A.D. Volkogon,
V.Yu. Harbuzova,
A.V. Ataman

ANALYSIS OF ANRIL GENE POLYMORPHISM RS4977574 ASSOCIATION WITH KIDNEY CANCER DEVELOPMENT IN UKRAINIAN POPULATION

Sumy State University
Department of Physiology and Pathophysiology with course of Medical Biology
Sanatorna str., 31, Sumy, 40018, Ukraine

ANRIL (Antisense Non-coding RNA in the INK4b-ARF-INK4a gene cluster. It is known that ANRIL overexpression is associated with development of oncological pathologies of different localization. In addition, there are a number of studies devoted to role of ANRIL genetic polymorphism in emergence and progression of tumors, including tumors of genitourinary system. The aim of the study was to check the possible association between ANRIL gene polymorphism rs4977574 and kidney cancer development in representatives of Ukrainian population. Whole venous blood of 101 patients with clear cell renal cell carcinoma (CCRCC) (42 women and 59 men) and 100 patients without oncology history (34 women and 66 men) was used in the study. DNA from blood white cells was extracted using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). Genotyping of rs4977574 ANRIL gene polymorphic locus was performed using real-time polymerase chain reaction (real-time PCR) method in the presence of TaqMan assay C_31720978_30. The mathematical data were processed using the SPSS software package (version 17.0). P values <0.05 were considered as statistically significant. It was found that difference in rs4977574-genotype distribution between patients with CCRCC and control persons was absent in general group (P=0.216). At the same time, the statistical analysis stratified by smoking habits statistically significant association between rs4977574 ANRIL gene polymorphism and risk of kidney cancer development was detected in male subjects under superdominant inheritance model (P=0.049). It was revealed that heterozygotes (AG-genotype) have 2.17-fold higher risk of CCRCC development (95% CI=1.005-4.695) compared to patients with AA- and GG-genotypes. In summary, this is the first report about ANRIL gene polymorphisms association with kidney cancer. Obtained results revealed that rs4977574 is related to kidney cancer risk only in Ukrainian men. Male individuals with AG-genotype have higher risk of CCRCC development compared to AA- and GG-genotype carriers.

Key words: long non-coding RNA, ANRIL, gene polymorphism, kidney cancer

Abstract. Analysis of ANRIL gene polymorphism rs4977574 association with kidney cancer development in Ukrainian population. Volkogon A.D., Harbuzova V.Yu., Ataman A.V. ANRIL (Antisense Non-coding RNA in the INK4b-ARF-INK4a gene cluster. It is known that ANRIL overexpression is associated with development of oncological pathologies of different localization. In addition, there are a number of studies devoted to role of ANRIL genetic polymorphism in emergence and progression of tumors, including tumors of genitourinary system. The aim of the study was to check the possible association between ANRIL gene polymorphism rs4977574 and kidney cancer development in representatives of Ukrainian population. Whole venous blood of 101 patients with clear cell renal cell carcinoma (CCRCC) (42 women and 59 men) and 100 patients without oncology history (34 women and 66 men) was used in the study. DNA from blood white cells was extracted using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). Genotyping of rs4977574 ANRIL gene polymorphic locus was performed using real-time polymerase chain reaction (real-time PCR) method in the presence of TaqMan assay C_31720978_30. The mathematical data were processed using the SPSS software package (version 17.0). P values <0.05 were considered as statistically significant. It was found that difference in rs4977574-genotype distribution between patients with CCRCC and control persons was absent in general group (P=0.216). At the same time, the statistical analysis stratified by smoking habits statistically significant association between rs4977574 ANRIL gene polymorphism and risk of kidney cancer development was detected in male subjects under superdominant inheritance model (P=0.049). It was revealed that heterozygotes (AG-genotype) have 2.17-fold higher risk of CCRCC development (95% CI=1.005-4.695) compared to patients with AA- and GG-genotypes. In summary, this is the first report about ANRIL gene polymorphisms association with kidney cancer. Obtained results revealed that rs4977574 is related to kidney cancer risk only in Ukrainian men. Male individuals with AG-genotype have higher risk of CCRCC development compared to AA- and GG-genotypes carriers.

Reферат. Аналіз зв’язку поліморфізму гена ANRIL із розвитком раку нирок у українській популяції. Volkogon А.Д., Гарбузова В.Ю., Атаман О.В. ANRIL (антисенсова некодуюча РНК в локусі INK4) також відома як CDKN2B-AS1 – довга некодуюча РНК довжиною 3,8 кб, що транскрибується з антисмислового ланцюга генної кластеру INK4b-ARF-INK4a. Відомо, що надміра експресія ANRIL пов’язана із розвитком онкологічних патологій різної локалізації. Крім того, існує ряд досліджень, присвячених ролі генетичного поліморфізму ANRIL у виникненні та прогресії злоякісних пухлин, включаючи пухлини сечово-статевої системи. Метою дослідження було встановлення можливого зв’язку між rs4977574-поліморфізмом гена ANRIL та розвитком раку нирок у представників українського населення. У дослідженні було використано цільну венозну кров 101 пацієнта зі світлоклітинним нирково-клітинним раком (СККР) (42 жінки та 59 чоловіків) та 100 пацієнтів без онкологічного анамнезу (34 жінки та 66 чоловіків). ДНК з лейкоцитів крові відбирали за допомогою наборів GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, США). Генотипування за поліморфним локусом rs4977574 гена ANRIL проводили за допомогою методу
ANRIL (Antisense Non-coding RNA in the INK4 Locus, also known as CDKN2B-AS1) – 3.8-kb long non-coding RNA (lncRNA) transcribed from antisense strand of INK4b-ARF-INK4a gene cluster [10]. It encodes the amino acid structure of three tumor suppressors: p14ARF, p15INK4b, and p16INK4a. These proteins play a key role in cell cycle arrest, affecting major cellular processes such as senescence, apoptosis, and stem cell self-healing [8].

Today there are a number of reports about relation between ANRIL and development of various oncological pathologies. ANRIL has been found to be overexpressed in gastric cancer [9], esophageal squamous cell carcinoma [12], prostate cancer [15], urinary bladder cancer [11], etc. However, the main molecular mechanism of ANRIL involvement in cancer emergence and progression remains ambiguous.

It is known that in normal cells ANRIL transcript production is required to inhibit the p14ARF, p15INK4b, and p16INK4a expression after DNA repair. However, abnormal ANRIL expression in cancer cells results in blocking of DNA damage response control, which finally leads to genomic instability and tumor progression [6]. Meseure et al. demonstrated that ANRIL mediates inhibition of INK4a transcription by interacting with CBX7 protein of PRC1 repressor complex (Polycomb repressive complex 1), which in turn leads to silencing [7]. Authors have also revealed significantly increased CBX7 and ANRIL amount and decreased INK4a concentration in breast cancer tissues.

ANRIL also influences cell proliferation by regulating target genes in trans. ANRIL has been shown to inhibit the activity of miR-99a/miR-449a in gastric cancer tissues, thereby increasing activity of target genes of these miRNAs – mTOR and CDK6 [9]. On the other hand, in esophageal squamous cell carcinoma ANRIL has been shown to affect cell growth through repression of TGFβ/Smad signaling pathway [12], although the exact molecular mechanisms of interaction between ANRIL and TGFβ1 remain unclear.

Today, there are lot of studies devoted to role of ANRIL genetic polymorphism in different tumors occurrence and progression [2, 4, 14, 16], including malignant tumors of genitourinary system [3]. However, studies about association between ANRIL gene single-nucleotide variants and risk of kidney cancer development are currently absent.

The aim of the study was to check the possible association between ANRIL gene polymorphism rs4977574 and kidney cancer development in representatives of Ukrainian population.

**MATERIALS AND METHODS OF RESEARCH**

The whole venous blood of 101 patients (42 women and 59 men) with clear cell renal cell carcinoma (CCRCC) and 100 patients (34 women and 66 men) without oncology history was used. Patients were treated at Sumy Regional Clinical Oncology Hospital from 2005 to 2016. The morphological diagnosis of CCRCC was established according to European Association of Urology (EAU) Guidelines [17]. All patients had II stage of cancer according to TNM classification of malignancies.

The study was conducted in compliance with Council of Europe Convention on Human Rights and Biomedicine, the Declaration of Helsinki, and Order of the Ministry of Health of Ukraine № 690 (23.09.2009) [1]. All participants signed the informed consent for venous blood sampling for genetic test. The study protocol was approved by the Ethic Committee of the Medical Institute of Sumy State University (number № 3/05.12.11).

DNA from venous blood leukocytes was extracted using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA).

Genotyping of ANRIL gene rs4977574 polymorphic locus was performed by real time polymerase chain reaction (Real-Time PCR) using TaqMan Assay C_31720978_30. The reaction was conducted in Quant Studio 5 DX Real-Time instrument (“Applied Biosystems, USA) using PCR Real-Time kit (“Thermo Fisher Scientific”, USA). The amplification reaction consisted of initial 10-minute
denaturation (95 °C) followed by 45 cycles of amplification for 15 s (95°C) and 30 s (60°C).

The mathematical data were processed using SPSS software package 17.0.1 version (SPSS Inc. 2147483647). Analysis of rs4977574-genotypes distribution between comparison groups was performed using Pearson's $\chi^2$ test. To check the deviation of rs4977574-genotypes distribution from Hardy-Weinberg equilibrium WpCalc online resource was used (https://wpcalc.com/en/equilibrium-hardy-weinberg/). The risk of CCRCC development, depending on specific rs4977574 genotype, was calculated by logistic regression under dominant (AG+GG vs. AA), recessive (GG vs. AA+AG), and super-dominant (AG vs. AA+GG) models of inheritance. Sex, age, body mass index and smoking status were used as covariates in multivariable regression. HaploReg v4 resource was used for bioinformatics analysis [18]. All tests were two-sided. p values <0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

After genotyping of comparison groups by ANRIL gene rs4977574-locus we tested the correspondence of AA-, AG- and GG-genotypes frequency to Hardy-Weinberg equilibrium. It was found that both in CCRCC patients and in control subjects rs4977574-genotypes distribution did not deviate from expected by Hardy-Weinberg law (p=0.368 and p=0.252, respectively).

The frequency of rs4977574-genotypes in comparison groups are presented in Table 1. It was found that difference in AA-, AG- and GG-genotypes distribution between CCRCC patients and control group was absent in general group (p=0.216). At the same time statistical analysis stratified by gender showed that rs4977574-genotypes frequency also did not significantly differ between comparison groups both in female (p=0.526) and male (p=0.160).

| Group    | n     | AA (%) | AG (%) | GG (%) | p   |
|----------|-------|--------|--------|--------|-----|
| Total    |       |        |        |        |     |
| CCRCC    | 101   | 22 (21.8) | 55 (54.5) | 24 (23.8) | 0.216 |
| Control  | 100   | 32 (32.0) | 44 (44.0) | 24 (24.0) |     |

| Group    | n     | AA (%) | AG (%) | GG (%) | p   |
|----------|-------|--------|--------|--------|-----|
| Female   |       |        |        |        |     |
| CCRCC    | 42    | 10 (23.8) | 22 (52.4) | 10 (23.8) | 0.526 |
| Control  | 34    | 11 (32.4) | 18 (52.9) | 5 (14.7) |     |

| Group    | n     | AA (%) | AG (%) | GG (%) | p   |
|----------|-------|--------|--------|--------|-----|
| Male     |       |        |        |        |     |
| CCRCC    | 59    | 12 (20.3) | 33 (55.9) | 14 (23.7) | 0.160 |
| Control  | 66    | 21 (31.8) | 26 (39.4) | 19 (28.8) |     |

Note. CCRCC – clear cell renal cell carcinoma; n – number of persons in the subgroups.

Then to test the possible link between lncRNA ANRIL genetic polymorphism and risk of kidney cancer development binary and multivariable logistic regression under different models of inheritance was used (Table 2). Significant association between rs4977574-locus and CCRCC occurrence without adjusting for covariates was not found in general group (p >0.05) as well as in individuals of different gender (p >0.05). However, after adjusting for age, body mass index, smoking habits statistically significant relation between ANRIL polymorphism rs4977574 and kidney cancer risk was detected in male subjects under superdominant model ($p_{adj}=0.049$). Thus, male heterozygotes (AG-genotype) have higher risk of CCRCC development ($OR_{adj}=2.172$; 95% CI=1.005-4.695) compared to subjects with AA- and GG-genotype.

As of January 2020, 32759 polymorphic sites are located in the ANRIL gene (according to NCBI: https://www.ncbi.nlm.nih.gov/snp/?term=CDKN2B-
AS1). Some ANRIL gene polymorphisms have significant association with tumor development. Thus, the ANRIL TCGA-haplotype (rs1333045, rs1333048, rs4977574 and rs10757278) has been shown to increase breast cancer risk in Iranian women [2]; rs2151280 polymorphism is highly correlated with optic glioma development in neurofibromatosis type 1 patients [14] and linked to relapse in multiple myeloma subjects [16]; wherein locus rs1011970 is related to lung cancer susceptibility and treatment [4]. The authors believe that these nucleotide variations may alter expression of different ANRIL splicing variants and, as a consequence, dysregulate the INK4b-ARF-INK4a locus expression.

Taheri M. et al. tested the link between prostate cancer, benign prostate hyperplasia and four ANRIL gene polymorphisms (rs1333045, rs4977574, rs1333048, and rs10757278) in Iranian patients [3]. It was shown that rs4977574, rs1333048, and rs10757278 are associated with prostate tumors occurrence origin.

Polymorphism rs4977574 is located within 16 intron of ANRIL gene (103785th gene position). Functional studies devoted to its role in diseases development are currently absent, but bioinformatic analysis using HaploReg v4 resource [18] revealed that rs4977574 polymorphism can change nucleotide sequence of transcription factor C-ets-1 and glucocorticoid receptor binding sites. Studies have shown significant role of these proteins in the origin and progression of kidney cancer [5, 13]. Thus, it can be assumed that ANRIL polymorphism rs4977574 is able to promote cancer development through the modulation of impact of mentioned transcription factors on lncRNA ANRIL expression.

In our study we firstly established the ANRIL rs4977574-genotypes distribution among Ukrainian population and examined the association of this polymorphism with kidney cancer development. The link between rs4977574 single-nucleotide polymorphism and CCRCC has been found only in men. After adjusting for covariates it was revealed that men with genotype rs4977574AG have higher risk of CCRCC development compared to men with rs4977574AA- and rs4977574GG-genotypes.

**CONCLUSION**

In summary, this is the first report about ANRIL gene polymorphisms association with kidney cancer. Obtained results revealed that locus rs4977574 is related to kidney cancer risk only in Ukrainian men.
Male individuals with AG-genotype have higher risk of CCRCC development compared to AA- and GG-genotypes carriers.

REFERENCES

1. [About the Approving of Conducting of Medicines Clinical Trials Procedure and Expertise of Clinical Trials Materials, and the Model Regulations on Ethics Committees: Order of the Ministry of Health of Ukraine No. 690]. 2009. September 23. Ukrainian.

2. Khosravi H, Taheri M, Noroozi R, Sarrafzadeh S, Sayad A, Ghafoori-Fard S. ANRIL Genetic Variants in Iranian Breast Cancer Patients. Cell J. 2017;19(Suppl 1):72-78. doi: https://doi.org/10.22074/cellj.2017.4496

3. Taheri M, Pouresmaeili F, Omrani MD, Habibi M, Sarrafzadeh S, Noroozi R, et al. Association of ANRIL gene polymorphisms with prostate cancer and benign prostatic hyperplasia in an Iranian population. Biomark Med. 2017;11(5):413-22. doi: https://doi.org/10.2217/bmm-2016-0378

4. Gong WJ, Yin J, Li XP, Fang C, Xiao D, Zhang W, et al. Association of well-characterized lung cancer IncRNA polymorphisms with lung cancer susceptibility and platinum-based chemotherapy response. Tumour Biol. 2016;37(6):8349-58. doi: https://doi.org/10.1007/s13277-016-3460

5. Czarnecka A, Niedzwiecka M, Porta C, Szczyllic K. Hormone signaling pathways as treatment targets in renal cell cancer (Review). Int J Oncol. 2016;48(6):2221-35. doi: https://doi.org/10.3892/ijo.2016.3460

6. Diamatpour A, Ghafoori-Fard S. The Role of Long Non Coding RNAs in the Repair of DNA Double Strand Breaks. Int J Mol Cell Med. 2017;6(1):1-12.

7. Meseure D, Vacher S, Alsibai KD, Nicolas A, Chehissi D, Natacci F. Non-Coding RNA and Tumor Development in Neurofibromatosis Type 1: ANRIL rs2151280 Is Associated with Optic Glioma Development and a Mild Phenotype in Neurofibromatosis Type 1 Patients. Genes (Basel). 2019;10(11):E892. doi: https://doi.org/10.3390/genes10110892

8. Zhu H, Li X, Song Y, Zhang P, Xiao Y, Xing Y. Long non-coding RNA ANRIL is up-regulated in bladder cancer and regulates bladder cancer cell proliferation and apoptosis through the intrinsic pathway. Biochem Biophys Res Commun. 2015;467(2):223-28. doi: https://doi.org/10.1016/j.bbrc.2015.10.002

9. Zhao B, Lu Y, Yang Y, Hu L, Bai Y, Li R, et al. Overexpression of IncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating the let-7a/TGF-β1/Smad signaling pathway. Cancer Biomark. 2016;21(3):613-620. doi: https://doi.org/10.3233/CBM-170683

10. Poj M, Li J, Sborov D, VanGundy Z, Cho Y, Lamprecht M, et al. Polymorphism in ANRIL is associated with relapse in patients with multiple myeloma after autologous stem cell transplant. Mol Carcinog. 2017;56(7):1722-32. doi: https://doi.org/10.1002/mc.22626

11. Powles T, Albiseg E, Staepler M, Bensalah K, Dabestani S, Giles R. Updated European Association of Urology Guidelines Recommendations for the Treatment of First-line Metastatic Clear Cell Renal Cancer. Eur Urol. 2017;pii: S0302-2838(17)31001-1. doi: https://doi.org/10.1016/j.eururo.2017.11.016

12. Ward LD, Kellis M. HaploReg v4: systematic mining of putative causal variants, cell types, regulators and target genes for human complex traits and disease. Nucleic Acids Res. 2016;44(D1):D877-881. doi: https://doi.org/10.1093/nar/gkv1340

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1. Association of ANRIL gene polymorphisms with prostate cancer and benign prostatic hyperplasia in an Iranian population / M. Taheri et al. Biomark Med. 2017. Vol. 11, No.5. P. 413-422.
2. Association of well-characterized lung cancer IncRNA polymorphisms with lung cancer susceptibility and platinum-based chemotherapy response / W. Gong et al. Cell J. 2017. Vol. 19, No. 1. P. 72-78. DOI: https://doi.org/10.22074/cellj.2017.4496
3. Association of ANRIL gene polymorphisms with prostate cancer and benign prostatic hyperplasia in an Iranian population / M. Taheri et al. Biomark Med. 2017. Vol. 11, No.5. P. 413-422. DOI: https://doi.org/10.2217/bmm-2016-0378
4. Association of well-characterized lung cancer IncRNA polymorphisms with lung cancer susceptibility and platinum-based chemotherapy response / W. Gong et al. Cell J. 2017. Vol. 19, No. 1. P. 72-78. DOI: https://doi.org/10.22074/cellj.2017.4496
5. Czarnecka A., Niedzwiedzka M., Porta C., Szczylik C. Hormone signaling pathways as treatment targets in renal cell cancer: review. Int J Oncol. 2016. Vol. 48, No. 6. P. 2221-2235.
6. Dianiapour A., Ghafari-Fard S. The Role of Long Non Coding RNAs in the Repair of DNA Double Strand Breaks. Int J Mol Cell Med. 2017. Vol. 6, No. 1. P. 1-12.
7. Expression of ANRIL-Polycomb Complexes-CDKN2A/B/ARF Genes in Breast Tumors: Identification of a Two-Gene (EZH2/CBX7) Signature with Independent Prognostic Value / D. Meseure et al. Mol. Cancer Res. 2016. Vol. 467, No. 2. P. 223-228.
8. Gamelli C., Gainsberg D., Haupt S., Haupt Y. New insights on the regulation of INK4/ARF locus expression. Oncotarget. 2017. Vol. 8, No. 63. P. 106147-106148. DOI: https://doi.org/10.18632/oncotarget.22258
9. Knockdown of long non-coding RNA ANRIL inhibits tumorigenesis in human gastric cancer cells via microRNA-99a-mediated down-regulation of BMI1 / P. Liu et al. Braz J Med Biol Res. 2018. Vol. 51, No. 10. e6839. DOI: https://doi.org/10.1590/1414-431x20186839
10. Kong Y., Hsieh C., Alonzo L. ANRIL: A IncRNA at the CDKN2A/B Locus With Roles in Cancer and Metabolic Disease. Front Endocrinol (Lausanne). 2018. Vol. 9. P. 405. DOI: https://doi.org/10.3389/fendo.2018.00405
11. Long non-coding RNA ANRIL is up-regulated in bladder cancer and regulates bladder cancer cell proliferation and apoptosis through the intrinsic pathway / H. Zhu et al. Biochem Biophys Res Commun. 2015. Vol. 467, No. 2. P. 223-228. DOI: https://doi.org/10.1016/j.bbrc.2015.10.002
12. Non-Coding RNAs in gastroesophageal cancers / G. Fanelli et al. Noncoding RNA Res. 2018. Vol. 3, No. 4. P. 195-212. DOI: https://doi.org/10.1016/j.ncrna.2018.10.001
13. MiR-532-5p suppresses renal cancer cell proliferation by disrupting the ETS1-mediated positive feedback loop with the KRAS-NAP1L1/P-ERK axis / W. Zhai et al. Br J Cancer. 2018. Vol. 119, No. 5. P. 591-604. DOI: https://doi.org/10.1038/s41416-018-0196-5
14. Overexpression of IncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF-β1/Smad signaling pathway / B. Zhao et al. Cancer Biomark. 2018. Vol. 21, No. 3. P. 613-620. DOI: https://doi.org/10.3233/CBM-170683
15. Overexpression of IncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF-β1/Smad signaling pathway / B. Zhao et al. Cancer Biomark. 2018. Vol. 21, No. 3. P. 613-620. DOI: https://doi.org/10.3233/CBM-170683
16. Polymorphism in ANRIL is associated with relapse in patients with multiple myeloma after autologous stem cell transplant / M. Poi et al. Mol Carcinog. 2017. Vol. 56, No. 7. P. 1722-1732. DOI: https://doi.org/10.1002/mc.22626
17. Updated European Association of Urology Guidelines Recommendations for the Treatment of First-line Metastatic Clear Cell Renal Cancer / T. Powles et al. Eur Urol. 2017. pii: S0302-2838(17)31001-1. DOI: https://doi.org/10.1016/j.eururo.2017.11.016
18. Ward L. D., Kellis M. HoploReg v4: systematic mining of putative causal variants, cell types, regulators and target genes for human complex traits and disease. Nucleic Acids Res. 2016. Vol. 44, No. 1. P. 877-881. DOI: https://doi.org/10.1093/nar/gkv1340

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