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Original Article

Expression quantitative trait loci in long non-coding RNA ZNRD1-AS1 influence cervical cancer development

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Abstract: *Zinc ribbon domain containing 1 (ZNRD1)* may play integral roles in immune response against HPV infection and cervical cancer. Its antisense transcript, *ZNRD1-AS1*, is an important regulator of *ZNRD1*. By bioinformatics analyses, we identified that several single nucleotide polymorphisms (SNPs) in *ZNRD1-AS1* may be expression quantitative trait loci (eQTLs) for *ZNRD1*. So we hypothesized that these eQTLs SNPs in *ZNRD1-AS1* may influence the susceptibility of cervical cancer through influencing *ZNRD1* expression. We designed a population-based case-control study containing 1486 cervical cancer patients and 1536 controls to test the associations of three *ZNRD1* eQTLs SNPs (rs3757328, rs6940552 and rs9261204) in *ZNRD1-AS1* with the risk of cervical cancer. Logistic regression analyses in additive genetic model showed that all the three eQTLs SNPs decreased the risk of cervical cancer. Compared with those carrying "0" variant allele, subjects carrying "1-6" variant alleles had a 20% decreased risk of cervical cancer. Moreover, the haplotype containing variant alleles of these three SNPs significantly decreased the risk of cervical cancer when compared with the most frequent haplotype. In conclusion, *ZNRD1* eQTLs SNPs in *ZNRD1-AS1* could have a predisposition for the development of cervical cancer.

Keywords: *ZNRD1*, *ZNRD1-AS1*, *eQTLs*, cervical cancer

Introduction

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer deaths among women worldwide [1]. It has been well established that infection with genital high-risk human papillomavirus (HR-HPV) is a necessary cause of cervical cancer [2, 3]. However, high-risk HPV infection alone has been found to be insufficient to induce tumor progression. Although studies have shown that up to 22% of general population worldwide are HPV positive [4], less than 10% HPV infections become persistent and fewer than 4% of individuals with HPV positivity develop premalignant lesion [5, 6], and even fewer develop invasive cancer [7].

Human zinc ribbon domain containing 1 (ZNRD1), a zinc finger-related protein involved in transcription regulation of human leukocyte antigens (HLA) [8-10], has been reported for governing host immune responses [11] and affecting pathophysiology of virus infection [12]. Studies have shown that ZNRD1 depletion could impair virus replication [13, 14]. In addition, ZNRD1 also implicates in the occurrence and development of cancers by playing a role in the process of DNA damage and repair [15], and suppressing cell proliferation [16, 17].

ZNRD1 is located on chromosome 6p21.3 and cloned from the *HLA* region. In the upstream region of the *ZNRD1*, there is a long non-coding RNA (lncRNA) gene, coding the *ZNRD1* gene

antisense RNA, known as *ZNRD1-AS1*. The long antisense transcripts are able to hybridize to their corresponding spliced mRNAs and result in the formation of dsRNAs that are cleaved by Dicer to endogenous siRNAs (endo-siRNAs). The coding mRNA is thus consumed to generate endo-siRNAs that may direct RNA-induced silencing complex to cleave additional copies of the mRNA transcript, resulting in further down-regulation of the protein-coding gene [18-20]. Therefore, *ZNRD1-AS1* may be involved in the regulating of the *ZNRD1*. By bioinformatics analyses, we identified that several single nucleotide polymorphisms (SNPs) in ZNRD1-AS1 may be expression quantitative trait loci (eQTLs) for ZNRD1 (http://www.regulomedb.org) [21, 22].

Recently, our study found that ZNRD1 regulatory SNPs may be susceptibility makers for risk of both chronic HBV infection and hepatocellular carcinoma [23], which further support that *ZNRD1-AS1* may regulate *ZNRD1* and be involved in both virus infection progress and consequent carcinogenesis. In view of the facts that cervical cancer is another most typical virus infection-related cancer, we hypothesized that *ZNRD1* eQTLs SNPs in *ZNRD1-AS1* that regulate *ZNRD1* expression may also contribute to the risk of cervical cancer occurrence. To test this hypothesis, we performed a population-based case-control study including 1486 cervical cancer cases and 1536 controls to evaluate the association between *ZNRD1* eQTLs SNPs (rs3757328, rs6940552 and rs9261204) in *ZNRD1-AS1* and the risk of cervical cancers.

Materials and methods

Study subjects

The methods were carried out in accordance with the approved guidelines. This study was approved by the ethics committee of Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CICAMS) and Nanjing Medical University. All the cervical cancer cases in the study were recruited between 2006 and 2010. And population-based controls were selected from a population-based cohort of more than 30,000 participants for non-communicable chronic diseases (NCDs) screening conducted in China.

All subjects met the following criteria: (1) Both cases and controls were genetically unrelated Han Chinese women with the history of sexual activity; (2) all the cervical cancer cases in the study were newly diagnosed and histologically confirmed; (3) all the control subjects were no self-reported pre-invasive disease and cancer history and autoimmune disorders such as HIV etc., and frequency matched to the cases by age (± 5 years) and residential areas (urban and rural). The subject was excluded if no signing of informed consent or refused to be interviewed for baseline information with standardized questionnaire for both cases and controls.

Data collection

All study participants donated peripheral blood samples. In-person interview was conducted to both cases and controls to collect demography information and exposure information by using a standardized questionnaire.

SNPs genotyping

Genomic DNA was extracted from a leukocyte pellet by traditional proteinase K digestion and followed by phenol-chloroform extraction and ethanol precipitation. All SNPs were genotyped using the Sequenom MassARRAY iPLEX platform (Sequenom Inc). The information on primers was shown in Table S1.

All the SNPs were genotyped by experienced researchers, blinded to the status of cases and controls. For the purpose of quality control, two blank (water) samples were added in each 384-well plate. 10% repeated samples were randomly selected from both cases and controls for SNP genotyping, which yielded complete consistency. The success rates of genotyping for the three SNPs were all above 97%.

Statistical analysis

Student's t test (for continuous variables) or χ^2 tests (for categorical variables) were used to evaluate differences in the distributions of demographic and exposure characteristics between the cases and controls, as well as SNPs genotypes. Unconditional logistic regression model was used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). Heterogeneity of associations between sub-

Table 1. Association between 3 eQTL SNPs in *ZNRD1-AS1* and cervical cancer susceptibility

Genotype	Cases (n = 1486) N (%)	Controls (n = 1536) N (%)	OR (95% CI)	*P*	OR (95% CI)[a]	*P*[a]
rs3757328						
GG	946 (70.4)	950 (66.2)	1.00		1.00	
GA	363 (27.0)	435 (30.3)	0.85 (0.72-1.00)	0.047	0.83 (0.70-0.99)	0.033
AA	35 (2.6)	51 (3.6)	0.71 (0.46-1.08)	0.111	0.66 (0.42-1.03)	0.068
Dominant			0.83 (0.71-0.97)	0.022	0.81 (0.69-0.96)	0.013
Additive			0.85 (0.74-0.97)	0.015	0.83 (0.72-0.95)	0.008
rs6940552						
GG	872 (64.8)	867 (59.9)	1.00		1.00	
GA	413 (30.7)	495 (34.2)	0.83 (0.71-0.97)	0.017	0.82 (0.70-0.97)	0.021
AA	61 (4.5)	86 (5.9)	0.64 (0.46-0.90)	0.009	0.67 (0.48-0.95)	0.026
Dominant			0.81 (0.70-0.94)	0.006	0.80 (0.69-0.94)	0.005
Additive			0.83 (0.74-0.94)	0.004	0.82 (0.72-0.94)	0.003
rs9261204						
AA	821 (62.4)	819 (57.2)	1.00		1.00	
GA	438 (33.3)	521 (36.4)	0.83 (0.71-0.97)	0.017	0.84 (0.71-0.99)	0.032
GG	57 (4.3)	91 (6.4)	0.64 (0.46-0.90)	0.009	0.60 (0.42-0.85)	0.005
Dominant			0.80 (0.69-0.93)	0.003	0.80 (0.69-0.94)	0.005
Additive			0.82 (0.72-0.92)	0.001	0.81 (0.71-0.92)	0.001

[a]Logistic regression analyses adjusted for age, smoking status, menopausal status, family history of cancer and parity.

groups was assessed by the χ^2-based *Q* test. The Cochran-Armitage test was used for trend analysis. Haploview was employed to analyze linkage disequilibrium (LD) parameters (i.e., D' and r^2). PHASE software (v2.1) was used to estimate the haplotype frequencies based on the observed genotypes. Statistical analyses were conducted using R package and Stata Version 10.0 software (Stata, College Station, TX). $P < 0.05$ was the criterion of statistical significance, and all statistical tests were two-sided.

Results

Total of 1486 cervical cases and 1563 controls were included in the present study. The demographic characteristics of the all cervical cases and controls have been summarized in Table S2. Significantly higher prevalence of premenopausal ($P = 0.002$), smoking ($P < 0.001$), and higher parity ($P = 0.001$) were shown in cases than in controls. However, there were no significant differences in the distribution of age, age at natural menopausal and prevalence of family history of cancer between cases and controls (Table S2).

The genotype distributions of rs3757328, rs6940552 and rs9261204 in cervical cancer patients and control subjects were described in **Table 1**. The observed genotype frequencies for the three SNPs among the controls were all in Hardy-Weinberg equilibrium ($P = 0.798$ for rs3757328, $P = 0.129$ for rs6940552 and $P = 0.532$ for rs9261204). Multiple logistic regression analysis showed that all of these three SNPs (rs3757328, rs6940552 and rs9261204) in *ZNRD1-AS1* were significantly associated with the risk of cervical cancer in both dominant model (rs3757328: adjusted OR = 0.81, 95% CI = 0.69-0.96; rs6940552: adjusted OR = 0.80, 95% CI = 0.69-0.94; rs9261204: adjusted OR = 0.80, 95% CI = 0.69-0.94) and additive genetic model (rs3757328: adjusted OR = 0.83, 95% CI= 0.72-0.95; rs6940552: adjusted OR = 0.82, 95% CI = 0.72-0.94; rs9261204: adjusted OR = 0.81, 95% CI = 0.71-0.92) (**Table 1**).

Then, we evaluated combined effects of variant alleles (3757328-A, rs6940552-A and rs9261204-G) on the risk of cervical cancer. As shown in **Table 2**, risk of cervical cancer was significantly decreased with the increasing number of variant alleles of these three SNPs in a dose-dependent manner (*P* for trend = 0.002). Compared with those carrying "0" vari-

Table 2. Cumulative effects of rs3757328-A, rs6940552-A and rs9261204-G on cervical cancer susceptibility

Variables	Cases (n = 1486) N (%)	Controls (n = 1536) N (%)	OR (95% CI)	*P*	OR (95% CI)[a]	*P*[a]
0	890 (62.6)	866 (57.3)	1.00		1.00	
1-3	468 (32.9)	554 (36.7)	0.82 (0.70-0.96)	0.013	0.83 (0.71-0.98)	0.024
4-6	63 (4.4)	91 (6.0)	0.67 (0.48-0.94)	0.021	0.63 (0.44-0.90)	0.011
Trend			*P*[b] = 0.002		*P*[b] = 0.002	
0	890 (62.6)	866 (57.3)	1.00		1.00	
1-6	531 (37.4)	645 (42.7)	0.80 (0.69-0.93)	0.003	0.80 (0.69-0.94)	0.005

[a]Logistic regression analyses adjusted for age, smoking status, menopausal status, family history of cancer and parity. [b]*P* value of Cochran-Armitage's trend test.

Table 3. Results of haplotype association analysis

Haplotype	Cases N (%)	Controls N (%)	OR (95% CI)[a]	*P*[a]
GGA	2345 (78.9)	2302 (74.9)	1.00	
AAG	473 (15.9)	587 (19.1)	0.78 (0.67-0.89)	< 0.001
Others[b]	154 (5.2)	183 (6.0)	0.81 (0.64-1.03)	0.084

SNPs order: rs3757328, rs6940552 and rs9261204. [a]Logistic regression analyses adjusted for age, smoking status, menopausal status, family history of cancer and parity. [b]Haplotypes with a frequency less than 5% were combined as others.

ant allele, subjects carrying "1-3" variant alleles had a 17% decreased risk of cervical cancer (95% CI = 0.71-0.98) and subjects carrying "4-6" variant alleles had a 37% decreased risk of cervical cancer (95% CI = 0.44-0.90). A total of 20% decreased risk of cervical cancer (95% CI = 0.69-0.94) was found among subjects carrying at least one variant allele compared to the subjects carrying "0" variant alleles (**Table 2**). Subgroup analyses stratified by selected variables, including age, menopausal status, parity, family history of cancer, and histological type, were conducted for the association of combined effects of these three SNPs with cervical cancer risk. Similar associations between these three SNPs (rs3757328, rs6940552 and rs9261204) in *ZNRD1-AS1* and the risk of cervical cancer were shown in each sub-group (all *P* value for heterogeneity test > 0.05) (Table S3).

LD information of these three SNPs was shown in Table S4 and additional insight into these associations by haplotype analysis was provided in **Table 3**. When compared with the most frequent GGA haplotype, the haplotype containing variant alleles of these three SNPs (AAG) increased the risk of cervical cancer significantly (adjusted OR = 0.78, 95% CI = 0.67-0.89) (**Table 3**), which was consistent with the combined analysis.

Discussion

In this study, we investigated the associations between *ZNRD1* eQTLs SNPs in *ZNRD1-AS1* and the risk of cervical cancer. We found that all the three SNPs (rs3757328, rs6940552 and rs9261204) in *ZNRD1-AS1* were related to the susceptibility of cervical cancer. To the best of our knowledge, this is the first study to explore the association between e-QTL SNPs in lncRNA and the risk of cancer.

The major steps known to be necessary for cervical carcinogenesis include HPV infection, persistence of that infection and eventually invasion. Although HPV infection is so common among general population (i.e., 15% among urban and rural Chinese females [24, 25]), the great majority of infected women (more than 90%) resolve the infection spontaneously and the infection persists in only a small fraction of women [26] as a result of cell-mediated immunity [27]. Therefore, host genetic variations related to immune response is essential to cervical cancer development after HPV acquisition.

ZNRD1 is a transcription-associated gene in *HLA* region spanning more than 3.65 kb. The association between genetic variants of the genes in *HLA* region and the risk of cervical cancer showed in our previous study [28] and other researches [29, 30] suggested that the genetic heritability in *HLA* region and the consequent changes of gene expression might

have potential function on virus clearance and cancer occurrence. Recent study revealed that ZNRD1 depletion could impair virus replication and have effect on infection progression [13, 14]. In addition to its effects on virus replication and progression, studies found that ZNRD1 also has effect on carcinogenesis. Up-regulation of ZNRD1 could significantly inhibit the growth of cells, reduce tumor microvessel densities, and inhibit the vascular endothelial growth factor (VEGF) production [17] and play a role in DNA damage and repair [15]. Meanwhile, ZNRD1 mediates the expression of microRNA-214 [17] which has been revealed to play important roles in the pathogenesis of cervical cancer [31]. As an antisense transcript of *ZNRD1*, LncRNA *ZNRD1-AS1* is located in the upstream region of *ZNRD1*. In *ZNRD1-AS1*, several SNPs have been identified as eQTLs SNPs, which were associated with the expression of ZNRD1 [21]. The latest study in our group found that ZNRD1 regulatory SNPs might be susceptibility markers for risk of both chronic HBV infection and hepatocellular carcinoma [23]. Therefore, based on the function of ZNRD1 on virus infection prognosis and carcinogenesis, the genetic variations related to *ZNRD1* expression having effect on the risk of cervical cancer is biologically relevant.

In conclusion, certain alleles of ZNRD1 eQTLs SNPs in lncRNA *ZNRD1-AS1* could have a predisposition for cervical cancer development. More studies with different ethnic background and biological functional researches will help to further understand the role of immune regulation in HPV infection-related cervical cancer carcinogenesis.

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Disclosure of conflict of interest

None.

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Table S1. Information of primers for Sequenom MassARRAY iPLEX

SNP	Primer	Sequence (5'-3')
rs3757328	2nd-PCR Primer	ACGTTGGATGTTGGAGGTGGTGGAACAGAG
	1st-PCR Primer	ACGTTGGATGTTGACTACTGCTAGCCTCAC
	Extend Primer	TTCCTCTCCTTCGACT
rs6940552	2nd-PCR Primer	ACGTTGGATGTGAACAGTGACATCTGTGCC
	1st-PCR Primer	ACGTTGGATGTAATCCCATCCTAGGTGGAG
	Extend Primer	GTCAGGGGCAGTGATTC
rs9261204	2nd-PCR Primer	ACGTTGGATGAAAGCAGGAACATTTCTACG
	1st-PCR Primer	ACGTTGGATGGCTGTTTCTTTACCAGCCAC
	Extend Primer	GGATGAACATTTCTACGACTGTC

Table S2. Demographic and selected variables between cervical cancer cases and controls

Variables	Cases (n = 1486)	Controls (n = 1536) N (%)	*P*
Age, year (mean ± SD)	53.85±12.70	53.21±11.91	0.159
Age at natural menopausal, year (mean ± SD)[a]	49.67±2.98	49.85±3.12	0.234
Menopausal status			0.002
Premenopausal	608 (41.70)	598 (38.93)	
Natural menopause	769 (52.74)	878 (57.16)	
Unnatural menopause	81 (5.56)	60 (3.91)	
Parity			0.001
0~1	620 (42.29)	731 (48.31)	
2	406 (27.69)	405 (26.77)	
> 2	440 (30.01)	377 (24.92)	
Smoking status			< 0.001
Smoker	62 (4.23)	22 (1.43)	
Non-smoker	1404 (95.77)	1514 (98.57)	
Family history of cancer			0.335
Yes	278 (18.98)	313 (20.38)	
No	1187 (81.02)	1223 (79.62)	
Histological types			
Squamous cell carcinoma	1380 (92.87)		
Adenocarcinomas	77 (5.18)		
Adenosquamous carcinoma	26 (1.75)		
Others	3 (0.20)		
Stage			
CIN3	10 (0.7)		
I	366 (24.6)		
II	769 (51.7)		
III	187 (12.6)		
IV	45 (3.0)		
Unclassified	109 (7.3)		

[a]Information was available in 875 cases and 746 controls.

Table S3. Stratified analyses on combined variant alleles with cervical cancer susceptibility

Variables	Cervical cancer susceptibility (0/1-3/4-6)			
	Cases N	Controls N	OR (95% CI)[a]	*P*[b]
Age				
≤ 53	450/240/30	454/314/46	0.81 (0.67-0.97)	0.926
> 53	440/228/33	412/240/45	0.82 (0.69-0.99)	
Menopausal status				
Premenopausal	357/193/23	311/228/30	0.82 (0.67-1.00)	1.000
Postmenopausal	464/244/34	500/307/57	0.82 (0.69-0.97)	
Parity				
0~1	357/208/19	400/279/38	0.84 (0.69-1.02)	0.715
≥ 2	522/253/42	453/265/53	0.80 (0.67-0.95)	
Family history of cancer				
No	709/374/49	691/442/71	0.81 (0.70-0.93)	0.774
Yes	169/87/12	175/112/20	0.85 (0.63-1.14)	
Histological types				
Squamous cell carcinoma	824/438/60	866/554/91	0.82 (0.72-0.94)	0.746
Adenocarcinomas	49/22/3	866/554/91	0.76 (0.49-1.18)	

[a]Logistic regression analyses adjusted for age, smoking status, menopausal status, family history of cancer and parity. [b]*P* value for the heterogeneity test.

Table S4. Linkage disequilibrium (LD) information of ZNRD1-AS1 variations

SNP	rs3757328	rs6940552	rs9261204
rs3757328	--	1.000[a]	0.997[a]
rs6940552	0.791[b]	--	1.000[a]
rs9261204	0.696[b]	0.884[b]	--

[a]D'; [b]R^2.