation of a larger RNA-protein complex,[33] or alter the localization of the protein within the cell. [15] Long ncRNAs can be processed to produce miRNAs, piRNAs, and other poorly characterized small RNAs.

Methods

RNA isolation from cell line

Cells from different cell types such as fetal liver, bone marrow, lung, colon, skeletal muscle, stomach,and prostate are grown under cell culture then after cells reach 80-90% density, they are performed for RNA isolation under appropriate conditions. RNA was extracted from cell culture using a High Pure RNA Isolation (Roche, Mannheim, Germany) kit. The RNA isolation protocol is as follows; - The cells with appropriate density are removed with Trypsin, and DMEM containing FCS is added to stop the effect of trypsin. Cells are centrifuged at 3500 rpm for 5 min. The supernatant is removed without touching the pellet. The remaining pellet is resuspended in 200 µl PBS. Add 400 µl of Lysis Buffer to this mixture and vortex for 15 seconds.

The whole mixture is transferred to filter tubes, and centrifuged for 30 seconds at 9200 rpm. The lower part is discarded. Add 100 µl (10 µl DNAse and 90 µl DNAse incubation buffer) to the filtered tubes and wait at room temperature for 45 minutes. Add 500 µl Wash Buffer I and centrifuge at 9200 rpm for 30 seconds. The lower part is discarded. Add 500 µl Wash Buffer II and centrifuge at 9200 rpm for 30 seconds. The bottom tube is replaced with a new one. Add 200 µl of Wash Buffer II and centrifuge for 2 minutes at 11800 rpm. The lower tube is discarded and a new tube is inserted.

Add 50 µl of Elution Buffer and wait for 1 minute at room temperature and centrifuge at 9200 rpm. The filtered tube is discarded measurements are made on the Nano Drop 1000 to determine the amount of RNA. RNAs are stored at -80 ºC until the working period.

RNA Quantitation: Determination of the quantity and quality of obtained RNA samples was done by detecting the A260/A280 ratio using a Nanodrop spectrophotometer. For PCR reactions, RNA was diluted according to its density.

cDNA components and their amounts: Single-stranded cDNA synthesis from RNA-isolated samples and tissue RNAs was performed with Maxima H Minus First Strand cDNA Synthesis Kit # K1652 (Thermo Scientific). From the cell culture samples, cDNA synthesis was performed by adding RNA at a final concentration of 2 µg / µl RNA and tissue RNA at a final concentration of 1 µg / µl RNA.

Statistical analysis: All result data statistically was measured by GraphPad Prisma version[8].

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Results

The expression level of ARID3A was analyzed in twenty different human tissues. Both fractional-quantity PCR and Real-Time PCR methods performed gene expression analyses. ARID3A is most expressed in the placenta among normal tissues and it has also been shown to be overexpressed in fetal liver, bone marrow, lung, and prostate tissues. However, ARID3A was found to show low expression in the colon, skeletal muscle, stomach, and liver tissues(Figure 1).

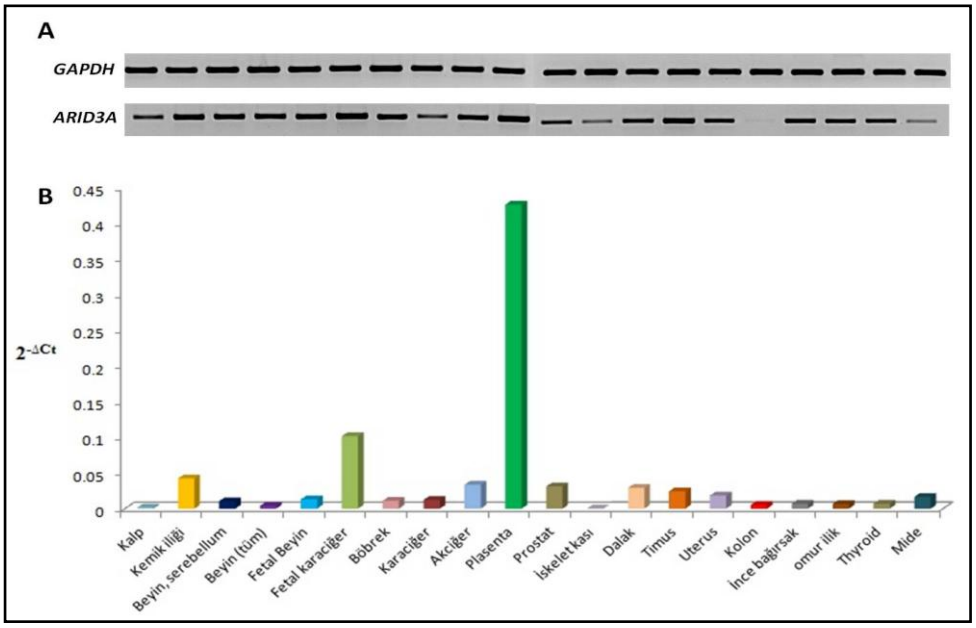

Figure 1: Expression levels of ARID3A and GAPDH genes in different tissues are shown.
A. Demonstration of expression levels of ARID3A and GAPDH genes by RT-PCR method.
B. Expression level of ARID3A gene as a result of qPCR.

We examined ARID3A expression in twenty distinct human cell lines. Both fractional-quantity PCR and real-time PCR techniques were used to analyse gene expression. ARID3A is overexpressed in the fetal liver, bone marrow, lung, and prostate, however, it is highest expressed in the placenta among normal cell lines. Onthe other hand, ARID3Apoor expressionwas seenin the colon, skeletal muscle, stomach, and liver cell lines (Figure 2).

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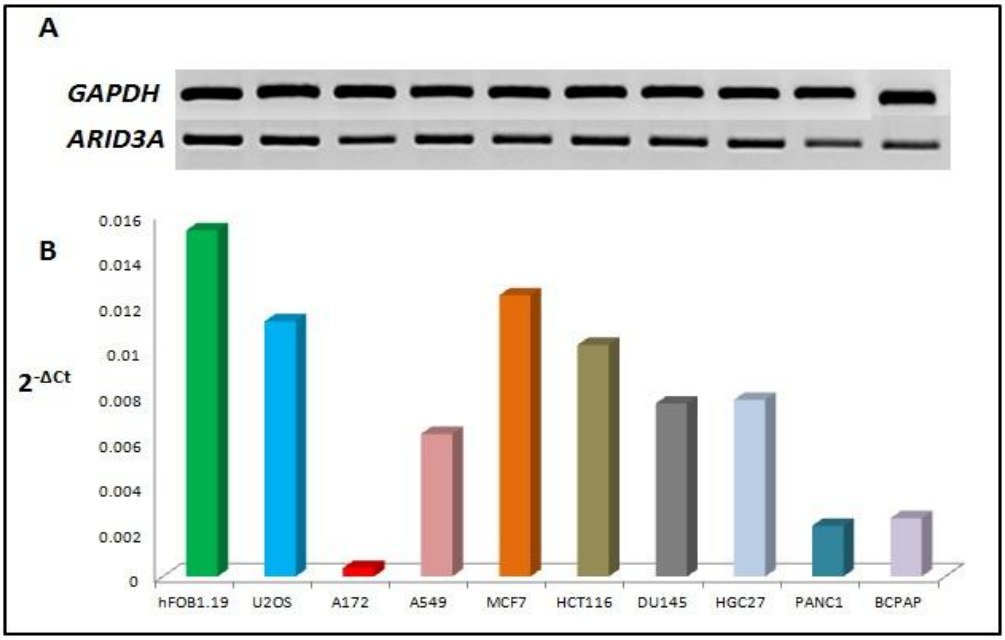

Figure 2: Demonstration of expression levels of ARID3A and GAPDH genes in different cell lines.
A. Demonstration of expression levels of ARID3A and GAPDH genes by RT-PCR method.
B. Expression level of ARID3A gene as a result of qPCR.

After increasing ARID3A expression, its effect on the migration of cells was observed on three consecutive days. Increasing the expression of ARID3A increased cell migration in U2OS cells. The migration of U2OS cells was found to be more pronounced compared to the negative and blank control (Figure3). The wound width formed was less for overexpressed ARID3A at 24, 48, and 72 hours. Overexpression of ARID3A confirmed that it is an important promoter of wound closure in U2OS cells. After three days, there is a gap that is almost invisible to the controls used. Increasing the expression of ARID3A significantly accelerated migration in cells (Figure 4).

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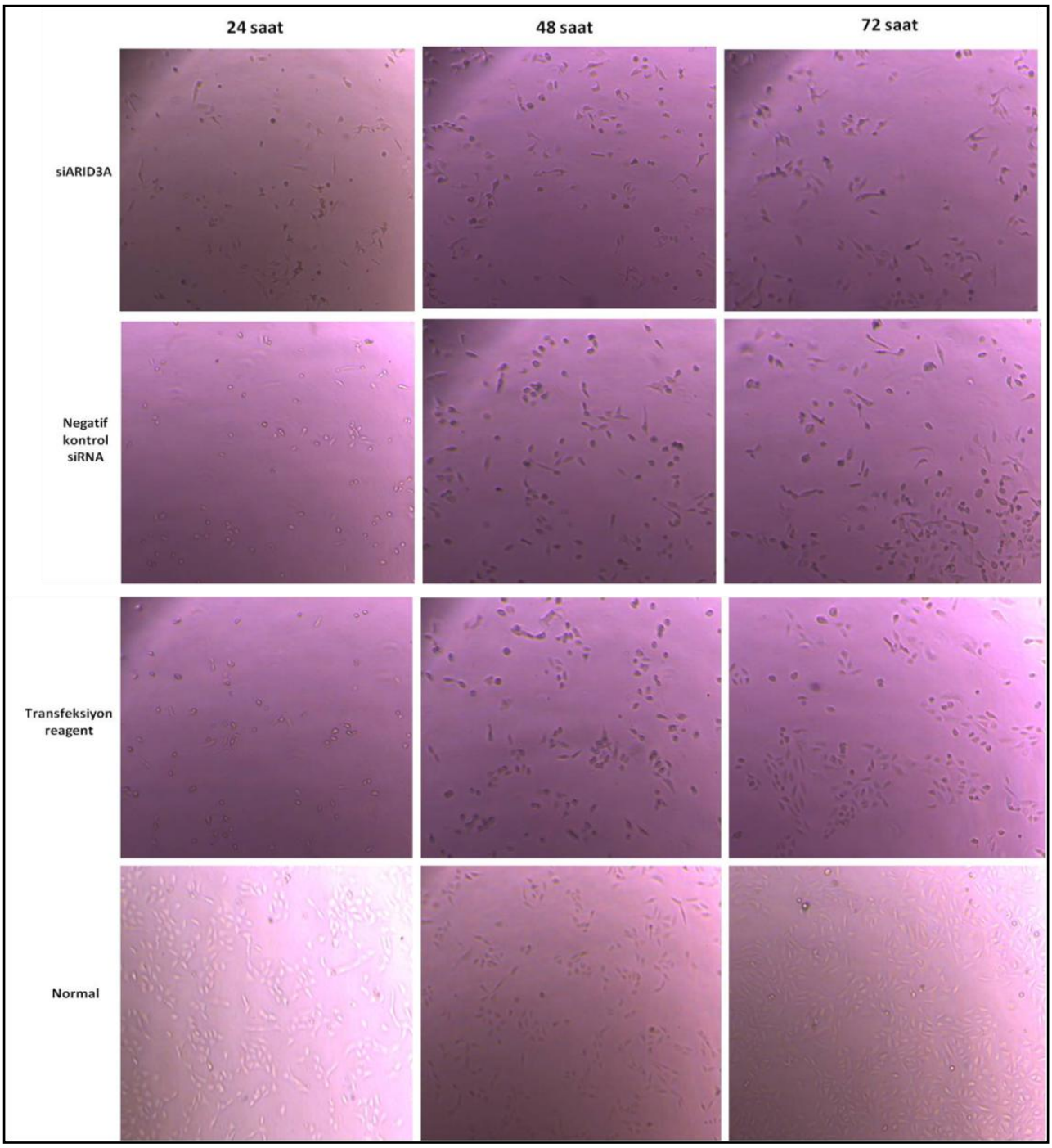

Figure3: Morphology and cell density of U2OS cell line transfected with siARID3A, negative control siRNA, transfection chemical only, and normal (non-transfected) cells at 24, 48, and 72 hours.

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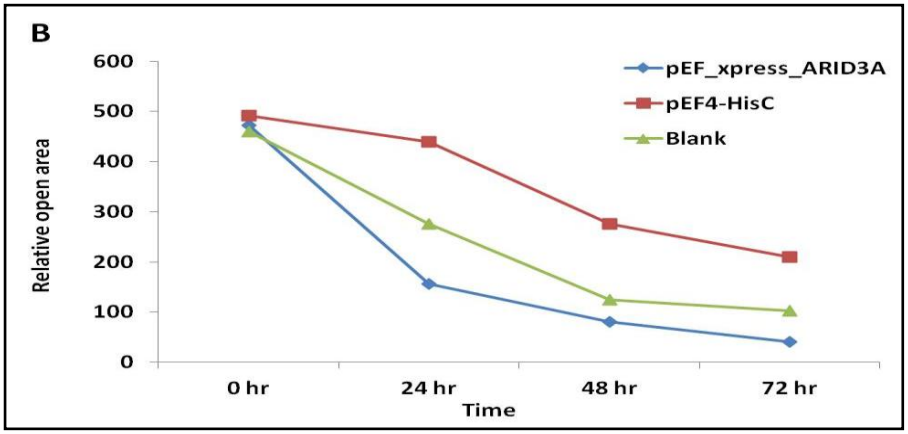

Figure4: Migration of U2OS cells after overexpression of ARID3A compared to positive control and blank control.

After silencing ARID3A, its effects on ERICD expression were determined. Gene expression analysis of ERICD was performed using both RT-PCR and qPCR methods and compared with non-transfected normal cells. It was found that ERICD expression level decreased as a result of silencing ARID3A. Additionally, the t-test showed that these results were statistically significant for 24 ($p < 0.0021$), 48 ($p < 0.0002$), and 72 ($p < 0.0001$) hours (Figure5). The lowest expression level of ERICD was observed on the third day, which was the most significant result found 72 hours after transfection (Figure 5).

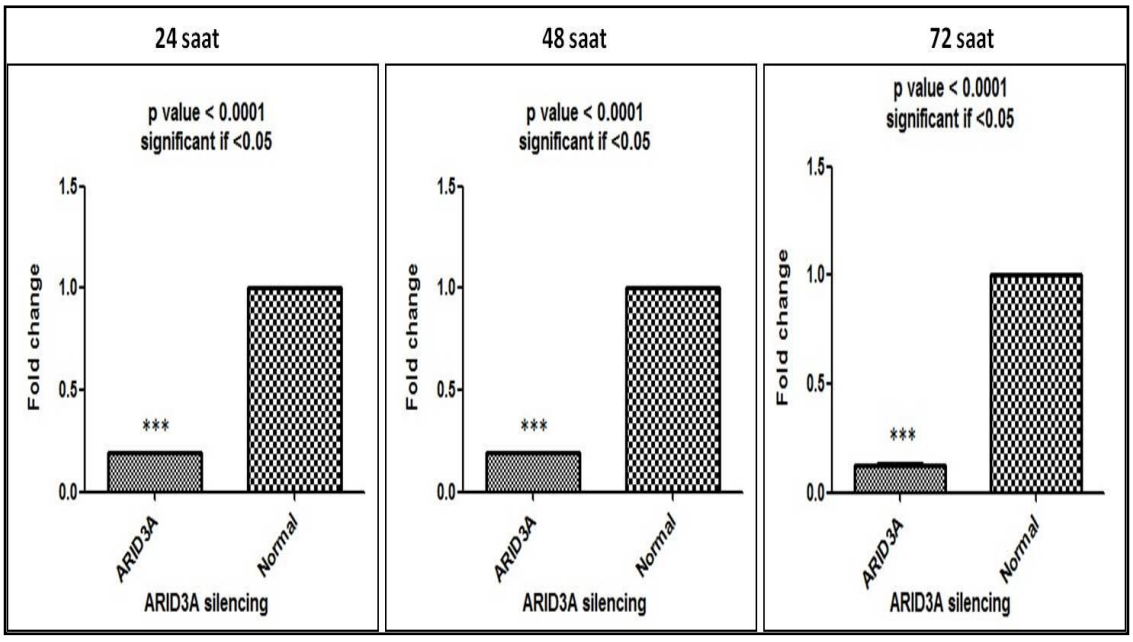

Figure 5: Demonstration of ARID3A silencing in the U2OS cell line by qPCR. Gene expression analyses were performed for 24, 48, and 72 hours.

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Discussion

Protein-centered dogma considered genome regions that did not code for proteins as "garbage". Many lncRNAs, including transposons, pseudogenes, and biologically important functional regulator simple repeats, are transcribed from so-called 'junk' regions of the genome.16,17 Long non-coding RNAs (lncRNAs) are a large and diverse class of non-protein-coding transcribed RNA molecules with a length of more than 200 nucleotides. LncRNAs are becoming increasingly important as a research topic related to cancer. Many lncRNA molecules that have roles in cancers have been identified so far. Some lncRNAs act as tumor suppressors, while others act as oncogenes. For example, lncRNA ANRIL has an oncogenic role in leukemia and prostate cancer by suppressing the expression of p15 and thus cell proliferation (senescence).18,19 Many lncRNAs such as MALAT1, H19, and HOTAIR have been identified that act as oncogenes in many types of cancer such as lung cancer, colon cancer, liver cancer, and breast cancer.20Many lncRNAs that act as tumor suppressors have been identified. MEG3 functions as a tumor suppressor in many types of cancer.21 PTCSC3 (Papillary Thyroid Carcinoma Susceptibility Candidate 3) is a tumor suppressor lncRNA associated with thyroid cancer.22 ERICD (E2f1-regulated inhibitor of Cell Death) is a recently discovered lncRNA. It is located on chromosome 8 (chr8:141646242-141648531) and consists of two exons. The transcript size is 1745 bp. ERICD is regulated by E2F (transcription factor 1) and modulates the cellular response to DNA damage.23 By analysis of the human genomic sequence upstream of ERICD, a putative E2F binding site was identified at -221/-214. This suggests that endogenous E2Fs bind upstream of ERICD.24 Because it promotes survival in the face of DNA damage, ERICD may have cancer-promoting effects as well as conferring chemoresistance.

ARID3A is a member of the AT-rich interaction domain (ARID) DNA-binding protein family that is involved in chromatin remodeling and regulation of gene expression. These proteins are characterized by the ARID DNA binding domain. ARID3A has a consensus sequence binding “AATTAA”.44 ARID3A enables the E2F software agent and E2F-dependent software.25 ARID3A prevents Ras-induced premature senescence in primary murine fibroblasts.26 ARID3A is transcriptionally activated by p53 and DNA damage, and ectopic expression of ARID3A has been reported to promote the growth of p53-deficient Saos-2 cells while suppressing growth in U2OS cells expressing normal p53.27 Additionally, high gene expression of ARID3A was found to be associated with good prognosis in colorectal carcinoma.28 ARID3A has an important role in E2F target gene expression. siRNA-mediated silencing of ARID3A has been shown to inhibit the transcription of E2F target genes such as E2F1, p107, CDC2 and CDC6. ARID3A silencing has also been found to attenuate S phase entry of normal human dermal fibroblasts (NHDFs) and suppress the growth of human tumor cell lines. Therefore, they

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are thought to have tumor suppressor roles.29 So far, whether ARID3A functions as an oncogene or tumor suppressor is still a matter of debate. Many lncRNAs are known to target DNA-binding proteins in many biological processes.30,31 LncRNAs act together with DNA-binding proteins to epigenetically regulate the transcription of DNA.32Interaction of ARID3A with long non-coding RNAs has not yet been demonstrated.

In this study, we aimed to find the possible interaction between ARID3A (AT-rich interaction domain 3A) and lncRNA ERICD (E2F1-Controlled Cell Inhibitor). Both are known to have E2F putative binding sites that are regulated by E2F and have been reported to play a role in various biological processes. ARID3A and ERICD have opposing roles in apoptosis during DNA damage and are regulated by the cell cycle. This makes us think that they play opposite roles in cellular processes. Based on these findings, we aimed to find possible interactions between the two. For this, we aimed to perform expression profile analysis for ERICD, as it is not yet available in the literature. To compare the results, expression profile analysis of ARID3A was also performed in different cancer cell lines and normal tissue samples. Silencing and overexpression experiments were performed to find possible possible interactions.

References

1. Rinn, J. and M. Guttman, *RNA Function. RNA and dynamic nuclear organization.* Science, 2014. 345(6202): p. 1240-1.
2. Gong, C. and L.E. Maquat, *lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3' UTRs via Alu elements.* Nature, 2011. 470(7333): p. 284-8.
3. Mariner, P.D., et al., *Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock.* Mol Cell, 2008. 29(4): p. 499-509.
4. Gutschner, T., M. Hammerle, and S. Diederichs, *MALAT1 -- a paradigm for long noncoding RNA function in cancer.* J Mol Med (Berl), 2013. 91(7): p. 791-801.
5. Matouk, I.J., et al., *The H19 non-coding RNA is essential for human tumor growth.* PLoS One, 2007. 2(9): p. e845.
6. Brooks, C.L. and W. Gu, *p53 regulation by ubiquitin.* FEBS Lett, 2011. 585(18): p. 2803-9.
7. Adams, J.M. and S. Cory, *The Bcl-2 apoptotic switch in cancer development and therapy.* Oncogene, 2007. 26(9): p. 1324-37.
8. Lowe, S.W., E. Cepero, and G. Evan, *Intrinsic tumour suppression.* Nature, 2004. 432(7015): p. 307-15.
9. Gupta, R.A., et al., *Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis.* Nature, 2010. 464(7291): p. 1071-6.
10. Dorak, M.T. and E. Karpuzoglu, *Gender differences in cancer susceptibility: an inadequately addressed issue.* Front Genet, 2012. 3: p. 268.
11. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2012.* CA Cancer J Clin, 2012. 62(1): p. 10-29.
12. Gutschner, T. and S. Diederichs, *The hallmarks of cancer: a long non-coding RNA point of view.* RNA Biol, 2012. 9(6): p. 703-19.

https://doi.org/10.15218/hcchs.2023.01

13. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. 100(1): p. 57-70.
14. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. 144(5): p. 646-74.
15. Hollstein, M., et al., *p53 mutations in human cancers.* Science, 1991. 253(5015): p. 49-53.
16. Levine, B. and G. Kroemer, *Autophagy in the pathogenesis of disease.* Cell, 2008. 132(1): p. 27-42.
17. Mizushima, N., *Autophagy: process and function.* Genes Dev, 2007. 21(22): p. 2861-73.
18. Galluzzi, L. and G. Kroemer, *Necroptosis: a specialized pathway of programmed necrosis.* Cell, 2008. 135(7): p. 1161-3.
19. Zong, W.X. and C.B. Thompson, *Necrotic death as a cell fate.* Genes Dev, 2006. 20(1): p. 1-15
20. Xu, C., et al., *MALAT-1: a long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis.* Int J Oncol, 2011. 39(1): p. 169-75.
21. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. 140(6): p. 883-99.
22. Jendrzejewski, J., et al., *The polymorphism rs944289 predisposes to papillary thyroid carcinoma through a large intergenic noncoding RNA gene of tumor suppressor type.* Proc Natl Acad Sci U S A, 2012. 109(22): p. 8646-51.
23. Feldstein, O., et al., *The long non-coding RNA ERIC is regulated by E2F and modulates the cellular response to DNA damage.* Mol Cancer, 2013. 12(1): p. 131.
24. Cabili, M.N., et al., *Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses.* Genes Dev, 2011. 25(18): p. 1915-27
25. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat Rev Genet, 2005. 6(8): p. 611-22.
26. Shay, J.W. and W.E. Wright, *Hayflick, his limit, and cellular ageing.* Nat Rev Mol Cell Biol, 2000. 1(1): p. 72-6.
27. Schmidt, L.H., et al., *The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth.* J Thorac Oncol, 2011. 6(12): p. 1984-92.
28. Meyne, J., R.L. Ratliff, and R.K. Moyzis, *Conservation of the human telomere sequence (TTAGGG)n among vertebrates.* Proc Natl Acad Sci U S A, 1989. 86(18): p. 7049-53.
29. Benetatos, L., G. Vartholomatos, and E. Hatzimichael, *MEG3 imprinted gene contribution in tumorigenesis.* Int J Cancer, 2011. 129(4): p. 773-9.
30. Counter, C.M., et al., *Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity.* EMBO J, 1992. 11(5): p. 1921-9.
31. Bobbs, A., et al., *ARID3B Directly Regulates Ovarian Cancer Promoting Genes.* PLoS One, 2015. 10(6): p. e0131961.
32. Molenaar, C., et al., *Visualizing telomere dynamics in living mammalian cells using PNA probes.* EMBO J, 2003. 22(24): p. 6631-41.