

Genetic Variants in microRNA Target Sites of 37 Selected Cancer-Related Genes and the Risk of Cervical Cancer

Yang Mi¹, Lijuan Wang^{2*}, Lu Zong², Meili Pei², Qingyang Lu³, Pu Huang^{2*}

1 Obstetrical department, Maternal and Child Health Hospital of Shaanxi Province, Xi'an, ShaanXi, Peoples' Republic of China, **2** Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xi'an Jiaotong University College of Medicine, Xi'an, ShaanXi, Peoples' Republic of China, **3** Department of Pathology, Liaocheng People's Hospital, Liaocheng, ShanDong, Peoples' Republic of China

Abstract

Objectives: Single nucleotide polymorphisms (SNPs) in putative microRNA binding sites (miRSNPs) modulate cancer susceptibility via affecting miRNA binding. Here, we sought to investigate the association between miRSNPs and cervical cancer risk.

Methods: We first genotyped 41 miRSNPs of 37 cancer-related genes in 338 patients and 334 controls (Study 1), and replicated the significant associations in 502 patients and 600 controls (Study 2). We tested the effects of miRSNPs on microRNA-mRNA interaction by luciferase reporter assay.

Results: Five SNPs displayed notable association with cervical cancer risk in Study 1. Only *IL-16* rs1131445 maintained a significant association with cervical cancer (CT/CC vs. TT, adjusted OR = 1.51, $P = 0.001$) in Study 2. This association was more evident in the combined data of two studies (adjusted OR = 1.49, $P = 0.00007$). We also found that miR-135b mimics interacted with *IL-16* 3'-UTR to reduce gene expression and that the rs1131445 T to C substitution within the putative binding site impaired the interaction of miR-135b with *IL-16* 3'-UTR. An ELISA indicated that the serum IL-16 of patients with cervical cancer was elevated (vs. controls, $P = 0.001$) and correlated with the rs1131445 genotype. Patients who carried the rs1131445 C allele had higher serum IL-16 than non-carriers ($P < 0.001$).

Conclusions: These results support our hypothesis that miRSNPs constitute a susceptibility factor for cervical cancers. rs1131445 affects *IL-16* expression by interfering with the suppressive function of miR135b and this variant is significantly associated with cervical cancer risk.

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* E-mail: ljwangdr@gmail.com (L.J.W); pu_huang_xjtu@163.com (P.H)

Introduction

Cervical cancer, the third most common cancer in women [1], is mainly caused by human papillomavirus (HPV) infection [2]. Although HPV infections are prevalent in sexually active women, only a minority of infections persist and develop into cervical intraepithelial neoplasia grade 2/3 (CIN 2/3) or even cervical cancer [3]. Several cofactors affect the transition from initial HPV infection to cervical cancer, including life-style, host immune response, and genetic susceptibility [4,5]. The important roles of genetic factors in cervical cancer pathogenesis are prompted by the findings that cervical cancer exhibits a significant familial clustering and the first degree relatives of patients have double risk of developing cancer [6]. A few risk-modulating variants for cervical cancer have been identified by a candidate gene association study. The genes that are associated with cervical cancer susceptibility are involved in DNA repair, cellular cycle and apoptosis, cell proliferation and differentiation, the human leukocyte antigen system and immune responses [7,8].

Recent compelling evidence indicates that polymorphisms in microRNA (miRNA) binding sites of cancer-related genes are an important source that harbors the causative genetic variants of cancer [9]. miRNA, often 19–25 nucleotide-length noncoding RNA, binds within the 3' untranslated region (UTR) of transcriptions of targeted genes to inhibit and even abolish its translation to protein. It is estimated that approximately 30% of human genes are regulated by miRNAs [10]. Gene deregulation is one of the key mechanisms by which cells can progress to cancer [11]. miRNA can participate in the carcinogenesis via post-transcriptional regulation of many cancer-related genes, including oncogenes and tumor suppressor genes. The aberrant expression of miRNAs contributes to the etiology of multiple cancers, including cervical cancer [12]. miRNA profile analysis of cervical cancer revealed that miRs-9, 21, 135b, 146a, 199a, 203 and 205 are frequently overexpressed in cancer tissues or cell lines [12]. Moreover, miR-205 acts as an oncogene to promote cell proliferation and migration of human cervical cancer cells [13]. In contrast, miR-214 directly targets the GALNT7 gene to

Table 1. The characteristics of patients with cervical cancer and cancer-free controls.

Characteristics	Study 1		<i>P</i> ^b	Study 2		<i>P</i>	All subjects		<i>P</i>
	Controls (n = 334)	Cases (n = 338)		Controls (n = 600)	Cases (n = 502)		Controls (n = 934)	Cases (n = 840)	
Age^a, year (Mean ± SD)	47.2 ± 10.8	46.5 ± 8.7	0.355	48.4 ± 11.2	47.5 ± 9.5	0.262	48.0 ± 11.1	47.1 ± 9.2	0.065
Race, n (%)									
Han Chinese	290 (86.8)	285 (84.3)	0.355	538 (89.7)	453 (90.2)	0.753	828 (88.7)	738 (87.9)	0.604
Hui Chinese	44 (13.2)	53 (15.7)		62 (10.3)	49 (9.8)		106 (11.3)	102 (12.1)	
Smoking									
Never	305 (91.3)	313 (92.6)	0.540	562 (93.7)	448 (89.2)	0.008	867 (92.8)	761 (90.6)	0.088
Ever	29 (8.7)	25 (7.4)		38 (6.3)	54 (10.8)		67 (7.2)	79 (9.4)	
Education									
JHS and below	189 (56.6)	201 (59.5)	0.550	314 (52.3)	282 (56.2)	0.717	503 (53.9)	483 (57.5)	0.488
SHS and above	145 (43.4)	137 (40.5)		286 (47.7)	220 (43.8)		431 (46.1)	357 (42.5)	
Age at primiparity									
≤24 years	206 (63.6)	219 (66.6)	0.424	390 (67.0)	365 (73.1)	0.031	596 (65.8)	575 (70.5)	0.038
>24 years	118 (36.4)	110 (33.4)		192 (33.0)	131 (26.9)		310 (34.2)	241 (29.5)	
Missing or nulliparous	10	9		18	15		28	24	
Marital status									
Never married	6 (1.9)	4 (1.2)	0.195	15 (2.7)	11 (2.3)	0.145	21 (2.4)	15 (1.8)	0.035
Married only once	259 (83.5)	264 (79.3)		453 (80.3)	370 (76.0)		712 (81.5)	634 (77.4)	
≥2 times	45 (14.5)	65 (19.5)		96 (17.0)	106 (21.7)		141 (16.1)	171 (20.9)	
Missing	24	5		36	15		60	20	
HPV infection status									
HPV +	106 (31.7)	296 (87.6)	<0.001	229 (38.2)	420 (83.7)	<0.001	335 (35.9)	716 (85.2)	<0.001
HPV -	228 (68.3)	42 (12.4)		371 (61.8)	82 (16.3)		599 (64.1)	124 (14.8)	
Clinical stage^c									
I		247 (73.1)			365 (72.7)			612 (72.9)	
II		69 (20.4)			118 (23.5)			187 (22.3)	
II I/IV		22 (6.5)			19 (3.8)			41 (4.9)	
Histology									
SC		280 (82.8)			398 (79.3)			678 (80.7)	
Adenocarcinoma		44 (13)			75 (14.9)			119 (14.2)	
Other		14 (4.1)			29 (5.8)			43 (5.1)	

^aAge of diagnosis for cases and age when they participated in this study for controls.

^bStatistically significant results with an FPRP value <0.2 are marked in bold.

^cBased on International Federation of Gynecology and Obstetrics classification system.

Abbreviation: SD, standard deviation; JHS, junior high school; SHS, senior high school; SC: Squamous carcinoma.

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suppress growth and invasiveness of cervical cancer cells by functioning as a tumor suppressor a tumor suppressor [14]. The base pair change within the miRNA-target region of cancer-related genes could destroy or create a binding site or alter the binding affinity and post-transcriptional control of mRNA by miRNA and, therefore, could affect the transformation from normal cells to cancer cells [9].

Bioinformatics analysis has identified many SNPs that are located in the putative miRNA binding sites of cancer candidate genes and that significantly affect the binding energy of putative miRNA:mRNA duplexes [15]. A small fraction of them have been further proved to alter the expression of target genes by the luciferase reporter assay and display a significant association with susceptibility to cancer [16,17]. Landi et al. screened out 57 SNPs within miRNA-binding sites from 104 candidate genes for

colorectal cancer, found eight SNPs of them showing various Gibbs free energy of binding between the two alleles of each SNP, and finally demonstrated a SNP, rs17281995, within the miRNA-binding sites of CD86, as the risk-modulating variant for colorectal cancer [16]. Using a similar research strategy, SNPs within miRNA binding sites of other important cancer candidate genes, such as DNA repair genes [17], integrin genes [18] and caspase genes [19], have also been identified and further evaluated as susceptibility variants for bladder cancer, breast cancer, colorectal cancer, and head and neck cancer risk. Recently, Shi et al. demonstrated that the rs11064 G allele within the *TNFAIP8* gene weakens the binding affinity of miR-22 to the *TNFAIP8* 3'-UTR and is associated with an increased risk of cervical cancer [20], which suggests that miRNA-binding sites are also the hot spots that harbor cervical cancer susceptibility variants.

In the present study, we sought to identify new risk variants for cervical cancer among miRNA-target sites in cancer-related genes. By reviewing the literature and bioinformatics databases, we found 37 cancer-related genes with variations in putative 3'-UTR miRNA binding sites. The association strength of these variants with cervical cancer was assessed by a two-stage case-control study. For the associated variant, its functional influence on miRNA-mediated expression regulation of the target genes was investigated using the luciferase reporter assay.

Materials and Methods

1 Study population

We recruited 840 patients with cervical cancer from the patients who received inpatient treatment in three hospitals between May 2008 and April 2012. Among them, 338 subjects from Liaocheng People's Hospital (LCPH) were used in first stage study (Study 1), which investigated all of the selected SNPs, and 502 subjects from The First Affiliated Hospital of Xi'an Jiaotong University (TFAHXJTU) and Maternal and Child Health Hospital of Shaanxi Province (MCHHSP) were used in second stage study (Study 2), which was to replicate the noteworthy findings ($P < 0.05$) from Study 1. All of the patients were diagnosed by histological examination of biopsy under colposcope and/or resected tissues. The clinical stage and histological type of cervical cancer of patients were collected from medical records and are shown in Table 1. A total of 934 healthy controls were recruited from the women who routinely attended physical examination in these three hospitals, 334 subjects from LCPH in Study 1 and 600 subjects from TFAHXJTU and MCHHSP in Study 2. All of the controls had no history of neoplastic or cancer-associated illness, and they had normal cervical cytology in at least two consecutive annual examinations. All of the subjects were genetically unrelated, and their age, race, smoking status, socio-economic status, and marital status were also investigated by reading medical records or by interviews. This study was approved by the Medical Ethics Committees of LCPH, TFAHXJTU, and MCHHSP and all of the participants gave informed written consent.

2 Polymorphism selection and genotyping

Previous studies have systematically screened the SNPs in miRNA binding sites of many cancer-related genes. Briefly, they first screened cancer-related genes by database and literature searches, and then, they identified the putative miRNA target sites in these genes by specialized algorithms (such as miRBase, miRanda, PicTar and TargetScan) and selected the SNPs that reside on the miRNA-binding sites by dbSNP search and BLAST, finally identifying the SNPs whose alleles make the target sequence have significantly differential binding energy to miRNA in a computer predictive model [16,18]. Because many cancer-related genes are shared by multiple cancers [21], we selected the SNPs from the previous literature that covers the topics about miRNA-binding site/miRNA target sites, polymorphisms and cancer/tumor. The MedLine/PubMed database was searched to find literature that was published from 2000 January to 2013 January. We lastly selected 41 SNPs in predicted miRNA-binding sites within 37 cancer-related genes with minor allele frequencies greater than 0.1 in the Chinese Han population (Table 2). Genomic DNA was extracted from peripheral blood leukocytes using the Blood DNA Midi Kit (Omega Biotech, Norcross, USA), according to the manufacturer's protocol. The selected SNPs were genotyped in patients and controls using MALDI-TOF within the MassARRAY system (Sequenom Inc., San Diego, CA, USA) [22].

3 HPV genotyping

Human HPV from cervical scraping smear and/or resected tissues was genotyped using the HPV GenoArray test kit (HybriBio Ltd, Guangdong, China). First, HPV DNA was amplified with the L1 consensus HPV primers MY09/MY11 and HMB01. Then, the PCR product processed flow-through hybridization in the manner of a macroarray format with a nylon membrane onto which HPV genotype-specific oligonucleotide probes were immobilized. This technique can identify 21 HPV genotypes, including 5 low-risk types (6, 11, 42, 43, and 44), 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and 2 intermediate-risk types (CP8304 and 53).

4 Serum IL-16 levels

The patients' and controls' blood samples were collected between 8 and 10 a.m. The sera were centrifuged at 3000 rpm for 10 min and frozen at -80°C . Serum IL-16 levels were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (PIERCE Endogen) according to the manufacturer's instructions. The minimum level of detection for IL-16 was 0.10 pg/ml. No cross-detection of other cytokines was observed. The intra-assay variation was $<10\%$.

5 Cell culture

Human cervical cancer cell lines, HeLa [23] and SiHa [24], were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco modified Eagle medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum and were grown at 37°C in a humidified 5% carbon dioxide incubator.

6 Transient transfections and luciferase assays

The 3'-UTR of IL-6 mRNA, including the C allele or T allele of rs1131445, were amplified using the primers of 5'-TGGCCTGGGCCTCCTCACAA-3' (forward) and 5'-CTCCACCACCCTTCCCTA-3' (reverse). The amplified fragments were then cloned into the downstream of the firefly luciferase gene in the pGL3-basic vector (Promega, Madison, USA). These recombination plasmids were sequenced to confirm their accuracy. For the luciferase reporter assays, HeLa and SiHa was plated in 24-well dishes to be co-transfected with 100 ng of pGL3-C or pGL3-T constructs, 50 pmol/ μL of chemically synthesized miRNAs miR-135b (GenePharma, Shanghai, China) or negative control miRNA using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA). In each transfection, 20 ng of pRL-TK plasmid (Promega, Madison, USA) was used to correct for transfection efficiency. Each transfection was conducted in triplicate. Cells were collected 48 hours after transfection, and luciferase activity was measured with a dual-Luciferase reporter assay system (Promega, Madison, USA) and normalized against the activity of the Renilla luciferase gene.

7. Statistical analysis

We calculated the statistical power as previously described [25]. A χ^2 test was performed to make a comparison of the categorical variables, including the genotype frequency and some demographic characteristics. Student's t-test, Mann-Whitney U-test or analysis of variance were conducted to compare the continuous variables, such as the age, serum IL-16 level and reporter expression level, when appropriate. The associations between the IL-16 variants and the cervical cancer risk were estimated by computing the ORs and their 95% CIs from multivariate logistic

Table 2. The candidate cancer-related genes, the selected SNPs, the miRNAs whose binding to target mRNAs could be altered by the corresponding base substitution, and the frequency distributions of selected SNPs in the cases and controls in Study 1.

Gene	Function involved	dbSNP ID	Chromosome/ Position	The binding miRNA ^a	Homozygous major allele (controls/ cases)	Heterozygote (controls/cases)	Homozygous major allele (controls/cases)	Chi-square value	P value ^b
REV3L	DNA metabolism and repair	rs465646 A/G	6/111620758	miR-25	225/253	98/80	10/3	7.216	0.027
RYR3	Regulation of cytosolic calcium	rs1044129 A/G	15/34158266	miR-367	116/110	173/188	44/39	1.06	0.589
BIRC5	Regulation of apoptosis	rs2239680 T/C	17/76219783	miR-335	206/183	116/127	10/25	8.27	0.016
NBS1	DNA repair	rs2735383 G/C	8/90947269	miR-629	109/115	150/162	74/60	2.061	0.357
RAP1A	Oncogene	rs6573 C/A	1/112253389	miR-196a	196/205	118/113	18/20	0.362	0.835
FGF2	Mitosis, angiogenesis and tumor growth	rs1476215 T/A	4/123818271	miR-196a	277/259	50/56	5/23	12.463	0.002
TGFA	Cell proliferation and differentiation	rs473698 G/C	2/70411736	miR-495	165/165	130/141	39/32	1.113	0.573
DGCR8	microRNAs biogenesis	rs3757 G/A	22/20099331	miR-523	222/230	100/99	10/9	0.146	0.93
ITGA6	Cell proliferation, migration and survival	rs17664 G/A	2/173369231	miR-152	195/213	112/108	27/16	3.667	0.16
ITGB3	Cell proliferation, migration and survival	rs3809865 A/T	17/45388586	miR-26b	201/213	110/115	23/10	5.557	0.062
ITGB5	Cell proliferation, migration and survival	rs2675 A/C	3/124482113	miR-504	233/246	94/83	5/7	1.346	0.51
LIG3	DNA Repair	rs4796030A/C	17/33330150	miR-612	123/127	156/172	55/39	3.544	0.17
BRCA1	DNA Repair	rs12516 C/T	17/41196408	miR-874	159/153	133/153	42/32	2.842	0.242
BRCA1	DNA Repair	rs8176318 C/T	17/41197274	miR-328	138/162	151/142	45/34	3.704	0.157
XPC	DNA repair	rs2229090 C/G	3/14187345	miR-1225-3p	167/173	135/134	32/31	0.102	0.95
RPA1	DNA repair	rs9914073 A/G	17/1801592	miR-548c-3p	135/147	157/158	42/33	1.57	0.456
RPA2	DNA repair	rs7356 A/G	1/28218100	miR-3149	87/96	178/170	68/72	0.704	0.703
GTF2H1	DNA repair	rs4596 G/C	11/18388128	miR-518a-5p	101/88	180/191	53/59	1.518	0.468
ERCC4	DNA Repair	rs4781563 G/A	16/14045399	miR-2355-3p	195/206	122/108	17/21	1.573	0.455
CASP3	Apoptosis	rs1049216 C/T	4/185550089	miR-181	187/193	131/131	16/14	0.204	0.903
CASP6	Apoptosis	rs1042891 C/T	4/110610086	miR-944	96/105	155/151	83/82	0.438	0.804
CASP7	Apoptosis	rs1127687 G/A	10/115490109	miR-136	191/189	120/125	23/24	0.11	0.946
CASP7	Apoptosis	rs10787498 T/G	10/115489650	miR-19a	163/179	149/144	20/15	1.495	0.474
CASP7	Apoptosis	rs12247479 G/A	10/115490060	miR-345	258/271	69/61	7/6	0.865	0.649
CASP7	Apoptosis	rs4353229 T/C	10/115489589	miR-224	113/111	175/183	46/44	0.217	0.897
E2F1	Cell proliferation and apoptosis	rs3213180 G/C	20/32263624	miR-1182	178/154	128/135	26/49	8.922	0.012
IL16	Immune response/inflammation	rs1131445 T/C	15/81601782	miR-135a	126/100	169/180	39/58	7.036	0.030
PTGER4	Immune response/inflammation	rs16870224 G/A	5/40692940	miR-9,	243/234	83/86	7/17	4.366	0.113
NOD2	Immune response/inflammation	rs31355000 G/A	16/50766886	miR-158	164/178	129/132	41/28	3.033	0.219
PLA2G2A	Phospholipid metabolism	rs11677C/T	1/20301964	miR-187	213/196	109/121	12/20	3.319	0.19
ALOX15	Linoleic acid metabolism	rs916055 T/T	17/4138208	miR-588	130/122	155/151	49/65	2.528	0.283
IL-23 R	Immune response/inflammation	rs10889677 A/C	1/67725120	Let-7e	189/180	131/141	14/17	0.854	0.653
TNFAIP2	Apoptosis	rs8126 T/C	14/103603569	miR-184	144/147	138/158	52/33	5.606	0.061

Table 2. Cont.

Gene	Function involved	dbSNP ID	Chromosome/ Position	The binding miRNA ^a	Homozygous major allele (controls/ cases)	Heterozygote (controls/cases)	Homozygous major allele (controls/cases)	Chi-square value	P value ^b
MTMR3	Apoptosis	rs12537 C/T	22/30423460	miR-181a	224/233	94/93	16/12	0.73	0.694
HOBX5	Cellular morphogenesis and differentiation	rs9299 A/G	17/46669430	miR-7	92/101	174/188	68/49	4.023	0.134
TYMS	DNA replication and repair	rs699517 T/C	18/673016	miR-498	163/155	130/153	41/30	3.751	0.153
EFNA1	Tumor development and maintenance	rs12904 A/G	1/155106697	miR-200c-3p	210/218	115/106	9/14	1.579	0.454
MYCL1	A highly plausible oncogene	rs3134615 C/T	1/40362066	miR-1827	271/283	57/52	6/3	1.466	0.481
TGFBR1	Cell proliferation, migration and apoptosis	rs334348 A/G	9/101912471	miR-628-5p	104/91	152/143	78/104	4.832	0.089
TP53BP2	Apoptosis	rs17739 C/T	1/223967953	miR-129-5p	182/161	127/147	25/30	3.176	0.204
TP53INP1	A tumor suppressor	rs7760 T/G	8/95938422	miR-330-5p	241/254	81/74	12/10	0.816	0.665

^aThe binding miRNAs that are supported by functional experiment, such as the luciferase reporter assay, are in boldface.

^bP value was calculated using a χ^2 test with 2 d.f. $P \leq 0.05$ are in boldface.

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regression analyses with an adjustment for the age, body mass index, smoking status and family history of cancer. To avoid a spurious association caused by multiple tests, the false positive report probability (FPRP) was calculated according to the method described by Wacholder et al. [26]. We set 0.2 as an FPRP threshold and assigned a prior probability of 0.01 to detect an OR of 1.50 (for the risk effects) or 0.67 (for the protective effects) for an association with the SNPs or clinico-demographic features. The Hardy-Weinberg equilibrium (HWE) of the SNPs among the controls was evaluated by the χ^2 test. All of the statistical analyses were performed by SPSS (version 13.0, Chicago, IL, USA). A value of $P < 0.05$ was considered to be significant (two-tailed).

Results

1 Population characteristics

We analyzed altogether 840 patients with cervical cancer and 934 controls in Studies 1 and 2. The demographic and clinical characteristics of patients and controls are presented in Table 1. There was no significant difference in the distribution of the age, race, smoking status, educational level, and marital status between the controls and the cases in Study 1, Study 2 and all of the subjects combined. However, the cases were more likely to be younger at age at first delivery ($P = 0.038$) and with HPV infection ($P < 0.001$) than the controls. HPV infection still remained significantly associated with cervical cancer after considering the FPRP that was less than 0.2. These two variables were further adjusted for any residual confounding effect in later multivariate logistic regression analyses.

2 Association of SNPs within miRNA-binding sites of cancer-related genes with cervical cancer risk

More than 99% of the samples were genotyped successfully for each SNP, and the replicate experiment for the randomly selected 300 samples acquired completely consistent genotype data with the original analysis. Table 2 shows the list of selected SNPs within the miRNA-binding sites in the 3'-UTR of 37 cancer-related genes, the binding miRNA that they potentially affect, and their genotype frequencies and associations with cervical cancer in Study 1. All of the observed genotype distributions among the controls of Study 1 were in line with the HWE. Among the 41 SNPs that were included in Study 1, five SNPs (rs465646 in *REV3L* gene, rs2239680 in *BIRC5* gene, rs1476215 in *FGF2* gene, rs3213180 in *E2F1* gene, rs1131445 in *IL-16* gene) exhibited a markedly different genotype distribution between the cases with cervical cancer and the controls ($P = 0.003 \sim 0.030$). To determine whether true significant associations existed or whether there were spurious results, we performed a replicate to analyze these SNPs in another population that resides in Shaan Xi province of Northern China. In Study 2, their observed genotype frequencies were also consistent with HWE (Table 3). Moreover, we found that only *IL-16* rs1131445 continued to show a significant association with cervical cancer. The subjects with its TC or CC allele had an increased risk for cervical cancer (adjusted OR = 1.51, 95% CI 1.18–1.93, $P = 0.001$) with an adjustment for age, smoking status, educational level, age at primiparity, marital status, and HPV infection status. The association between rs1131445 and cervical cancer was more significant in data that combined Study 1 and 2 (adjusted OR = 1.49, 95% CI 1.22–1.81, $P = 0.00007$) with adjustment for the aforementioned variables. We then calculated the FPRP values for all of the observed significant associations. When the assumption of prior probability was 0.01, the association with the *IL-16* rs1131445 (TC/CC vs.TT) was still noteworthy in both Study 1 subjects (FPRP = 0.171) and all subjects

Table 3. Association of the selected SNPs from the Study 1 and an additional SNP in *IL-16* with the risk for cervical cancer in the Study 2.

Variant	Genotype	Study 2				Combined Study 1 and 2				
		Controls (n = 600) No. (%)	Cases (n = 502) No. (%)	P value (2df) ^a	Logistic regression Adjusted OR (95%CI)	Controls (n = 934) No. (%)	Cases (n = 840) No. (%)	P value (2df) ^a	Logistic regression Adjusted OR (95%CI)	P value ^b
REV3L	AA	432 (72.0)	353 (70.6)	0.741	1.00 (referent)	657 (70.4)	606 (72.5)	0.599	1.00 (referent)	
rs465646	AG	153 (25.5)	131 (26.2)		1.05 (0.8~1.38)	251 (26.9)	211 (25.2)		0.91 (0.74~1.12)	0.392
	GG	15 (2.5)	16 (3.2)		1.31 (0.64~2.69)	25 (2.7)	19 (2.3)		0.82 (0.45~1.51)	0.532
	AG+GG	168 (28.0)	147 (29.4)		1.07 (0.82~1.39)	276 (29.6)	230 (27.5)		0.91 (0.73~1.11)	0.334
BIRC5	TT	353 (58.8)	277 (55.3)	0.199	1.00 (referent)	559 (60)	460 (55)	0.008	1.00 (referent)	
rs2239680	TC	215 (35.8)	185 (36.9)		1.10 (0.86~1.41)	331 (35.5)	312 (37.3)		1.14 (0.94~1.40)	0.179
	CC	32 (5.3)	39 (7.8)		1.56 (0.95~2.55)	42 (4.5)	64 (7.7)		1.85 (1.23~2.79)	0.004
	TC+CC	247 (41.2)	224 (44.7)		1.16 (0.91~1.47)	373 (40)	376 (45)		1.22 (1.01~1.47)	0.035
FGF2	TT	452 (75.3)	401 (79.9)	0.166	1.00 (referent)	729 (78.2)	660 (78.6)	0.018	1.00 (referent)	
rs1476215	TA	134 (22.3)	89 (17.7)		0.75 (0.55~1.01)	184 (19.7)	145 (17.3)		0.98 (0.78~1.23)	0.26
	AA	14 (2.3)	12 (2.4)		0.97 (0.44~2.12)	19 (2)	35 (4.2)		0.87 (0.68~1.11)	0.012
	TA+AA	148 (24.7)	101 (20.1)		0.77 (0.57~1.03)	203 (21.8)	180 (21.4)		2.03 (1.15~3.58)	0.858
E2F1	GG	325 (54.2)	277 (55.2)	0.869	1.00 (referent)	503 (54)	431 (51.3)	0.104	1.00 (referent)	
rs3213180	GC	224 (37.3)	180 (35.9)		0.95 (0.73~1.22)	352 (37.8)	315 (37.5)		1.04 (0.85~1.27)	0.669
	CC	51 (8.5)	45 (9.0)		1.03 (0.68~1.59)	77 (8.3)	94 (11.2)		1.42 (1.02~1.97)	0.032
	GC+CC	275 (45.8)	225 (44.8)		0.96(0.76~1.21)	429 (46)	409 (48.7)		1.11 (0.92~1.34)	0.264
IL16	TT	243 (40.5)	156 (31.1)	0.002	1.00 (referent)	369 (39.5)	256 (30.5)	0.00004	1.00 (referent)	
rs1131445	TC	284 (47.3)	258 (51.4)		1.42 (1.09~1.85)	453 (48.5)	438 (52.1)		1.40 (1.14~1.72)	0.0014
	CC	73 (12.2)	88 (17.5)		1.88 (1.31~2.73)	112 (12)	146 (17.4)		1.88 (1.4~2.52)	0.00002
	TC+CC	357 (59.5)	346 (68.9)		1.51(1.18~1.93)	565 (60.5)	584 (69.5)		1.49 (1.22~1.81)	0.00007

^aP value was calculated using a χ^2 test with 2 d.f.

^bAdjusted for age, smoking status, education level, age at primiparity, marital status, and HPV infection status. Bold characters indicate the corresponding P values, and the ORs were statistically significant after considering FPRP.

Abbreviations: CI, confidence interval; OR, odds ratio.
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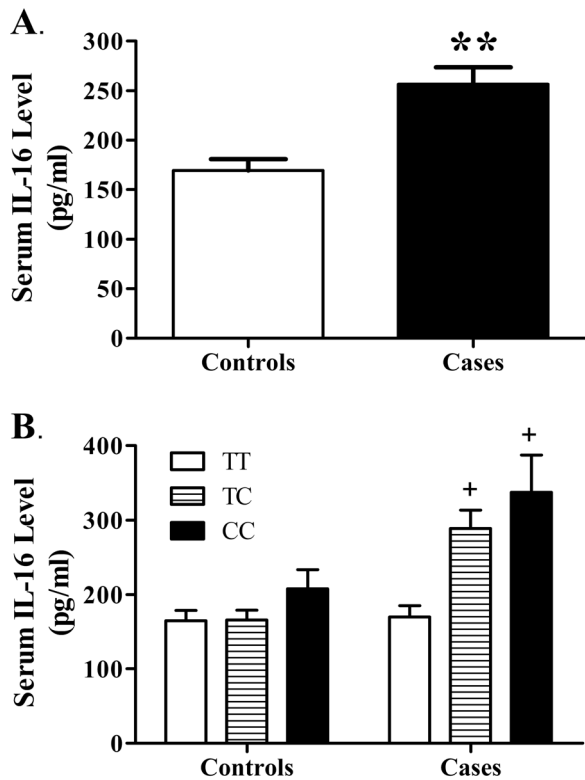


Figure 1. Elevated serum IL-16 levels in patients with cervical cancer and their correlation with the rs1131445 genotype. A. Serum IL-16 levels in controls and patients with cervical cancer, as measured by ELISA. B. Correlations of serum IL-16 levels with genotype in controls and patients. Data were expressed as the mean \pm SEM. * P <0.05 vs. controls group. + P <0.05 vs. patients with TT genotype. doi:10.1371/journal.pone.0086061.g001

(FPRP = 0.011). Given an OR of 1.5 at a nominal P = 0.05 for genotype frequencies ranging from 0.20 to 0.40, the statistical power of our study to detect an association of each SNP with cervical cancer in Study 2 and combined Study 1 and 2 was estimated at 81.0–91.5% and 95.0–98.9%, respectively. The stratification analysis did not reveal a significant interaction of the demographic and clinical characteristics and the rs1131445 genotype on the cervical cancer risk (data not shown). Additionally, there were not significant correlations between the genotypes of rs1131445 and the clinical stage and histological type of cervical cancer (data not shown).

3 Serum IL-16 level and its correlation with the *IL-16* rs1131445 genotype

Given the observed notable association between the rs1131445 and cervical cancer risk, we further investigate the serum IL-16 levels in controls and patients with cervical cancer as well as the potential regulatory effects of the rs1131445 genotype on serum IL-16. We selected 100 patients with cervical cancer and 80 controls from the subjects of Study 2 and examined their serum IL-16 levels by ELISA. The serum IL-16 of patients with cervical cancer significantly increased compared to controls (P = 0.001, Figure 1A). Moreover, in patients with cervical cancer, there was a marked correlation between the serum IL-16 and rs1131445 genotype (P = 0.001). The rs1131445 C allele-carrying patients had a higher serum IL-16 than the non-carriers (P < 0.05, Figure 1B). Furthermore, we did not note any statistically

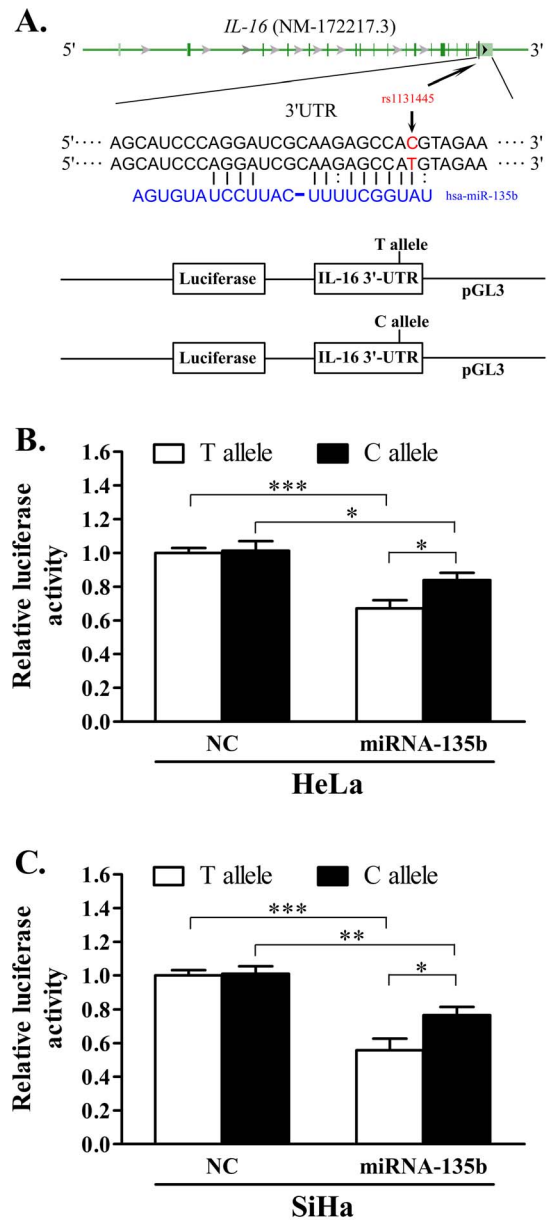


Figure 2. The effect of SNP rs1131445 on the interaction between the *IL-16* 3'-UTR and miR-135b. A. Schema of *IL-16* 3'-UTR harboring a putative miR-135b binding site, the position of rs1131445, and the construct of pGL3-*IL-16*-3'-UTR-T/C. B and C. Relative luciferase activity in the presence of miR-135b or the negative control miRNA is shown for the *IL-16* 3'-UTR with the T allele and C allele in the HeLa (B) and SiHa (C) cancer cells; data are shown as the percentage relative to the luciferase activity of the cells transfected with the NC and T allele; the error bar represents s.e. from three independent experiments; * P <0.05, ** P <0.05, *** P <0.01. NC represents the negative control. doi:10.1371/journal.pone.0086061.g002

significant association of serum IL-16 with the clinical stage and histological type of the cervical cancer (data not shown).

4 The effect of rs1131445 on the interaction between miR-135b and *IL-16* 3'-UTR

A putative binding site within the 3'-UTR of *IL-16* for miR-135b has been identified using miRBase, miRanda, and PicTar software. It was also predicted that the substitution from the T to the C allele in rs1131445 could decrease the Gibbs binding free

energy of miRNA-mRNA by 3.93 KJ/mol [16], which suggests that this SNP could affect the *IL-16* gene expression by altering the miRNA-mRNA binding affinity (Figure 2A). To test this hypothesis, a luciferase assay was performed in the HeLa and SiHa cervical cancer cell lines, which transfected with the pGL3-C-allele or pGL3-T-allele constructs accompanied by chemically synthesized miRNAs miR-135b or a negative control miRNA (Figure 2A). For both constructs, in the presence of miR-135b mimics, the expression of luciferase was significantly reduced (Figure 2B-C), which confirms the functional potential of the miRNA-mRNA interaction. In contrast, the negative control miRNA with no predicting binding site in the *IL-16* 3'-UTR has no effect on the luciferase expression (Figure 2B-C). Moreover, we found that the C allele resulted in a modest but statistically significant increase of luciferase expression compared to that of the T allele (Figure 2B-C).

Discussion

In the present study, we screened out a cervical cancer susceptibility variant from the miRNA-binding site SNPs within 37 cancer-related genes by a two-stage study. We found that the SNP rs1131445 C allele in the 3'-UTR of *IL-16* was associated with an increased risk of cervical cancer. Using a luciferase assay, we found that miRNA-135b markedly decreased the luciferase expression of pGL3-*IL-16*-3'-UTR constructs in the HeLa and SiHa cervical cancer cell lines. More importantly, these inhibitory effects of miRNA-135b were weakened by the substitution from the T to the C allele in rs1131445. These results support the hypothesis that the variations in the putative miRNA target sites could constitute a susceptibility factor for cervical cancers.

IL-16 is synthesized by various immune and parenchymal cells, including CD4 and CD8 T cells, eosinophils, macrophage/dendritic cells, mast cells, bronchial epithelium, and fibroblasts [27]. The protein directly translated from *IL-16* mRNA is a precursor molecule and must be cleaved into two fragments by caspase-3. The cleaved bioactive IL-16 is released following stimulation by histamine or serotonin and acts as a chemoattractant factor to regulate the inflammatory response [27]. Recently, IL-16 has been recognized as an important promoting factor for tumors [28,29,30,31]. The production of IL-16 correlates with the onset and progression of various hematopoietic cancers and solid cancers [28]. The constitutive IL-16 expression has also been found to upregulate in gliomas [29] and prostate cancer [31]. Moreover, its expression in prostate cancer tissue was correlated to tumor aggressiveness and biochemical relapse of the disease [31]. Serum IL-16 level elevation has been observed in multiple cancers [32,33]. Serum IL-16 is a predictor for the development of distant metastases of breast cancer [32]. Moreover, in breast cancer, the upregulation of secreted IL-16 enhances the infiltration of monocytes into the tumor tissue [30].

The roles of IL-16 in cervical cancer pathophysiology remain largely unclear. Our findings of serum IL-16 elevation following cervical carcinogenesis and the notable association of *IL-16* with cervical cancer risk suggest that IL-16 could regulate cervical carcinogenesis. This hypothesis is supported by the carcinogenic effects of persistent inflammation on cervix uteri. Immune cell infiltration into tumors is a critical determinant for cervical carcinogenesis [30,34]. The macrophage infiltrating in cervix uteri increases linearly with cervical disease progression, from normal cervix, low-grade to high grade squamous intraepithelial lesions, to

invasive cancer [35]. Tumor-associated macrophages contribute to tumor progression by exerting their multiple functions in tumor cell proliferation, tumor cell invasion, and tumor angiogenesis [36]. Besides macrophages, CD4⁺ regulatory T cells can contribute to carcinogenesis by curtailing the efficacy of T cell immune responses against cancers [37]. In cervical cancer, HPV-specific CD4⁺ T cells suppress cytokine (IFN- γ , IL-2) production to interfere with the anti-tumor immune response [38]. IL-16 can be expressed by epithelial cells under inflammatory conditions [39] and serves to chemoattract monocytes/macrophages and CD4⁺ T cells to the infection site [40]. Moreover, secreted IL-16 stimulates monocytes/macrophages to produce various pro-inflammatory cytokines [41]. Therefore, it is plausible that the upregulation of IL-16 in HPV-induced inflammation could promote carcinogenesis by the hyperfunction of macrophages, HPV-specific CD4⁺ T cells, or other inflammatory cells.

Here, we provide the first evidence that rs1131445 in the miR-135b binding site of *IL-16* 3'-UTR, which can affect IL-16 protein expression by interfering with miR135b suppressive function, was significantly associated with the risk of cervical cancer. A functional assay in vitro indicated that the transfection of HeLa and SiHa cells carrying the *IL-16* 3'-UTR with miR-135b mimic significantly reduces the expression of luciferase, which suggests that IL-16 might be targeted by miR-135b. The rs1131445 T to C change inhibits the interaction of miR-135b with *IL-16* 3'-UTR and upregulates its expression. Consistent with this speculation, in vivo analysis revealed that patients who carry the rs1131445 C allele had a higher serum IL-16 than non-carriers, which led to an elevated serum IL-16 of patients compared to healthy controls. The upregulation of miR-135b has been found in cervical cancer [42], which could inhibit the expression of *IL-16* by targeting its 3'-UTR. The rs1131445 C allele impairs the binding of miR-135b to the target and leads to higher constitutive expression of *IL-16* and subsequent release to serum. Given the important regulatory roles of IL-16 release in the immune response and the consequent level of the local inflammatory microenvironment, individuals who carry the rs1131445 C allele would be expected to have elevated risk for cervical cancer. In conclusion, our data suggested that rs1131445, as a functional variant, could modulate the individual risk of cervical cancer likely by deregulating the post-transcriptional regulation of miRNA-135b on *IL-16* expression. Although our study used reasonable numbers of patients and controls and rigorous statistical methods, we could not completely rule out the possibility that spurious results emerged from our case-control association study design due to chance or population stratification. Therefore, further large scale replication study using different ethnic populations, or family-based analysis, or prospective cohort design is required to produce very conclusive results. Additional functional analyses are also required to clarify the exact roles of IL-16 in cervical cancer genesis and progression in the future.

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Author Contributions

Conceived and designed the experiments: IJW PH. Performed the experiments: YM LZ MLP QYL. Analyzed the data: YM IJW PH. Contributed reagents/materials/analysis tools: MLP QYL PH. Wrote the paper: YM IJW PH.

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