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A functional Cis-eQTL locus in lncRNA ZNRD1-AS1 contributes to the susceptibility of endometrial cancer

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Abstract. – OBJECTIVE: Endometrial cancer (EC) remains one of the most common gynecologic malignancies worldwide. However, the exact etiology is still unknown. Human Zinc ribbon domain containing 1 (ZNRD1) was involved in carcinogenesis and progression of multiple cancers, including EC. ZNRD1-AS1, a long noncoding RNA (lncRNA) located in the upstream of ZNRD1, has been reported as an essential component in carcinogenesis. However, the underlying relations of ZNRD1-AS1 with development of EC remain obscure. This study aims to evaluate the potential role of ZNRD1-AS1 and Cis-eQTL loci of ZNRD1 in the occurrence of EC.

PATIENTS AND METHODS: We first evaluated the expression of ZNRD1-AS1 and ZNRD1 among EC tissues and corresponding normal tissues using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Then, to reveal the underlying mechanisms, we investigated the associations between Cis-eQTL loci of ZNRD1 in ZNRD1-AS1 and the susceptibility of EC. Further, *in vitro* experiments were conducted to evaluate the regulation role of rs9261204 on the expression of ZNRD1gene.

RESULTS: Higher expression of ZNRD1-AS1 and lower expression of ZNRD1 were detected in the EC tissues, compared to the normal tissues. Minor allele of rs9261204 was significantly associated with increased risk of EC (OR: 1.33; 95% CI: 1.09-1.61; $p = 0.004$). Furthermore, *in vitro* experiments confirmed that Ishikawa cells with rs9261204 G allele had lower mRNA level of ZNRD1, compared to the A allele.

CONCLUSIONS: Our findings first showed the contribution of LncRNA ZNRD1-AS1 and Cis-eQTL loci of ZNRD1 to the development of EC. Further studies incorporating larger populations and functional assays are warranted.

Key Words:
Endometrial cancer, LncRNA, ZNRD1-AS1, SNP, Rs9261204.

Introduction

According to the World Cancer Report 2014, approximately 320,000 females were diagnosed with endometrial cancer (EC) worldwide and 76,000 died each year, making it the sixth most common cancer in women[1]. In China, 63.4 thousand incident cases and 21.8 deaths occurred annually, as reported by the National Office for Cancer Prevention and Control[2]. Environmental and genetic factors were both involved in the development of EC. Obesity, never having had a child, late menopause, high levels of estrogen, increasing age, diabetes mellitus, breast cancer, and use of tamoxifen, were known to be the most important factors to increase risk of EC[3-6]. Genome-wide association study (GWAS) have identified multiple loci associated with EC risk; however, still a large part of the etiology for EC remains unknown[7-9].

Human Zinc ribbon domain containing 1 (ZNRD1), which encodes a protein with similarity to the Saccharomyces cerevisiae Rpa12p subunit of RNA polymerase I, is a zinc finger-related protein involved in transcription regulation of human leukocyte antigens (HLA)[10]. ZNRD1 gene could suppress cell proliferation through cell cycle arrest in G1 phase[11-13]. ZNRD1 also implicates in the process of DNA damage and repair and suppresses cell proliferation[10-14]. It has been confirmed to be involved in carcinogenesis and development of multiple cancers[15]. Studies[16-18] also evaluated the association of expression quantitative trait loci (eQTL) of ZNRD1 in long non-coding RNA (LncRNA) ZNRD1-AS1, a LncRNA in the upstream region of ZNRD1 which could down-regulate the expression of ZNRD1, with the susceptibility and development of lung cancer, cervical cancer, and hepatocellular carcinoma. However, no study

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has tried to explore their associations with EC susceptibility. Here, we aim to evaluate the expression pattern of ZNRD1-AS1 and ZNRD1 in EC tissues, and the contribution of Cis-eQTL loci in ZNRD1-AS1 for the development of EC.

Patients and Methods

Patients

Eligible patients were recruited with a confirmed diagnosis of EC based on histopathological evaluation. Frequency-matched controls that admitted for benign gynecologic causes and routine control, not diagnosed as any type of cancer, and no family history of cancer, were included in this study as control group. Demographic parameters of patients and controls were collected by in-person questionnaires. 5 mL of peripheral venous blood specimens were collected at the time of the interview. This investigation was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. Signed written informed consents were obtained from all participants before the study.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA from fresh cultured EC cell lines, tissue samples, and whole blood, according to the manufacturer's instructions. The expression levels of ZNRD1-AS1 (Forward primer: TCCTAGGATTGCTGCAGGTC; reverse primer: CTATTGCCTGGATCCCATGT) and ZNRD1 (Forward primer: TCGATGTGGTCATGAAGGAA; reverse primer: ACCAGACAGGATGGACAAGG) among EC tissues and corresponding normal tissues of 30 cases were detected using qRT-PCR performed with SYBR Green 2x Master Mix (Life Technologies, Gaithersburg, MD, USA). Delta CTs were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, forward primer: GCACCGTCAAGGCTGAGAAC; reverse primer: TGGTGAAGACGCCAGTGGA) reference gene, and ΔΔCT analysis was performed to calculate the relative expression of RNA.

eQTL Determination and Genotyping

As previous publications[16-18], we selected rs3757328, rs6940552, and rs9261204 as the tagSNPs of the Cis-eQTL for ZNRD1 in ZNRD1-AS1 gene region using database RegulomeDB[19]. The genotyping was determined by the SNaPshot assay (ABI, Waltham, MA, USA) according to the manufacturer's instructions. For quality control, positive and negative controls were included in each genotyping plate, and 3% of the samples were repeatedly genotyped, with a concordance rate of 100% between the duplicates.

Cell Culture and Plasmid Construction

Ishikawa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Grand Island, NY, USA). Cultures were supplemented with 10% of fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The full-length insert of the common allele of ZNRD1-AS1 was cloned to pcDNA3.1 vector (Life Technologies, Gaithersburg, MD, USA). The minor allele expression plasmid was generated using QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA USA) and introducing SNP as point mutation in the insert. Ishikawa cells were transfected for 48 hours with vectors, common allele, or minor allele of ZNRD1-AS1 constructs.

Statistical Analysis

The expression levels of ZNRD1-AS1 and ZNRD1 between matched pairs of samples were analyzed using Wilcoxon matched-pairs signed rank test. Allele frequencies were confirmed by gene counting. Hardy-Weinberg equilibrium, genotype and allele frequency comparisons between groups were evaluated using the Chi-square tests. To evaluate the associations between the genotypes and EC risk, odds ratios (ORs) and 95% of confidence intervals (CIs) were calculated by using unconditional logistic regression analysis with adjustment for age and sex. The statistical packages Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism (San Diego, CA, USA) were used for the statistical analyses and graphing. Statistical differences were considered significant if $p<0.05$.

Results

ZNRD1-AS1 and ZNRD1 Expression in EC Tissues and Adjacent Normal Tissues

We first evaluated the expression levels of ZNRD1-AS1 and ZNRD1 among 30 EC tissues

and adjacent normal tissues using qRT-PCR. The results showed significantly increased expression of ZNRD1-AS1 and decreased expression of ZNRD1 in the EC tissues (Figure 1, p-value < 0.01). These findings suggest a tumor suppressor role for ZNRD1 gene and a tumor contributor role for LncRNA ZNRD1-AS1in the carcinogenesis of EC.

Association of tagSNPs of the Cis-eQTL for ZNRD1 in ZNRD1-AS1 Gene Region With EC Susceptibility

As shown in Table I, the genotype distributions of the three tagSNPs of the Cis- eQTL for ZNRD1 in ZNRD1-AS1 gene region (rs3757328, rs6940552, and rs9261204) in EC cases and healthy control subjects were displayed. We found G allele of rs9261204 was significantly associated with an increased risk of EC when compared with A allele (OR: 1.33; 95% CI: 1.09-1.61; p=0.004). The adjusted OR for the carriers with the GG genotype was 1.71 (95% CI: 1.11-2.64) and for those with the AG genotype was 1.29 (95% CI: 0.99-1.68) compared with the AA genotype. After adjusted for Bonferroni correction, the trend was still significant ($0.004 \times 3 = 0.012 < 0.05$).

Effect of SNP rs9261204 on ZNRD1 Expression in Ishikawa Cell Line

We evaluated the effect of SNP rs9261204 on ZNRD1 expression in the Ishikawa cell line. As shown in Figure 2, cells with rs9261204 allele G had the lower mRNA level of ZNRD1 compared with those with allele A (p=0.028). As expected, it is biologically plausible that the G allele at rs9261204 may result in lower expression of ZNRD1, hence promoting EC cell proliferation.

Discussion

Genetic disorders and gene defects of LncRNAs accounted for many diseases including cancers[20-25]. In this study, we investigated the expression of ZNRD1-AS1 and ZNRD1 in EC tissues and corresponding adjacent normal tissues, which found significantly increased expression of ZNRD1-AS1 and decreased expression of ZNRD1 in EC tissues. Then we investigated the associations between ZNRD1 eQTLs SNPs in ZNRD1-AS1 region and EC susceptibility and found that G allele of SNP rs9261204 was significantly associated with an increased risk of EC when compared with A allele. Furthermore, cell line expression analyses showed G allele at rs9261204 may result in lower expression of ZNRD1, hence promoting EC cell proliferation. This should be the first study aiming to explore the relation between LncRNA ZNRD1-AS1, its variants, and ZNRD1 and development of EC, suggesting their biological functions underlying the associations that await future studies.

ZNRD1 was first cloned from the human MHC class I region by Fan et al[10] in 2000. Then, Zhang et al[13] found that ZNRD1 antisense RNA transduction could reverse the MDR of human

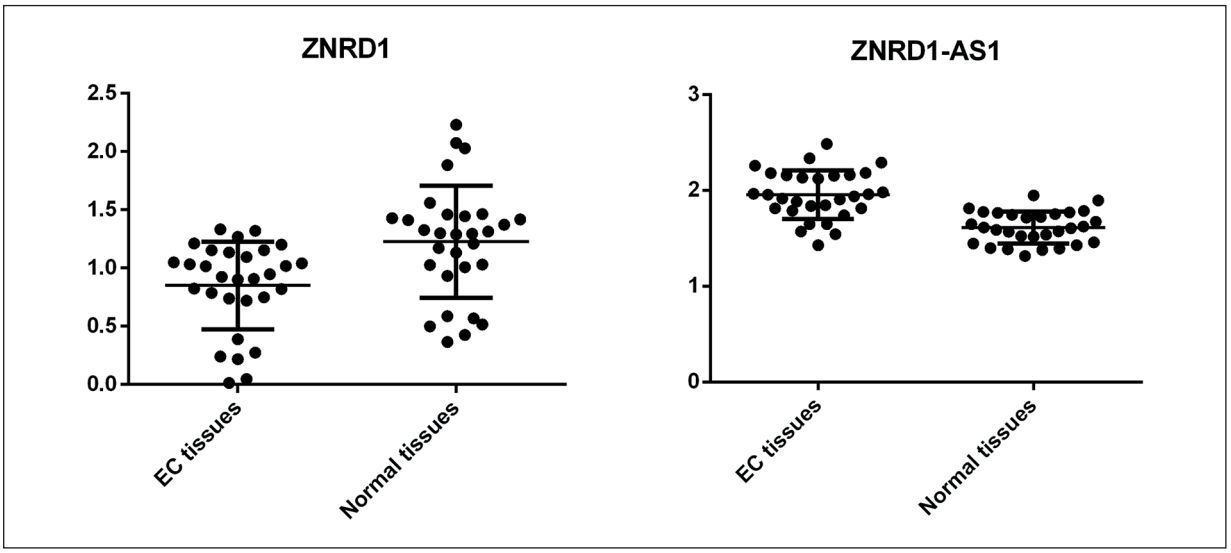

Figure 1. Quantification and statistical analysis of ZNRD1-AS1 and ZNRD1 expression in EC tissues and matched normal tissues.

Table I. Association between 3 eQTLs SNPs in ZNRD1-AS1 and bladder cancer susceptibility.

Genotype	Cases	Controls	Adjusted OR (95% CI)*
rs3757328			
GG	337	340	1.00 (reference)
AG	146	150	0.98 (0.74-1.29)
AA	17	10	1.71 (0.78-3.77)
Additive			1.07 (0.85-1.35)
p trend			0.556
rs6940552			
GG	274	289	1.00 (reference)
AG	175	169	1.09 (0.84-1.43)
AA	51	42	1.28 (0.82-1.98)
Additive			1.13 (0.93-1.38)
p trend			0.224
rs9261204			
AA	241	280	1.00 (reference)
AG	200	180	1.29 (0.99-1.68)
GG	59	40	1.71 (1.11-2.64)
Additive			1.33 (1.09-1.61)
p trend			0.004

*Adjusting for age, BMI

drug-resistant gastric cancer cell SGC7901/VCR to a degree. It was found to be down-regulated in human gastric cancer tissues as compared to the matched adjacent non-neoplastic tissues[12]. ZNRD1 spans the HLA region and several studies[26-33] have suggested that genetic variants of the genes in the HLA region contributed to the risk of cancers. ZNRD1 antisense RNA transduction could reverse the MDR of human drug-resistant gastric cancer cell SGC7901/VCR to a degree[13]. In the current study, we also found the decreased expression levels of ZNRD1 in EC tissues, compared with corresponding normal tissues. These findings above revealed the essential function of ZNRD1 in the carcinogenesis and tumorigenesis.

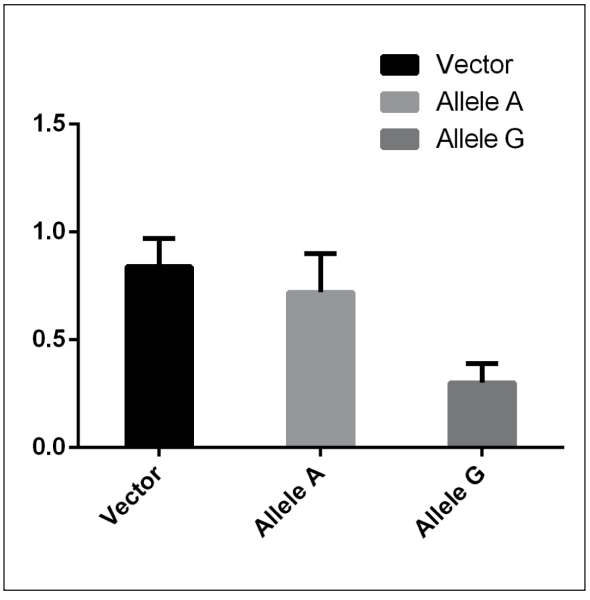

Figure 2. Effect of SNP rs9261204 on ZNRD1 expression in the Ishikawa cell line.

The research about the genetic variants and biological function of lncRNAs, which could act as the Cis-eQTL loci of downstream genes, is becoming investigative emphasis and hot spot in the studies of complex diseases. LncRNA ZNRD1-AS1 was located in the upstream region of ZNRD1. First, Wen et al[17] reported that rs9261204 and rs6940552 in ZNRD1-AS1 may influence both chronic HBV infection and hepatocellular carcinoma (HCC) development. Later, Guo et al[16] identified that rs3757328, rs6940552, and rs9261204 in ZNRD1-AS1 could influence cervical cancer development. Then, Li et al[18] reported rs9261204 contributing to the susceptibility of lung cancer. One meta-analysis also showed rs3757328, rs6940552, and rs9261204 in ZNRD1-AS1 were all associated with increased cancer risk in an Asian population[15]. In the current study, only SNP rs9261204 was significantly associated with an increased EC risk. Our cell line expression analyses also showed that cells with allele G at rs9261204 had the lower mRNA level of ZNRD1 compared with those with allele A. Using the UCSC Genome Browser, we found SNP rs9261204 falls within

several transcription factor ChIP regions including CTCF, SMC3, RAD21, ZNF143, TEAD4, PBX3, and FOXA1, which means the potential diverse regulation roles.

Conclusions

LncRNA ZNRD1-AS1, its variants, and ZNRD1 could contribute to the development of EC from different approaches, including *in vivo*, *in vitro*, and epidemiological investigations. The applicability of these results to other populations of EC patient needs to be explored before these markers can be used to guide the clinical practice. Further studies incorporating larger populations and functional assays are warranted to validate and extend our findings. However, these data still provide new insight into methods of identifying targets for EC prevention and guide the individualized choice of treatment methodologies based on molecular pathway alterations.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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