Association between polymorphisms in TLR4 gene targeted by microRNA-140 and cervical precancerous lesion in south Chinese women: a case control study

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ABSTRACT. Toll-like receptor 4 (TLR4), one of the key immune system effectors, plays a main role in immune recognition of cervical cancer. Micro-RNAs are involved in regulation of multiple important genes in the progression of cervical cancer. A case-control study of 592 people was conducted from Yun'an County, Yunfu City, Guangdong Province, China. Cervical fall off epithelia were collected to detect human papilloma virus (HPV), followed by Thin Prep cytology test (TCT). Moreover, extraction of DNA from peripheral blood were performed for genotyping from the 296 patients and another 296 age-matched healthy control subjects.

Logistic regression was used to determine the risk genotypes for susceptibility to cervical precancerous lesion, and multifactor dimensionality reduction (MDR) was further employed to preliminarily investigate the gene-environment interaction on risk of cervical precancerous lesion. Gene expression of miRNA-140 from serum was done by real-time PCR. We investigated whether target sites of TLR4 gene polytheisms (rs11536896 T>C, rs7873784 G>C) of miR-140 were associated with cervical precancerous lesion risk. The alleles C>A of SNP rs11536896 were significantly different in ASCUS in comparison of case group and control group with HPV infection. The presence of the allele C was associated with a higher risk of developing ASCUS lesion in HPV negative women (OR: 1.75, 95%CI:1.20-2.54, p = 0.003). There was statistically significant difference between the expression of miRNA 140 and the susceptibility to cervical precancerous lesion, in which there is down-regulation of the miRNA-140 in case group (T=6.73, P=0.007). Gene-environment interaction analysis by MDR software revealed an association among rs7873784 and hrHPV infection and more types of infected HPV (p < 0.0001, OR: 25.48; 95%Cl: 5.20-124.84). Collectively, these results suggested that rs11536896 and rs787378 from TLR4 gene were associated with risks of cervical precancerous lesion. Thus, this miRNA-140 and SNPs（ rs11536896rs787378） of TLR4 gene could be considered as a potential molecular mechanism and biomarker for detecting and diagnosing cervical cancer in early time.

KEY WORDS: Cervical precancerous lesion, HPV, TLR4,

miRNA-140

BACKGROUND

Cervical cancer is one of the most common malignancies in women, accounting for an estimated 52,800 new cases and 266,000 deaths globally in 2012(International agency for research on cancer. Globocan 2012) Accumulating evidence have showed that human papilloma virus (HPV) infection, especially high-risk types of HPV (hrHPV) infection, is an necessary cause in over 95% of cases (Walboomers et al. 1999). Most people who have had HPV infection, however, do not develop malignancy, only a part of them would ultimately suffer from cervical cancer (Winer et al. 2003). Therefore, HPV-induced carcinogenesis in the development of cervical cancer is highly complex, with its underlying molecular mechanisms remaining unclarified. It typically takes up to 20-30 years for precancerous lesions (CIN1-3) to further develop to cervical cancer following a hrHPV infection, during which period early diagnosis and intervention can be carried out. Therefore, it is worthwhile exploring and clarifying the problems of cervical precancerous lesions.

However, HPV infection alone is not sufficient to initiate cervical cancer. The transition from HPV infection to cervical cancer is affected by various factors, including harmful lifestyle, related immune response, and genetic susceptibility. While the majority of cervical cancers are caused by HPV infection, other contributing factors, such as immune systems and genetic susceptibility, also play an important role in dictating predisposition to cervical cancer (Wang et al. 2009; Zidi et al. 2015).

Toll-like receptors (TLRs), a family of important pattern recognition receptors in innate immune system, can identify conservative antigen molecules derived from pathogenic microorganisms, such as lipopolysaccharide (LPS), a pathogen nucleic acid (R. Yang et al. 2005). A large number of studies have already shown that HPV infection not only leads to an exception the expression of TLRs receptor，but also promotes its mediated signaling pathways, which is related to molecular interaction in cancer progression(Werner et al. 2012; Zhou, Zhu, & Cheng, 2013). During the process, TLR-4 contributes to recognize HPV16 Virus-like particles (VLPs) via TLR4 - Myd88 pathway to activate the Nuclear factor kB (NF-kB), subsequently inducing CD4+T cell activation, and producing a series of cytokines to eliminate viruses(Mai, Kang, & Pichika, 2013). A significant decrease was observed in the relative gene expression of TLR-4 in patients with cervical cancer (Aggarwal et

al. 2015). Taken together, we cautiously speculate that TLR-4 plays a key role in the occurrence and progression of cervical precancerous lesions.

More and more recent studies have reported an association among genetic susceptibility, non-coding RNAs and the formation, development of many tumors. Of note, microRNAs (miRNAs), a class of short non-coding RNAs, are highly efficient regulators of gene expression via an interaction with the 3′-UTR of their target mRNAs in various cellular processes (Zorc et al. 2012). miRNA-140, a newly discovered miRNA, played a role in osteoarthritis at first (Miyaki et al. 2009), further, miRNA-140 has been reported to be involved in the development of cervical cancer (Jing, Sa, & Xu, 2016; Su et al. 2016).

In addition, single-nucleotide polymorphisms (SNPs) located in the 3'-UTRs of genes may affect interactions with miRNAs, whose association with tumorigenesis is currently a focus of research. One miRNA targets numerous messenger RNAs (mRNAs), and one mRNA may be regulated by more than one miRNA. Therefore, functional variations, such as SNPs located in the 3'-UTRs of cancer-associated genes, may cause differential regulation of target gene expression and simultaneously alter numerous molecular pathways that are associated with tumorigenesis. However, SNPs of the 3'-UTR region of the TLR4 gene have rarely been investigated, which deserves further work. Thus, our study was designed to evaluate the possible association of the SNP in the miRNA-140 targeting site of TLR4 with the development of cervical lesions in women with HPV infection.

MATERIALS AND METHODS

Study population

Two hundred ninety-six sexually active women, with mean age of 42.07±8.45 years old (ranging from 18 to 68 years old), were collected in the study, who were screened in Family Planning Service Stations of Yun'an County, Yunfu City, Guangdong Province, China, between June, 2013 and August, 2014. All enrolled patients presented different grades of cervical intraepithelial neoplasia (ASCUS, LSIL, HSIL), which were confirmed by histological and cytological analysis. The Bethesda System was used for cytological diagnostic criteria and WHO (2014) was utilized for histologic diagnostic criteria.

Two hundred ninety-six age-matched healthy control subjects were healthy women, with mean age 43.10±7.28 years old (ranging from 14 to 70 years old), but without cervical lesions, who came to Family Planning Service Stations for the screening during the same period and of the same region. Those healthy subjects, without no history of lesions or tumors which were evaluated by the physician, were enrolled as controls, and provided written, informed consent.

A total of 592 participants finished a questionnaire, including demographic data (including age), BMI, family history of cancer, gynecological history, passive smoking status (day/week), age at menarche (year), age at first sexual intercourse (year), age at first pregnancy (year), pregnant frequency, abortive frequency, HPV infection, and numbers of infected HPV types. The protocol of this study was approved by the research ethics committee of the Family Planning Specialized Hospital of Guangdong Province and all participants provided with written, informed consent.

HPV Genotyping Text

The DNA of HPV was detected by nested PCR using two rounds of amplification, yielding a 150 bp PCR amplicon that included a conserved sequence of the viral genome. The primers for first round, and second round were MY09/11, and GP 05/06+, respectively. GP06 primers were biotinylated for later PCR enzyme immune assay. Protocol and cycling conditions for DNA amplification were described elsewhere. PCR products were electrophoresed in 2 % agarose gels, stained with SaferGreen™ (Invitrogen) and visualized in blue light by SafeImager™ (Invitrogen).

DNA extraction

Two SNPs, rs11536896 and rs7873784 in the TLR-4 gene were included in our study. Genomic DNA was extracted from EDTA anti-coagulated peripheral blood according to a standard proteinase K digestion and phenol chloroform extraction method (Javadi et al. 2014). Eventually, the extraction of DNA was resolved in Elution Buffer and stored at −20 °C.

RNA extraction and RT-PCR

The blood was mixed with a one-quarter volume of 2% dextran solution (MW 464 000 Da; Sigma-Aldrich, St Louis, MO, USA) and incubated at room temperature for 30 min. Total RNA, including miRNA, was extracted from the purified T cells using the mirVana miRNA isolation kit (Ambion,Austin, TX, USA), according to the manufacturer's protocol. The concentration of RNA was quantified using a NanoDrop Spectrophotometer. All the extracted miRNAs were converted into corresponding cDNAs Briefly, a 10μl reaction mixture containing miRNA-specific stem-loop RT primers (final concentration 2 nM each), 500 μM deoxyribonucleotide triphosphates (dNTPs), 0.5 μl Superscript III (Invitrogen, Carlsbad, CA, USA) and 1 μg total RNA were used for the reverse transcription (RT) reaction.The pulsed RT reaction was performed under the followingconditions: 16°C for 30 min, followed by 50 cycles at 20°C for 30 s, 42°C for 30 s and 50°C for 1s. After RT, the products were diluted 20-fold before further analysis. A real-time PCR-based method was used to quantify the expression levels of miRNAs using a protocol described previously. The prepared RT product (1μl) was used as the PCR template. Each PCR reaction contained 1 × SYBR Master Mix (Applied Biosystems, Foster City, CA, USA), 200 nM miRNA-specific forward primer and 200nM universal reverse primer. All reactions were performed in duplicate on an ABI 7500 fast real-time PCR system (Applied Biosystems). The conditions for quantitative PCR were 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 62°C for 32 s. There primers as follow: hsa-miR-140 F: 5'ACACTCCAGCTGGGCAGTGGTTTTACCCTATG

R: 5'CTCAACTGGTGTCGTGGA, U6 F：5'CTCGCTTCGGCAGCACA R：5'AACGCTTCACGAATTTGCGT.

HPV Typing

Cervical scraping smear was genotyped using the HPV Geno Array test kit (The Beijing Genomics Institute, BGI, Guangdong, China). The presence of various HPV types was amplified with the L! consensus HPV primers MY09/MY11 and HMB01. Then, the flow-through hybridization was performed using PCR product in the manner of a microarray format with a nylon membrane, on which, HPV genotype-specific oligonucleotide probes were immobilized (Yoshikawa et al. 1991). Sixteen HPV types can be detected by the technique, including HPV16, HPV18, HPV35, HPV31, HPV33, HPV51, HPV39, HPV45, HPV58, HPV59, HPV52, HPV56, HPV66, and HPV68 (all HR HPV), as well as HPV6 and HPV11 (both LR HPV) (Venturoli et al. 2002). All these procedures were performed in the clinical standard laboratory of BGI.

SNPs Screening

The information of two SNPs targeted by miRNA was detected from website in combines miR Base sequence database（http://www.ncbi.nlm.nih.gov/pubmed） and PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

Two predictable miRNA target genes of the TLR4 rs11536896, rs787378, were found by software, including miRanda (http://www.microrna.org/microrna/home.do) and Target Scan.

(http:/www.targetscan.org/index.html).The screening SNPs have to meet the following conditions, minimum allele frequency in the Chinese Han nationality to SNP minor allele frequency (MAF) > 0.05, and the SNPs located in the matching area of microRNAs with target genes.

LD analysis

A total of two SNPs across the TLR4 loci were analyzed. The linkage disequilibrium (LD plot) of the SNPs in the TLR4 genes of patients with cervical precancerous lesion was shown in Figure 1. Linkage disequilibrium (LD) analyses gave D' values and r2 values, suggesting that two of the TLR4 SNPs loci were in LD. The haplotype block was based on confidence intervals D'. Each diamond represented the pairwise magnitude of LD, with red indicating strong LD (D' > 0.8) and logarithm of odds score (LOD) ≥ 2.0 (D' = 0.92, LOD = 104.56, r2 = 0.83) (Figure1).

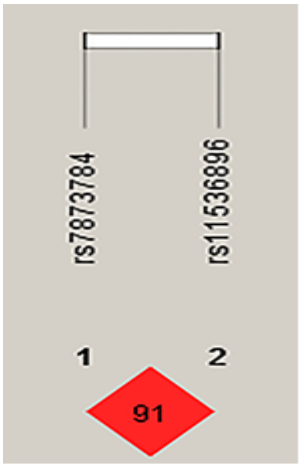

Figure 1. LD analysis of patients with cervical precancerous lesion

Statistical analysis

Initially, the departure of frequencies of two DNAs (rs11536896, rs7873784) polymorphisms from expectation under Hardy-Weinberg equilibrium (HWE) for tested genotype distributions was assessed using chi-square goodness of fit test in controls. Logistic regression was further used to establish the association between the gentic polymorphisms of miRNA target genes site of TLR-4 rs11536896 and rs7873784 and risk of pre-cervical cancer by calculating odds (ORs), 95% confidence intervals (95%Cl), and their corresponding p values. Moreover, gene-environment interaction in risk of cervical precancerous lesion was evaluated by multifactor dimensionality reduction (MDR, V3.0.2). Finally, joint effect of TLR4 gene polymorphisms in the analysis by the number of risk genotypes was evaluated using conditional logistic regression models. Haplotypes of the TLR4 SNPs were assessed using HaploView 4.2 Software (Barrett et al. 2005). A p value<0.05 was considered as statistically significant. All the statistical analyses were performed using the SPSS 17.0.

RESULT

General characteristics of population

There were no significant differences in distributions of age, family history of cancer, Gynecological history, age at first pregnancy, abortive frequency between cases and controls ($p > 0.05$) However, BMI, passive smoking status, age at menarche, age at first sexual intercourse, pregnant frequency, HPV infection and numbers of infected HPV types in case group were significantly different from those in control group($p < 0.05$)(shown in table1). Therefore, these variables were subsequently adjusted for any residual confounding effect in unconditional logistic regression analyses.

Table 1 Demographic characteristics

Characteristics	Case group		Control group		T/X^2	p-value
	No.	%	No.	%		
BMI						
<18.5	41	13.85	18	6.08	10.01	**0.007[a]**
18.5-25	220	74.32	238	80.41		
≥25	35	11.82	40	13.51		
Family history of cancer						
Positive	1	0.34	4	1.35		0.373[b]
Negative	295	99.66	292	98.65		
Gynecological history						
Positive	171	57.77	166	56.08	0.17	0.678[a]
Negative	125	42.23	130	43.92		
Passive smoking status(day/week)						
6-7	69	23.31	43	14.53	9.19	**0.027[a]**
4-5	30	10.14	36	12.16		
1-3	42	14.19	58	19.59		
<1	155	52.36	159	53.72		
Age at menarche (year)						
<16	167	56.42	199	67.23	7.33	**0.007[a]**
≥16	129	43.58	97	32.77		

Age at first sexual intercourse(year)						
<20	18	3.09	36	47.68	6.60	**0.010[a]**
≥20	278	6.17	260	43.05		
Age at first pregnancy(year)						
≤21	76	3.09	70	47.68	0.33	0.567[a]
＞21	220	6.17	226	43.05		
Pregnant frequency						
≤3	222	76.14	244	82.15	4.21	**0.040[a]**
＞3	73	23.86	53	17.85		
Abortive frequency						
Positive	238	40.82	261	44.77	3.26	0.071[a]
Negative	49	8.40	35	6.00		
HPV Infection						
Negative	82	27.70	204	68.92	101.65	**0.000[a]**
Infect low-risk HPV	3	1.01	3	1.02		
Infect high-risk HPV	211	71.28	89	30.07		
Type of infect HPV numbers						
Negative	79	26.69	204	68.92	105.85	**0.000[a]**
1	186	62.84	80	27.03		
＞2	31	10.47	12	4.05		
Age(year)						
Mean ± SD	42.07±8.45		43.10±7.28		1.74	0.082[c]

a: Chi-square test b: Fisher's exact test c: Student's t test

In this study, 296 cases and 296 controls from Southern China population were analyzed for the association between TLR4 polymorphisms and cervical precancerous lesions. The distribution of the two SNPs genotypes in TLR4 and their associations with cervical precancerous lesion risk were shown in Table 2. The genotypic distributions of the two studied SNPs were both in accordance with Hardy–Weinberg equilibrium ($p > 0.05$) in controls. There were no differences in the genotypic distribution of TLR4 rs11536896, rs7873784 between cases and controls. Further, logistic regression analyses were utilized, which failed to reveal any significant difference in any phenotypes and gene models after adjusting the variables, including BMI, passive smoking status, age at menarche, age at first intercourse, pregnant frequency.

Table2. Association between rs11536896 and rs7873784 in TLR4 gene and risk of cervical precancerous lesions

TLR4		**Cases**	**Controls**	**OR (95% CI)**	**P**	**OR (95% CI)[a]**	**P[a]**	**P[b]**
rs11536896								0.148
Codominant model	TT	232	223	1		1		
	TC	57	71	0.77(0.52-1.14)	0.197	0.77(0.52- 1.16)	0.211	
	CC	3	2	1.44(0.24-8.71)	0.690	1.94(0.31-11.98)	0.478	
Dominant model	TT	232	223	1		1		
	TC+CC	60	73	0.79（0.54-1.16）	0.234	0.80(0.54- 1.19）	0.273	
Recessive model	TT+TC	289	294	1		1		
	CC	3	2	1.53（0.25-9.20）	0.645	2.04(0.33-12.63）	0.442	
allele	T	521	517	1				
	C	63	75	1.20（0.84-1.71）	0.316			
rs7873784								0.054
Codominant model	GG	230	223	1		1		
	CG	61	72	0.82(0.56-1.21)	0.320	0.81(0.55- 1.21)	0.304	
	CC	2	1	1.94(0.18-21.54)	0.590	3.16(0.28-35.62)	0.353	
Dominant model	GG	230	223	1		1		
	CG+CC	53	73	0.84（0.57-1.22）	0.363	0.84(0.56- 1.24）	0.376	
Recessive model	GG+CG	291	295	1		1		
	CC	2	1	1.53（0.25-9.20）	0.645	3.30(0.29- 37.21）	0.334	
allele	G	521	618	1				
	C	65	73	0.96（0.67-1.37）	0.819			

a: Adjusted for BMI, passive smoking status, age at menarche, age at first intercourse, pregnant frequency. b： P value for Hardy-Weinberg equilibrium test for control group

Women with positive and negative results of TCT lesions were then stratified according to the status of HPV infection, and TLR4 SNPs were compared between the different subgroups, shown in Table3, Table S2 and Table S3. There were significant differences in the alleles C>A of SNP rs11536896 in ASCUS in comparison of case group and control group with HPV infection, and the presence of the allele C was associated with a higher risk of developing ASCUS lesion in HPV negative women (OR: 1.75, 95%CI:1.20-2.54, p = 0.003). However, no difference was observed in other allele, genotypes between case group and control group.

Table 3. TLR4 SNP Polymorphisms Frequencies in the Control Group and in Patients with ASCUS, LSIL and HSIL

TLR4	Controls		ASCUS		OR(95%CI)	P	LSIL		OR(95%CI)	P	HSIL.		OR(95%CI)	P
	n	%	n	%			n	%			n	%		
rs11536896														
TT	223	75.3	100	77.5	Ref		97	82.2	Ref		35	76.1	Ref	
TC	71	24.0	29	22.5	0.91(0.56-1.49)	0.138	19	16.1	0.62(0.35- 1.08)	0.087	9	19.6	0.81(0.37- 1.76)	0.705
CC	2	0.7	0	0.0	0.99(0.98-1.00)	1.000	2	1.7	2.30(0.32-16.56)	0.589	2	4.3	6.37(0.87-47.71)	0.097
Dominant model	73	100	29	100	0.89(0.54-1.45)	0.628	21	100	0.66(0.39-1.14)	0.132	11	100	0.96(0.46- 1.99)	0.913
Recessive model	294	100	129	100	0.99(0.98-1.00)	1	116	100	0.40(0.06-2.83)	0.322	44	100	0.15(0.02-1.09)	0.089
T	517	87.3	229	79.8	Ref		213	90.3			79	85.9	Ref	
C	75	12.7	58	20.2	**1.75(1.20-2.54)**	**0.003***	23	9.7	0.74(0.45-1.22)	0.240	13	14.1	1.13(0.60- 2.14)	0.697
rs7873784														
GG	223	75.3	97	75.2	Ref		97	80.8	Ref		36	81.8	Ref	
CG	72	24.3	32	24.8	1.02(0.63-1.65)	0.930	21	17.5	0.67(0.39- 1.15)	0.146	8	18.2	0.51(0.23- 1.14)	0.096
CC	1	0.3	0	0.0	1.00(0.99-1.00)	1.000	2	1.7	4.60(0.41-51.31)	0.223	0	0.0	0.99(0.98- 1.00)	1.000
Dominant model	73	100	32	100	0.99（0.62-1.62）	0.98	23	100	1.32（0.82-2.34）	0.228	8	100	1.47（0.46-3.31）	0.346
Recessive model	295	100	129	100	1.00（0.99-1.00）	1.00	118	100	0.20（0.02-2.23）	0.201	44	100	1.00（0.99-1.00）	1.00
G	516	87.5	226	87.6	Ref		215	89.6	Ref		80	90.9	Ref	
C	74	12.5	32	12.4	0.99(0.63-1.54)	0.955	25	10.4	0.81(0.50- 1.31)	0.392	8	9.1	0.70(0.32- 1.50)	0.354

*: Statistically significant P-value.

The expression of miRNA-140 in case and control groups was shown in Figure 2. There was statistically significant difference between miRNA-140 expression and the susceptibility to cervical precancerous lesion, in which there is down-regulation of the miRNA-140 in case group. Meanwhile, a significant correlation was found between miRNA-140 level and heterozygotes genotypes of TLR4 polymorphism (rs11536896, rs7873784) in cervical precancerous lesion group (Table 4). However, there are not differences in rs11536896, rs7873784 mutants.

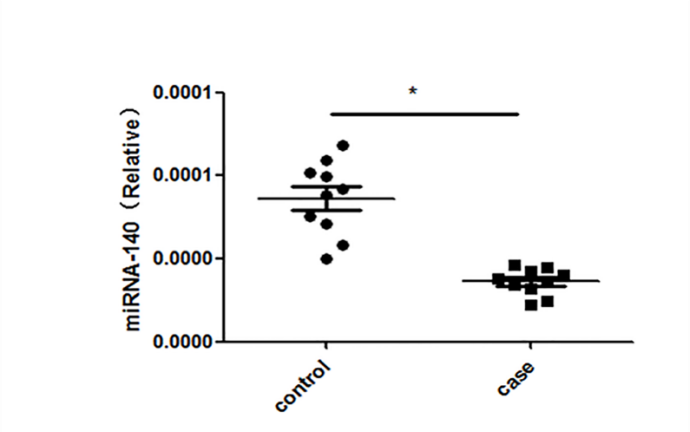

Figure 2. The miRNA-140 expression in cases and controls. Results are expressed as mean ± S.D. *P<0.05.The results were expressed as mean ± SD.

Table 4. Comparisons of relative in microRNA-140 indifferent genotyping

Allele	N	MiRNA-140 relative		P- value
		Mean	SD	
rs11536896				
TT(Wild)	12	0.000073523	0.0000338413	
TC(heterozygotes)	7	0.000044576	0.0000075206	**0.003**
CC (mutant)	1	0.000031814		
rs7873784				
GG(Wild)	11	0.000077129	0.0000329872	
CG(heterozygotes)	7	0.000044576	0.0000075206	**0.008**
CC (mutant)	2	0.000032837	0.0000014481	0.225

Relative in microRNA -140 was significantly associated with both heterozygots genotype

A variety of factors were executed with MDR analysis, including two SNPs of TLR4 (rs11536896, rs7873784), family history of cancer, passive smoking status, age at menarche, age at first sexual intercourse, pregnant frequency, age at first pregnancy, abortive frequency, HPV infection, numbers of infected HPV types, BMI and Gynecological history (Table 5).

Table 5. MDR models of rs11536896, rs7873784 those are association between SNPs and environmental factors

model	Training bal. acc	Testing bal. acc	CV consistency	X^2	P	OR
Infect HPV	0.7128	0.6702	5/10	7.13	<0.001	4.38(1.46- 13.10)
Infect HPV, BMI	0.7219	0.6640	5/10	114.43	<0.001	6.79(4.71- 9.79)
Infect HPV, Types of infect HPV numbers, rs7873784	0.7755	0.7625	9/10	18.39	<0.001	25.48(5.20-124.84)
Infect HPV, Types of infect HPV numbers, BMI, rs7873784	0.8044	0.7522	6/10	15.94	<0.001	14.31(3.84- 53.38)

Consequently, rs7873784, HPV infection and numbers of infected HPV types were the strongest cervical cancer prediction model, with a maximum cross-validation consistency (CVC) of 9/10, relatively higher testing balance accuracy (TBA) of 0.7625, OR of 25.48(95% CI: 5.20-124.84), which suggested that the interaction of rs7873784, HPV infection and numbers of infected HPV types probably increased the risk of cervical cancer.

DISCUSSION

To date, various studies have investigated the association between SNPs in miRNA binding sites and cervical cancer risk. These studies shown that SNP in the 3' UTR of target genes may be significant to establishing the cancer risk of certain individual. However, no studies have yet shown that a link between genetic variations of the 3'UTR of the TLR4 gene to developing cervical precancerous lesions. In this study, we first study to investigate the possible association between the polymorphisms of miRNA-140 binding site of TLR4 gene and the presence of cervical lesions under various environmental factors in females from Yun'an County, Yunfu City, Guangdong Province, China.

In the TLRs family, TLR4 is expressed in some epithelial cells and plays an essential role in the defense against microbes by recognizing structurally conserved bacterial molecules. As reported by Wang et al. TLR4 expression may interact with HPV16 infection in the progression of cervical cancer, while LPS had no effect on cell cycle distribution (Y. Wang et al. 2014). Sabrina Zidi et al. reported TLR4 increased the risk of cervical neoplasm progression to advanced stages of cervical cancer when compared to the early stages (I+ II) (Zidi et al. 2015).

To the best of our knowledge, miR-140 was recognized as a tumor suppressor to regulating growth and metastasis of hepatocellular carcinoma by controlling NF-κB activity (H. Yang et al. 2013). miRNA-140 has been found to be down-regulated in multiple types of cancer, and suppresses cancer by targeting several important oncogenes like SOX2, DNMT1, TGFBR1 and FGF9(Su et al. 2016). Meanwhile, we predicted that the TLR4 gene exists miRNA-140 target regulation site by bioinformatics methods. Thus, we aimed to study the expression of miRNA-140 and its target gene TLR4 polymorphism (rs11536896, rs7873784) in pre-cancer

patients in comparison to control subjects and to correlate these results with clinical data of patient group to explain their role in pathogenesis.

In our study, the alleles C in rs11536896 in ASCUS were significantly different in case group and control group with HPV infection, while the presence of the allele C was associated with a higher risk of developing ASCUS lesion in HPV negative women (OR: 1.75, 95%CI:1.20-2.54,P = 0.003). Increasing evidence has suggested that SNPs in the miRNA target gene sites may disturb or obstruct miRNAs function, subsequently affecting regulation of target genes expression, which is likely to involve in susceptibility to certain diseases, including cervical cancer and precancerous lesion (Xiong et al. 2011). In the present results, the minor C allele in TLR4- rs11536896 enhance the risk of cervical precancerous lesions. Considering SNPs located in the miRNA seed‑match region, affect the binding affinity of miRNA-140 to target mRNAs, causing differential regulation of target gene expression and altering numerous molecular pathways. The minor C allele may lead to changes in the TLR4 expression. The change of expression in TLR4 have been reported by a variety of studies (He et al. 2017; Jiang et al. 2017),, which may participate in carcinogenesis and tumor progression during chronic inflammation by shaping the tumor microenvironment. The mechanism requires further investigation in functional studies.

We found that there was a statistically significant association between miRNA-140 and the susceptibility to cervical precancerous lesion, that there is down-regulation of the miRNA-140 in case group. Our findings are consist with the study fromYang, H. et al. (Yang et al. 2013), in which miRNA-140 expression is frequently down-regulated in cervical cancer tissue and plasma samples compared to the non-tumor counterparts. In our study, miRNA-140 might reduce TLR4 activity by SNPs site. Another study revealed that miRNA-140 reduced cell migration and invasion of hypopharyngeal carcinoma cell by targeting down regulating ADAM10 expression (Jing et al. 2016).

Further, the two SNPs were statistically significant in two groups before we adjusted high-risk environmental factors. In recent report, TLR4 may serve as a candidate HPV-induced oncogene in cervical cancer and HR-HPVs may gain an advantage in inducing carcinogenesis by reshaping the environment of the cellular miRNA composition and target gene expression to benefit the virus (Jin et al. 2017). Therefore, the present study hypothesized that there is an interaction between HPV and each SNPs in the TLR4 gene, which is associated with the risk of cervical precancerous lesions. Studies showed that many environmental factors consumption were the risk in population, and we found environmental risk factors, which were not consistent to previous reports (Chang et al. 2014; L. D. Wang et al. 2014; White & Wong, 2015). This might be interpreted by the fact that gene susceptibility was variable in different ethnics. Hence, MDR model was used to analyze interaction between rs11536896, rs7873784 and environmental factors in the study, which indicated an interaction between HPV infection, numbers of infected HPV types and rs7873784. From the results, we concluded that the individuals with HPV infection, numbers of infected HPV types and rs7873784 were at high risk for developing cervical precancerous lesions, who deserve more attention in clinical practice.

Despite certain claims that the association between the SNPs of TLR4 gene related sites (including rs11536896 and rs7873784) and cancer development demands in-depth research, no other loci have been found to have significant correlations with cancer (Kutikhin, 2011; Weng et al. 2014). To the best our knowledge, it was the first study to investigate the association between two SNPs of TLR4 gene targeted by miR-140 and gene-environment interaction with cervical precancerous lesions risk. Cervical carcinoma is a complex disease, characterized by an intricate interplay of both genetic polymorphism and environmental factors (Yin et al. 2015). MDR analysis indicated that gene-environment interactions were likely to exist between rs7873784 and HPV infection, numbers of infected HPV types affecting the risk of cervical precancerous lesion, with synergistic effects on risk of cervical precancerous lesion. When it comes to the underlying mechanism, rs787384 may probably not only increase the risk of cervical precancerous lesion, but also enhance susceptibility of infecting high-risk HPV and more types of HPV. However, interactions between rs11536896 and other environmental factors were not found. Further, environmental factors and SNPs were pooled into two groups (high- and low-risk group) in MDR models, falling off the multifactor criterion predictors from impactive n dimensions to one dimension (Choudhury & Ghosh, 2015), which was a new method to investigate potential interactions of gene-environment models in human genetics and biological studies. The model overwhelms the others by reducing change of type 1 error better than traditional account methods in the multiple testing (Milne et al. 2008; Motsinger-Reif et al. 2008; Xu et al. 2014). The confounding bias from age was excepted in the study, because the groups of case and control were 1:1 matched by age ($p > 0.05$). According to Hardy-Weinberg equilibrium, result distribution of the genotype in the control groups as well as the selection bias were further avoided, which altogether made the findings in our study persuasive.

Nevertheless, there are certain limitations in the work. Firstly, miR-140 as a targeted microRNA with rs11536896, rs7873784 has not been proven by a religious experiment design or other research support.

Anyhow, it provides a good idea to test miR-140 as a new function in cervical neoplasm progression. Secondly, the sample size is relatively small to find a convincing association between SNPs and cervical precancerous lesion, which influences the reliability of the results. For example, there were only three participants in case group and two participants in control group, who harboured two SNPs CC (T to C and G to C). Finally, certain problems similar to other retrospective studies are not avoided in the study. Therefore, cohort study with a larger sample size are warranted, more data from laboratory should be collected to further explore the mechanisms of TLR4 in the cervical lesion.

CONCLUSION

The alleles C>A of SNP rs11536896 were significantly different in ASCUS in comparison of case group and control group with HPV infection. The presence of the allele C was associated with a higher risk of developing ASCUS lesion in HPV negative women (OR: 1.75, 95%CI: 1.20-2.54, P=0.003). there was statistically significant difference between miRNA-140 expression and the susceptibility to cervical precancerous lesion, in which there is down-regulation of the miRNA-140 in case group (T=6.73, P=0.007). Further, miRNA-140 may affect pre-cervical cancer development by binding affinity of miRNAs to target mRNAs, causing differential regulation of target gene expression and altering numerous molecular pathways. MDR analysis demonstrated an interaction between genetic variation rs7873784 in TLR4 gene and HPV infection, numbers of infected HPV types, which might be due to the increased risk of cervical precancerous lesion as well as the enhanced susceptibility of infection with high risk HPV and more types of HPV by rs787384. In conclusion, this miRNA-140 and it is targets SNPs (rs11536896, rs7873784) of TLR4 gene could be a potential molecular mechanism and biomarker.

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