Cancer Immunol Immunother (2012) 61:2153–2159
DOI 10.1007/s00262-012-1280-y

ORIGINAL ARTICLE

Genetic polymorphisms in the *ITPKC* gene and cervical squamous cell carcinoma risk

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Received: 23 March 2012 / Accepted: 1 May 2012 / Published online: 18 May 2012
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Abstract Cervical cancer is caused primarily by infection with oncogenic types of human papillomavirus (HPV). However, HPV infection alone is not sufficient for the progression to cervical cancer. Host immunogenetic factors may involve in the development of this disease. Inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) is recently shown to act as a negative regulator of T-cell activation. We aim to study if polymorphisms in the *ITPKC* gene are associated with the risk of cervical cancer in Taiwanese women. *ITPKC rs28493229 C/G*, *rs890934 G/T*, *rs2303723 C/T*, and *rs10420685 A/G* polymorphisms were genotyped in a hospital-based study of 465 women with cervical squamous cell carcinoma (CSCC) and 800 age-matched healthy control women. The presence and genotypes of HPV in CSCC were determined. The frequency of *G/G* genotype and *G* allele of the *ITPKC rs28493229* polymorphism was significantly higher in patients with CSCC compared with controls (OR = 1.81, 95 % CI 1.20–2.73, $P = 0.005$, $P_c = 0.02$; OR = 1.70, 95 % CI 1.14–2.54, $P = 0.008$, $P_c = 0.03$, respectively). No significant associations were found for other 3 polymorphisms. Haplotype analysis revealed the distribution of haplotype *CGTA* was significantly reduced in women with CSCC (OR = 0.59, 95 % CI 0.40–0.89, $P = 0.01$, $P_c = 0.04$). In conclusion, we found the *G/G* genotype and *G* allele of the *ITPKC rs28493229* polymorphism may contribute to the risk of CSCC in Taiwanese women. This finding provides new insights into the mechanisms of immune activation in cervical cancer.

Keywords Cervical cancer · HPV · Immunity · ITPKC · Polymorphism

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Introduction

Cervical cancer is one of the most prevalent female malignancies worldwide. It is a serious health problem in Taiwan, with 2,700 women developing this disease each year, second in incidence only to breast cancer [1]. Persistent infection of high-risk human papillomavirus (HPV) is strongly associated with the development of cervical cancer [2]. But only a small fraction of those infected

develop cervical cancer, suggesting that other factors are also important determinants of disease risk. Along with environmental and lifestyle factors, familial aggregation studies indicate that host genetic factors are likely to involve in cervical cancer pathogenesis [3].

Cell-mediated immunity is believed to be critical in controlling both HPV infections and HPV-associated cancers. High incidence of persistent HPV infections and subsequent HPV-related cancers have been observed among immunosuppressed individuals [4, 5]. In addition, significant reduction of CD4+ T-cell population in peripheral blood mononuclear cells is found in patients with cervical intraepithelial neoplasia (CIN) and cervical cancer [6, 7]. The cell-mediated immune responses to specific HPV E6 and E7 peptides correlate significantly with the resolution of HPV infection and regression of CIN [8]. The infiltrating CD4+ T cells and macrophages have also been observed in spontaneously regressing genital warts [9]. In contrast, partial or complete failure of HPV-16-specific CD4+ T-cell responses and decreased numbers of CD4+ T cells in the cervical mucosa are found in women with prevalent HPV-16 infections [10]. The Th1 cytokines interleukin-2 (IL-2) and interferon-γ are crucial regulators of cell-mediated immunity and their production by peripheral blood mononuclear cells reduced significantly in patients with CIN and cervical cancer [6, 10, 11]. These findings clearly demonstrate that host immunity is associated with HPV infections and progression to cervical cancer. Therefore, genetic variants of immunologically relevant genes may be potential determinants for host susceptibility to cervical cancer.

Inositol 1,4,5-trisphosphate 3-kinase C (ITPKC), encoded by the *ITPKC* gene on chromosome 19q13.2, is an important negative regulator of T-cell activation [12]. When the T-cell receptors bind a ligand, the enzyme phospholipase C-γ is activated and leads to phospholipid hydrolysis that yields diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3, a second-messenger molecule, then binds to its receptor on endoplasmic reticulum (ER) membrane and causes calcium ions (Ca^{2+}) release into the cytoplasm [13]. Depletion of the ER calcium stores further triggers store-operated calcium influx. Increased intracellular free Ca^{2+} eventually results in the T-cell activation via the Ca^{2+}/nuclear factor of activated T cells (NFAT) signaling pathway. ITPKC is able to phosphorylate IP3 to IP4, which in turn attenuates the Ca^{2+}/NFAT signaling pathway and downregulates T-cell activation [14]. The *C* allele of the functional single nucleotide polymorphism (SNP) *rs28493229* in the *ITPKC* gene has been found to reduce mRNA expression of *ITPKC* by altering splicing efficiency [12]. This SNP has also been confirmed to strongly associate with susceptibility to Kawasaki disease [12], a pediatric disorder characterized by hyperactive T-cell phenotype.

Given the pivotal role of ITPKC in regulating the cell-mediated immunity, we tested the hypothesis that specific *ITPKC* SNPs are associated with cervical cancer risk in a hospital-based case–control study of 465 cervical squamous cell carcinoma (CSCC) patients and 800 sex- and age-matched healthy controls.

Patients and methods

Study subjects

The study included 465 patients with CSCC (mean ± SD age at diagnosis 53.2 ± 13.1 years) residing in northern Taiwan. The diagnosis of CSCC was confirmed by histological examinations of tissues from biopsies or resected specimens. The control group consisted of 800 sex- and age-matched healthy subjects (mean ± SD age at sampling 52.4 ± 12.1 years) who were enrolled from women attending the routine Pap screening and with normal Pap smear and no previous history of cervical dysplasia. The cases and controls were all genetically unrelated Taiwanese women. Informed written consent was obtained from all participants for use of their surgical resections, cervical scrapings, or blood for the study. The study protocol conformed to the ethical guidelines of the 1964 Declaration of Helsinki and was approved by the institutional review board of Mackay Memorial Hospital.

DNA extraction

Formalin-fixed, paraffin-embedded tissue blocks from 458 (98.5 %) of the 465 patients were sectioned and dewaxed, and genomic DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. No tissue samples were available in seven patients because they had had a hysterectomy in other hospitals before they were enrolled in this study. Therefore, genomic DNA was extracted from their donated blood. Genomic DNA of controls was extracted from cervical scrapings using Qiagen DNA extraction kit.

HPV detection and typing

The detection of HPV and genotyping on 458 cervical DNA samples were carried out by polymerase chain reaction (PCR). A pair of degenerate primers, GP61/MY11, designed according to the highly conserved domain, was used to amplify a 190-bp fragment in the L1 region of the HPV genome. The PCR product was then sequenced on an automated sequencer (ABI 377, Applied Biosystems, Foster City, CA) to determine the HPV

genotype. No HPV DNA testing was done for the 800 control subjects.

ITPKC genotyping

ITPKC rs28493229 C/G (Intron 1), *rs890934 G/T* (Intron 1), *rs2303723 C/T* (Intron 1), and *rs10420685 A/G* (Exon 3) polymorphisms were genotyped. These SNPs except *rs28493229 C/G* were chosen using Haploview's Tagger program with a minor allele frequency of over 20 % [15]. Another rationale to choose these SNPs was based on their potential functional significance. They were determined using the Pre-Developed TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA). Briefly, polymerase chain reactions (PCR) were carried out in a 96-well GeneAmp PCR System 9700 (Applied Biosystems) with mixes consisting of 10 ng of genomic DNA, 5 μl of TaqMan Universal PCR Master Mix, 0.5 μl of 20× Assay Mix, and ddH_2O to a final volume of 10 μl. Thermal cycle conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 92 °C for 15 s, and annealing and extension at 60 °C for 1 min. After PCR, the TaqMan assay plates were transferred to the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) where the endpoint fluorescence intensity in each well of the plate was read. The allele-specific fluorescence data from each plate were analyzed using the SDS v.1.1 software (Applied Biosystems) to automatically determine the genotype of each sample.

Statistical analysis

Genotype and allele frequencies of the *ITPKC rs28493229 C/G*, *rs890934 G/T*, *rs2303723 C/T*, and *rs10420685 A/G* SNPs were determined by direct counting. The Hardy–Weinberg equilibrium was assessed for each SNP in both the control and case groups by Chi-square analysis. The frequencies of *ITPKC* haplotypes as well as linkage disequilibrium between paired SNPs in controls and cases were estimated using the Haploview 4.2 program [15]. Haplotypes with a frequency of <2 % were grouped together.

Statistical differences in the genotype, allele, and haplotype distributions between controls and cases were performed by Chi-square test with Yates' correction where appropriate (one expected number <5). Odds ratios (OR) and 95 % confidence intervals (CI) were also calculated. The Bonferroni correction was used to correct for multiple comparisons where appropriate. P_c values of less than 0.05 (2-tailed) were considered to be statistically significant.

Prior to the study, statistical power to detect the effects of these *ITPKC* SNPs on susceptibility to CSCC was calculated using the Quanto Ver. 1.1 software (Department of Preventive Medicine, University of Southern California, CA, USA). We designed the study to have a power of 93.5 % to determine a relative risk of 1.7 conferred by the risk allele of *rs28493229 C/G* polymorphism at a significance level of 0.05, with an estimated prevalence of CSCC of 360/100,000 [16].

Results

HPV DNA was detected in 84.7 % of the 458 tumors tested, with 53.9 % positive for HPV type 16, 8.3 % for HPV type 18, and 22.5 % for other HPV types.

A total of 4 *ITPKC* SNPs were successfully genotyped in 800 controls, 465 patients with CSCC, and 247 patients with HPV-16-positive CSCC (Tables 1, 2, 3, 4). The genotype frequencies were in Hardy–Weinberg equilibrium in controls and patients for all the polymorphisms ($P > 0.05$). We found significant differences in the distribution of genotypes ($P = 0.005$) and alleles ($P = 0.008$) of *rs28493229 C/G* SNP between controls and all patients

Table 1 Genotype and allele frequencies of the *ITPKC* gene *rs28493229 C/G* polymorphism in controls and in all patients with CSCC and those with HPV-16-positive CSCC

	Controls ($N = 800$) No. (%)	All CSCC ($N = 465$) No. (%)	HPV-16-positive CSCC ($N = 247$) No. (%)	All CSCC		HPV-16-positive CSCC	
				P value (χ^2)	OR (95 % CI)	*P* value (χ^2)	OR (95 % CI)
Genotype				0.005 (10.5)		0.02 (5.35)	
C/C	0 (0.0)	1 (0.2)	0 (0.0)		5.17 (0.47–57.2)		
C/G	97 (12.1)	32 (6.9)	17 (6.9)		0.54 (0.35–0.81)		0.54 (0.31–0.92)
G/G	703 (87.9)	432 (92.9)	230 (93.1)		1.81 (1.20–2.73)		1.87 (1.09–3.19)
Allele				0.008 (6.94)		0.03 (5.04)	
C	97 (6.1)	34 (3.7)	17 (3.4)		0.59 (0.39–0.88)		0.55 (0.33–0.93)
G	1,503 (93.9)	896 (96.3)	477 (96.6)		1.70 (1.14–2.54)		1.81 (1.07–3.06)

ITPKC inositol 1,4,5-trisphosphate 3-kinase C, *CSCC* cervical squamous cell carcinoma, *HPV* human papillomavirus, *OR* odds ratio, *CI* confidence interval

with CSCC (Table 1). The differences remained significant after correction for multiple tests ($P_c = 0.02$ for genotype and $P_c = 0.03$ for allele frequencies). A significant increase in the *G/G* genotype (OR = 1.81, 95 % CI 1.20–2.73) and *G* allele (OR = 1.70, 95 % CI 1.14–2.54) was found in CSCC patients, whereas *C/G* genotype (OR = 0.54, 95 % CI: 0.35–0.81) and *C* allele (OR = 0.59, 95 % CI 0.39–0.88) decreased significantly.

In order to clarify if interactions between oncogenic HPV-16 infection and *ITPKC* polymorphisms would affect susceptibility or progression to CSCC, we further stratified all patients with CSCC by positivity of HPV type 16. The results showed that *rs28493229 C/G* genotype and allele frequencies in women with HPV-16-positive CSCC differed significantly from control individuals ($P = 0.02$ and 0.03, respectively), but did not survive the multiple tests ($P_c = 0.08$ and 0.12, respectively) (Table 1). For the other three polymorphic sites examined on the *ITPKC* gene, no significant difference in genotype and allele distribution was observed between all women with CSCC and controls or between women with HPV-16-positive CSCC and controls (Tables 2, 3, 4). Pairwise linkage disequilibrium analyses among these SNPs revealed strong inter-marker linkage disequilibriums in both controls (D' 0.98–1.00) and patients (D' 0.95–1.00).

We also analyzed the possible haplotypes constructed by *rs28493229 C/G*, *rs890934 G/T*, *rs2303723 C/T*, and *rs10420685 A/G* SNPs in controls, all women with CSCC, and those with HPV-16-positive CSCC (Table 5). Although the overall *P* value did not achieve statistical significance ($P = 0.06$), the frequency of haplotype *CGTA* was significantly reduced in all patients with CSCC (OR = 0.59, 95 % CI 0.40–0.89, $P = 0.01$, $P_c = 0.04$) but not in the HPV-16-positive CSCC patients ($P = 0.03$, $P_c = 0.12$). No significant differences were found in other haplotypes between any of the groups tested.

Discussion

In the study, we investigated the association of specific *ITPKC* SNPs and their haplotypes with the risk of cervical cancer in Taiwanese women. We found that *G/G* genotype

Table 2 Genotype and allele frequencies of the *ITPKC* gene *rs890934 G/T* polymorphism in controls and in all patients with CSCC and those with HPV-16-positive CSCC

	Controls ($N = 800$) No. (%)	All CSCC ($N = 465$) No. (%)	HPV-16-positive CSCC ($N = 247$) No. (%)	All CSCC		HPV-16-positive CSCC	
				P value (χ^2)	OR (95 % CI)	*P* value (χ^2)	OR (95 % CI)
Genotype				0.22 (3.06)		0.12 (4.24)	
G/G	240 (30.0)	130 (27.9)	64 (25.9)		0.91 (0.70–1.17)		0.82 (0.59–1.13)
G/T	391 (48.9)	217 (46.7)	116 (47.0)		0.92 (0.73–1.15)		0.93 (0.70–1.23)
T/T	169 (21.1)	118 (25.4)	67 (27.1)		1.27 (0.97–1.66)		1.39 (1.00–1.93)
Allele				0.13 (2.34)		0.05 (3.86)	
G	871 (54.4)	477 (51.3)	244 (49.4)		0.88 (0.75–1.04)		0.82 (0.67–1.00)
T	729 (45.6)	453 (48.7)	250 (50.6)		1.13 (0.97–1.33)		1.22 (1.00–1.50)

ITPKC inositol 1,4,5-trisphosphate 3-kinase C, *CSCC* cervical squamous cell carcinoma, *HPV* human papillomavirus, *OR* odds ratio, *CI* confidence interval

Table 3 Genotype and allele frequencies of the *ITPKC* gene *rs2303723 C/T* polymorphism in controls and in all patients with CSCC and those with HPV-16-positive CSCC

	Controls ($N = 800$) No. (%)	All CSCC ($N = 465$) No. (%)	HPV-16-positive CSCC ($N = 247$) No. (%)	All CSCC		HPV-16-positive CSCC	
				P value (χ^2)	OR (95 % CI)	*P* value (χ^2)	OR (95 % CI)
Genotype				0.27 (2.61)		0.42 (1.72)	
C/C	33 (4.1)	28 (6.0)	15 (6.1)		1.49 (0.89–2.50)		1.50 (0.80–2.82)
C/T	279 (34.9)	152 (32.7)	82 (33.2)		0.91 (0.71–1.16)		0.93 (0.69–1.26)
T/T	488 (61.0)	285 (61.3)	150 (60.7)		1.01 (0.80–1.28)		0.99 (0.74–1.32)
Allele				0.64 (0.22)		0.60 (0.27)	
C	345 (21.6)	208 (22.4)	112 (22.7)		1.05 (0.86–1.27)		1.07 (0.84–1.36)
T	1,255 (78.4)	722 (77.6)	382 (77.3)		0.95 (0.79–1.16)		0.94 (0.74–1.19)

ITPKC inositol 1,4,5-trisphosphate 3-kinase C, *CSCC* cervical squamous cell carcinoma, *HPV* human papillomavirus, *OR* odds ratio, *CI* confidence interval

Table 4 Genotype and allele frequencies of the *ITPKC* gene *rs10420685 A/G* polymorphism in controls and in all patients with CSCC and those with HPV-16-positive CSCC

	Controls ($N = 800$) No. (%)	All CSCC ($N = 465$) No. (%)	HPV-16-positive CSCC ($N = 247$) No. (%)	All CSCC		HPV-16-positive CSCC	
				P value (χ^2)	OR (95 % CI)	*P* value (χ^2)	OR (95 % CI)
Genotype				0.33 (2.22)		0.54 (1.22)	
A/A	489 (61.1)	287 (61.7)	152 (61.5)		1.03 (0.81–1.30)		1.02 (0.76–1.36)
A/G	278 (34.8)	151 (32.5)	81 (32.8)		0.90 (0.71–1.15)		0.92 (0.68–1.24)
G/G	33 (4.1)	27 (5.8)	14 (5.7)		1.43 (0.85–2.41)		1.40 (0.73–2.65)
Allele				0.75 (0.10)		0.79 (0.07)	
A	1,256 (78.5)	725 (78.0)	385 (77.9)		0.97 (0.80–1.18)		0.97 (0.76–1.23)
G	344 (21.5)	205 (22.0)	109 (22.1)		1.03 (0.85–1.26)		1.03 (0.81–1.32)

ITPKC inositol 1,4,5-trisphosphate 3-kinase C, *CSCC* cervical squamous cell carcinoma, *HPV* human papillomavirus, *OR* odds ratio, *CI* confidence interval

Table 5 Analysis of *ITPKC* haplotypes in controls and in all patients with CSCC and those with HPV-16-positive CSCC

Haplotype	Controls ($2N = 1,600$) No. (%)	All CSCC ($2N = 930$) No. (%)	HPV-16-positive CSCC ($2N = 494$) No. (%)	All CSCC		HPV-16-positive CSCC	
				P value (χ^2)	OR (95 % CI)	*P* value (χ^2)	OR (95 % CI)
GTTA	728 (45.5)	448 (48.2)	247 (49.9)	0.19 (1.69)	1.11 (0.95–1.31)	0.08 (3.07)	1.20 (0.98–1.47)
GGTA	430 (26.9)	240 (25.8)	119 (24.0)	0.56 (0.34)	0.95 (0.79–1.14)	0.22 (1.51)	0.86 (0.68–1.09)
GGCG	341 (21.3)	201 (21.6)	106 (21.5)	0.86 (0.03)	1.02 (0.84–1.24)	0.95 (0.00)	1.01 (0.79–1.29)
CGTA	96 (6.0)	34 (3.7)	17 (3.4)	0.01 (6.63)	0.59 (0.40–0.89)	0.03 (4.84)	0.56 (0.33–0.94)
Others[a]	2 (0.1)	6 (0.7)	5 (1.1)				

Haplotype inferred using Haploview 4.2 program, based on the order of *rs28493229 C/G*, *rs890934 G/T*, *rs2303723 C/T*, and *rs10420685 A/G* polymorphisms

P value for four haplotypes between all CSCC patients and controls: $P = 0.06$ ($\chi^2 = 7.47$, 3 *df*)

P value for four haplotypes between HPV-16-positive CSCC patients and controls: $P = 0.06$ ($\chi^2 = 7.35$, 3 *df*)

ITPKC inositol 1,4,5-trisphosphate 3-kinase C, *CSCC* cervical squamous cell carcinoma, *OR* odds ratio, *CI* confidence interval

[a] Others indicate combination of the remaining haplotypes with a frequency of less than 2 %

and *G* allele of the *ITPKC rs28493229* polymorphism conferred a risk of CSCC, whereas *C/G* genotype and *C* allele protected against disease development. In addition, CSCC risk increased only in women homozygous for *G* allele, indicating that the *G* allele conferred susceptibility in a recessive mode. Analysis of haplotype distribution showed the haplotype *rs28493229 C-rs890934 G-rs2303723 T-rs10420685 A* was associated with a decreased risk of CSCC. Our results indicate that the *ITPKC* gene is important in the pathogenesis of CSCC. The limitations of our study include a selection bias existing in the study of retrospective design and scarcity of screened SNPs. Therefore, a large-scale prospective study which investigates extensive SNPs of comprehensive immune-related genes is needed to confirm our findings.

The genetic variability of the host plays a role in the development of cervical cancer, especially genes controlling the immune response. Human leukocyte antigen (HLA) genes have been extensively studied because they are pivotal in the regulation of immune function. Studies have shown that HLA-DRB1*1501, DQB1*03 alleles, and DRB1*1501-DQB1*0602 haplotype are associated with an increased risk of cervical cancer to certain ethnic groups, whereas DRB1*13 alleles and DRB1*1301-DQB1*0603 haplotype are associated with a decreased risk of disease [17]. In addition, recent studies have reported that HLA-A*0301, *1104, B*4402, Cw*0501, DPB1*0202, and *1301 alleles confer susceptibility to cervical cancer, but protective effect against the disease is found in HLA-A*2402, B*1501, and Cw*0202 alleles [18–21]. For non-HLA immune-related genes, interleukins, interferon-γ, cytotoxic T-lymphocyte antigen-4, and tumor necrosis factor-α have been found to be associated with increased or decreased risk of cervical cancer [22–27]. However, the association between *ITPKC* polymorphisms and the risk of cervical cancer has not been investigated to date.

Ca^{2+} is an important second messenger in many cells, including cells of the immune system [28]. After an antigen binds to the T-cell receptor, NFAT protein is activated by elevated intracellular Ca^{2+} and leads to the transcription of

several genes including *IL2* [29]. ITPKC regulates Ca^{2+}/NFAT signaling pathway by modulating the amount of IP3. Overexpression of ITPKC in Jurkat cells results in reduced NFAT activation and *IL2* gene expression. On the contrary, ITPKC knockdown by short hairpin RNA enhances NFAT activity and *IL2* gene expression [12]. Therefore, it is reasonable to infer that any variation in the expression or function of ITPKC may change the delicate homeostasis of the immune system. Identification of ITPKC as a CSCC susceptibility gene could shed light on the mechanisms of immune regulation in the development and progress of cervical cancer and help innovating a diagnostic test and novel therapy.

The *G* allele of the *ITPKC rs28493229* polymorphism has been reported to result in higher ITPKC expression that might, in turn, lead to decreased activation of T cells [12]. The higher frequencies of the *G/G* genotype and *G* allele found in our patients with CSCC imply that T cells from these patients would have higher expression level of ITPKC following T-cell activation than would those from controls. The increased expression of ITPKC results in attenuation of the T-cell activation, which might then contribute to persistent HPV infections and increase the risk of CSCC. If further investigation bears out the utility of such a marker, it could be used to identify high-risk women who require more frequent cervical cancer screening or who are priority candidates for HPV vaccine prophylaxis.

In conclusion, we showed for the first time that the *G/G* genotype and *G* allele of the *ITPKC* SNP *rs28493229* is significantly associated with CSCC risk in Taiwanese women. In theory, this genotype might render a female more susceptible to persistent HPV infections and the development of CSCC.

Acknowledgments This research was supported by grants MMH 9781 and MMH E-99007 from Mackay Memorial Hospital, Taiwan.

Conflict of interest The authors declare that they have no conflict of interest.

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