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Research Paper

Single nucleotide polymorphisms and haplotypes of carbonic anhydrase 9 can predict invasive squamous cell carcinoma of uterine cervix

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Abstract

This study aimed to explore the involvement of carbonic anhydrase 9 (CA9) single nucleotide polymorphisms (SNPs) in the development of invasive cancer of uterine cervix for Taiwanese women. Ninety-seven patients with cervical invasive squamous cell carcinoma and 88 with preinvasive squamous cell lesions as well as 324 control women were recruited. Two CA9 SNPs in exons, including rs2071676 (+201, G/A) in exon 1 and rs3829078 (+1081, A/G) in exon 7, rs1048638 (+1584, C/A) in 3′-untranslated region of exon 11, as well as an 18-base pair deletion/insertion (376deltion393) in exon 1 were selected and their genotypic distributions were determined by real-time polymerase chain reaction. Haplotype was then constructed with rs2071676, 376del393, rs3829078 and rs1048638 in order. The results revealed that Taiwanese women with genotypes CA or CA/AA in CA9 SNP rs1048638 displayed a more risk in developing cervical invasive cancer, assigning wild genotype CC as a reference. AA in SNP rs2071676 tended to increase the risk of developing cervical invasive cancer, using GG/GA as a reference. When women had the diplotypes, carrying at least one haplotype A1AA (one mutant allele A in rs2071676, no deletion in 376del393, no mutant allele A in rs3829078 and one mutant allele A in rs1048638), they were significantly susceptible to cervical invasive cancer. In conclusion, CA9 SNP rs1048638 and haplotype A1AA are associated with the susceptibility of cervical invasive squamous cell carcinoma for Taiwanese women.

Key words: carbonic anhydrase 9, single nucleotide polymorphism, haplotype, invasive squamous cell carcinoma of uterine cervix

Introduction

Cervical invasive cancer was the second common type of gynecological cancer based on cancer registry annual report, 2013 Taiwan. Cytologic diagnoses of cervical dysplasia were categorized into low-grade and high-grade squamous intraepithelial lesions (LSILs and HSILs). Histologic diagnosis of LSILs was converted into cervical intraepithelial neoplasia 1 (CIN1; low-grade CIN) as well as HSIL was subdivided into CIN 2 and CIN 3 (high-grade CIN) [1]. About 20-30% of HSILs may progress to invasive cancer [2, 3]. Only approximately 10% of LSILs may develop to invasive cancer.

A unique characteristic of the tumor microenvironment of solid cancers is hypoxia, which results from an imbalance between the increasing demand for oxygen and nutrients by rapidly

proliferating tumor cells [4]. Hypoxic cancer cells undergo a metabolic reprogramming and switch to glycolytic metabolism to maintain cellular bioenergetics via Warburg effect, producing acidic metabolites and leading to acidic environment [5-8]. Cancer cells utilize carbonic anhydrases (CAs) to maintain a balance between intracellular alkalization for their proliferation and extracellular acidification of tumor microenvironment for their invasiveness by catalyzing the reversible hydration of CO2 to bicarbonate (HCO_3^-) and protons (H^+), serving their critical role in tumor progression [9-14]. The critical components for pH regulation, which cancer cells upregulate in hypoxia condition, include the membrane-associated CA9 and CA12 [11, 15]. CA9 is the most strongly expressed gene in response to hypoxia in human cancer cells [16, 17]. It is overexpressed in a variety of tumor types and is related to cancer progression [18-21].

Single nucleotide polymorphism (SNP) occurs if a single nucleotide in the shared sequence of a gene changes in more than 1% of a certain population. It is probably associated with the susceptibility of certain diseases such as cancers [22]. It may predict the risk of cancer such as oral cancer, by the analysis of genetic polymorphisms [23]. The CA9 gene is located on chromosome 9p13-p12 and comprises 11 exons [24]. SNPs of CA9 gene may have an impact on the expression of CA9 and then disease development via influencing the promoter area, exon and 3′-untranslated region (3′-UTR) [25]. To date, few studies correlate CA9 genetic polymorphisms with uterine cervical cancer. However, our previous study found that the CA/AA frequency of CA9 SNP rs1048638 is higher in patients with cervical cancer, as compared to control women in Taiwan [26]. Therefore, we investigated the distribution of CA9 gene SNPs and haplotypes among patients with invasive squamous cell carcinoma or preinvasive squamous lesions of uterine cervix and normal controls, and tried to predict cervical invasive cancer for Taiwanese women.

Materials and Methods

Description of the enrolled subjects

We consecutively enrolled five hundred and nine Taiwanese women, consisting of 97 patients with invasive squamous cell carcinoma and 88 patients with preinvasive squamous lesions of uterine cervix as well as 324 control Taiwanese women, into this study. The studied individuals all live in Central Taiwan. Patients with cervical invasive cancer received treatment protocols at the Department of Obstetrics and Gynecology in Chung Shan Medical University Hospital, Taiwan, between May 1, 1999 and April 30, 2011. Patients with preinvasive lesions were diagnosed to have high-grade CIN using colposcopy-directed cervical punch biopsy or loop electrosurgical excision procedure. They may subsequently receive large loop excision of transformation zone or total abdominal or vaginal hysterectomy. The histologic type of cervical invasive cancer and preinvasive lesions was squamous cell type, confirmed by pathologic report. Meanwhile, 324 recruited control women were further defined by colposcopy after they had normal Papanicolaou smear in general examination at Outpatient Department in Chung Shan Medical University Hospital. The mean ages of patients with cervical invasive cancer, those with preinvasive lesions and normal women were 53.6 (standard deviation [SD], 11.7), 41.9 (SD, 11.8) and 44.2 (SD, 10.2) years old, respectively. This study was approved by Chung Shan Medical University Hospital Institutional Review Board (CSMUH No: CS11152). Each subject completed the consent.

Acquirement of blood specimens and extraction of genomic DNA

We collected 97 blood samples from patients with cervical invasive cancer and 88 from preinvasive lesions. Meanwhile, 324 blood samples were obtained from control women. Genomic DNA was extracted from peripheral vein blood leukocytes, which was immediately placed into EDTA anticoagulated tube after the blood collection, using a QIAamp DNA blood mini kits (Qiagen, Valencia, Valencia, CA, USA) according to the manufacture's protocol [27, 28].

Selection and identification of CA9 genetic polymorphisms

Based on National Center for Biotechnology Information, database SNP, over 30 genetic polymorphisms have been found to exist in the 11 exons region of the CA9 gene. We selected two CA9 SNPs in exons, including rs2071676 (+201, G/A) in exon 1 and rs3829078 (+1081, A/G) in exon 7 based on their potential involvement in the various cancer types [26, 29-31]. Moreover, one in 3′-UTR of exon 11, i.e. rs1048638 (+1584, C/A), as well as an 18-base pair deletion/insertion 376deltion393 (376del393) in exon 1 according to the studies of Chien et al. [29], de Martino et al. [32], and Chinese HapMap (Han Chinese in Beijing, China) data. All of the minor allelic frequencies of these four CA9 genetic polymorphisms were $\geq$ 5%.

The genotypic frequencies of the CA9 SNPs rs2071676 (+201, G/A) (C_25472146_10), rs3829078 (+1081, A/G) (C_27507259_10) and rs1048638 (+1584,

C/A) (C_1294917_10) were detected by the ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). They were assessed by the TaqMan assay using SDS vers. 3.0 software (Applied Biosystems). The 376del393 polymorphism was checked using polymerase chain reaction and the products were electrophoresed through 3% agarose gels. Ethidium bromide was used to stain the products. The methods, primer sequences and probes for determination of the CA9 gene polymorphisms were described as our previous study [29].

Statistical analysis

ANOVA was used to analyze the age distribution of studied population, including patients with cervical invasive cancer or preinvasive lesion and control women. And then Scheffe method was used for post hoc analysis. Chi-square or Fisher's exact tests were used to examine the relationships among frequencies of CA9 gene SNPs, allele and incidence of cervical neoplasia (including invasive cancer and preinvasive lesions). Logistic regression or multiple logistic regression models were separately used to compare distributions of CA9 gene SNPs genotypes between patients with cervical neoplasia and control women or compare distributions of CA9 gene SNPs genotypes, alleles or haplotypes among patients with invasive cancer or preinvasive lesions and control women before and after controlling the age. Odds ratios (ORs) and adjusted odds ratios (AORs; controlling for age) and their 95% confidence intervals (CIs) were calculated by WinPepi software or SPSS. A significant difference was defined by $p<0.05$.

Results

There were significant differences in age distribution between patients with cervical invasive cancer and those with preinvasive lesion (53.6 ± 11.7 vs. 41.9 ± 11.8, $p<0.001$) as well as between those with cervical cancer and control women (53.6 ± 11.7 vs. 44.2 ± 10.2, $p<0.001$) but no significant difference between those with preinvasive lesions and control women (41.9 ± 11.8 vs. 44.2 ± 10.2, $p=0.231$). The age distribution of each SNP genotype for each CA9 SNP was not different ($p=0.246$ for rs2071676, $p=0.675$ for rs3829078, $p=0.434$ for rs1048638, $p=0.359$ for 376deletion393). Genotypic distributions of SNPs rs2071676, rs3829078 and rs1048638 conformed to Hardy-Weinberg equilibrium ($p=0.505$, $\chi2$ value: 0.445; $p=0.753$, $\chi2$ value: 0.100; and $p=0.330$, $\chi2$ value: 0.948, respectively).

Association of distribution of CA9 gene polymorphisms with cervical neoplasia

A significant difference was only found in the distribution of CA9 gene SNP rs1048638 ($p=0.019$) between women with cervical neoplasia and normal women (Table 1). No such difference was present in rs2071676, rs3829078 and 376deletion393. Genotypes CA/AA of CA9 SNP rs1048638 were found to be differently distributed between patients with cervical neoplasia and control women while assigning wild genotype CC as a reference ($p=0.016$). Women with genotype CA/AA still tended to have a more risk (AOR: 1.72, 95% CI: 1.00-2.94) in developing cervical neoplasia using CC as a reference after controlling for age.

After cervical neoplasia group was categorized into subgroups of invasive cancer and preinvasive lesions, a significant difference was revealed in the distribution of AA using GG/GA as a reference in CA9 SNP rs2071676 ($p=0.035$) among patients with invasive cancer or preinvasive lesions and control women (Table 2). However, AA in SNP rs2071676 increased the risk of developing cervical invasive cancer (OR: 1.65, 95% CI: 1.01-2.70) but did not increase the risk of preinvasive lesions (OR: 0.71, 95% CI: 0.39-1.30) using GG/GA as a reference. After control of the age, AA tended to have the risk of cervical cancer (AOR: 1.63, 95% CI: 0.94-2.87) while assigning GG/GA as a reference. Furthermore, women with CA or CA/AA displayed more risk to have cervical invasive cancer while assigning wild homozygote CC as a reference (OR: 2.57, 95% CI: 1.42-4.66 and OR: 2.42, 95% CI: 1.23-4.37, respectively; Table 2) but did not carry more risk of preinvasive lesions (OR: 1.38, 95% CI: 0.68-2.81 and OR: 1.30, 95% CI: 0.65-2.63, respectively) in CA9 SNP rs1048638. Even after controlling for the age, CA or CA/AA still displayed more risk of invasive cancer (AOR: 2.23, 95% CI: 1.13-4.39 and AOR: 2.15, 95% CI: 1.09-4.20, respectively; Table 2). However, no significant difference was found for genotypic distribution in rs3829078 and 376deletion393 among patients with invasive cancer or preinvasive lesions and controls.

Association of allelic distribution of CA9 gene polymorphisms among women with cervical invasive cancer or preinvasive lesions and normal women

The minor allelic frequencies of CA9 SNPs defined from control women in this study were 0.50 for rs2071676, 0.05 for rs3829078 and 0.06 for rs1048638, which were similar to those in HCB based on NCBI, dbSNP. Mutant allele A in CA9 SNP rs1048638 was the only one that increased the risk of cervical invasive cancer (AOR: 1.93, 95% CI: 1.02-3.64;

Table 3). Other CA9 genetic polymorphisms did not display this risk. In addition, CA9 allelic distribution was not associated with the development of cervical preinvasive lesions.

The constructed haplotypes and diplotypes of CA9 genetic polymorphisms and their involvement in cervical invasive cancer for Taiwanese women

Based on PHASE program, we constructed the phased haplotypes of four points (rs2071676, 376del393, rs3829078 and rs1048638) in CA9 gene. The haplotypes were showed rs2071676, 376del393, rs3829078 and rs1048638 in order. No mutant alleles and no deletion of haplotype (G1AC; 1, insertion) was used as a reference for analysis. We found that only haplotype A1AA tended to increase the risk of cervical invasive cancer (p=0.053; AOR: 2.01, 95% CI: 0.99-4.07; Table 4), assigning G1AC as a reference. Therefore, we compared the risk of CA9 diplotypes carrying at least one A1AA with other types of diplotypes to assess the risk of cervical cancer. We found that diplotypes carrying at least one A1AA significantly increase the risk of cervical invasive cancer in comparison with other diplotypes (p=0.035; AOR: 2.10, 95% CI: 1.05-4.20; Table 5). Diplotypes carrying at least one A1AA still did not increase the risk of preinvasive lesions (AOR: 1.58, 95% CI: 0.77-3.23)

Table 1. Genotypic distribution of single nucleotide polymorphisms of carbonic anhydrase 9 gene in patients with uterine cervical neoplasia and normal women.

Variables	Normal controls (n = 324)	Cervical neoplasia[b] (n = 185)	p value	OR (95% CI)	AOR (95% CI)[c]
rs2071676					
Co-dominant			0.739		
GG[d]	79	41		1.00	1.00
GA	168	95		1.09 (0.69-1.72)	0.98 (0.61-1.58)
AA	77	49		1.23 (0.73-2.06)	1.13 (0.66-1.96)
Dominant			0.570		
GG[d]	79	41		1.00	1.00
GA/AA	245	144		1.13 (0.72-1.79)	1.03 (0.65-1.62)
Recessive			0.494		
GG/GA[d]	247	136		1.00	1.00
AA	77	49		1.16 (0.74-1.78)	1.15 (0.74-1.78)
rs3829078					
Co-dominant			0.749		
AA[d]	294	168		1.00	1.00
AG	29	17		1.03 (0.55-1.92)	0.98 (0.50-1.93)
GG	1	0		u.a.	u.a.
Dominant			0.979		
AA[d]	294	168		1.00	1.00
AG/GG	30	17		0.99 (0.50-1.92)	0.94 (0.54-2.10)
Recessive			1.000		
AA/AG[d]	323	185		1.00	1.00
GG	1	0		u.a.	u.a.
rs1048638					
Co-dominant			0.019[a]		
CC[d]	289	151		1.00	1.00
CA	33	34		1.97 (1.18-3.31)	1.81 (1.05-3.12)
AA	2	0		u.a.	u.a.
Dominant			0.016[a]		
CC[d]	289	151		1.00	1.00
CA/AA	35	34		1.86 (1.08-3.20)	1.72 (1.00-2.94)
Recessive			0.536		
CC/CA[d]	322	185		1.00	1.00
AA	2	0		u.a.	u.a.
376deletion393					
Ins/ins[d]	246	144	0.714	1.00	1.00
Ins/del	76	39		0.88 (0.57-1.36)	0.82 (0.51-1.30)
Del/del	2	2		1.71 (0.24-12.26)	2.07(0.29-15.04)
Ins/ins[d]	246	144	0.624	1.00	1.00
Ins/del or del/del	78	41		0.90 (0.57-1.41)	0.85 (0.53-1.34)
Ins/ins or ins/del[d]	322	183	0.624	1.00	1.00
Del/del	2	2		1.76 (0.13-24.43)	2.17(0.30-15.63)

Statistical analysis: logistic regression model, chi-square or Fisher's exact tests, [a]$p < 0.05$. [b]Cervical neoplasia included preinvasive squamous lesions and invasive squamous cell carcinoma of uterine cervix. [c]The adjusted odds ratio with its 95% confident interval was estimated by logistic regression after controlling for age. [d]Used as references for comparison to evaluate the odds ratios of other genotypes. AOR, adjusted odds ratio; 95% CI, 95% confidence interval. Del, deletion; ins, insertion; u.a., unavailable.

Table 2. Genotypic distribution of single nucleotide polymorphisms of carbonic anhydrase 9 gene in patients with invasive cancer or preinvasive lesions of uterine cervix and normal women.

Variables	Controls (n=324)	Preinvasive lesions (n=88)	Invasive cancer (n=97)	*p* value	OR (95% CI)[b]	OR (95% CI)[c]	AOR (95% CI)[d]
rs2071676							
Co-dominant				0.068			
GG[e]	79	18	23		1.00	1.00	1.00
GA	168	54	41		1.41 (0.78- 2.56)	0.84 (0.47-1.49)	0.65 (0.34-1.26)
AA	77	16	33		0.91 (0.43-1.92)	1.47 (0.79-2.73)	1.22 (0.61-2.48)
Dominant				0.743			
GG[e]	79	18	23		1.00	1.00	1.00
GA/AA	245	70	74		1.26 (0.70-2.23)	1.04 (0.61-1.77)	0.82 (0.45-1.51)
Recessive				0.035[a]			
GG/GA[e]	247	72	64		1.00	1.00	1.00
AA	77	16	33		0.71 (0.39-1.30)	1.65 (1.01-2.70)	1.63 (0.94-2.87)
rs3829078							
Co-dominant				0.939			
AA[e]	294	79	89		1.00	1.00	1.00
AG	29	9	8		1.16 (0.53-2.54)	0.91 (0.40-2.07)	0.97 (0.38-2.45)
GG	1	0	0		u.a.	u.a.	u.a.
Dominant				0.897			
AA[e]	294	79	89		1.00	1.00	1.00
AG/GG	30	9	8		1.12 (0.51-2.45)	0.88 (0.39-1.99)	0.91 (0.36-2.31)
Recessive				0.751			
AA/AG[e]	323	88	97		1.00	1.00	1.00
GG	1	0	0		u.a	u.a	u.a
rs1048638							
Co-dominant				0.024[a]			
CC[e]	289	76	75		1.00	1.00	1.00
CA	33	12	22		1.38 (0.68-2.81)	2.57 (1.42-4.66)	2.23 (1.13-4.39)
AA	2	0	0		u.a.	u.a.	u.a.
Dominant				0.011[a]			
CC[e]	289	76	75		1.00	1.00	1.00
CA/AA	35	12	22		1.30 (0.65-2.63)	2.42 (1.34-4.37)	2.15 (1.09-4.20)
Recessive				0.564			
CC/CA[e]	322	88	97		1.00	1.00	1.00
AA	2	0	0		u.a.	u.a.	u.a.
376deletion393							
Ins/ins[e]	246	70	74	0.355	1.00	1.00	1.00
Ins/del	76	16	23		0.74 (0.41-1.35)	1.01 (0.59-1.72)	1.00 (0.54-1.86)
Del/del	2	2	0		3.51 (0.49-25. 40)	u.a.	u.a.
Ins/ins[e]	246	70	74	0.774	1.00	1.00	1.00
Ins/del or del/del	78	18	23		0.81 (0.46-1.45)	0.98 (0.58- 1.67)	0.98 (0.53-1.81)
Ins/ins or ins/del[e]	322	86	97	0.184	1.00	1.00	1.00
Del/del	2	2	0		3.75 (0.52-27.03)	u.a.	u.a.

Statistical analysis: multiple logistic regression or chi-square or Fisher's exact tests, [a]$p < 0.05$. [b]Comparison between patients with cervical preinvasive squamous lesions and control women. [c]Comparison between patients with cervical invasive squamous cell carcinoma and control women. [d]The adjusted odds ratio with its 95% CI was estimated by multiple logistic regression models after controlling for age between cancer patients and control women. [e]Used as references for comparison to evaluate the odds ratios of other genotypes. AOR, adjusted odds ratio; 95% CI, 95% confidence interval. Del, deletion; ins, insertion; u.a., unavailable.

Table 3. Allelic frequency of single nucleotide polymorphisms of carbonic anhydrase 9 in patients with invasive cancer or preinvasive lesions of uterine cervix and normal women.

Variables	Normal controls (n=324)	Preinvasive lesions (n=88)	Invasive cancer (n=97)	*p* value	AOR (95% CI)[b]	AOR (95% CI)[c]
rs2071676						
G[d]	326	90	87	0.361	1.00	1.00
A	322	86	107		0.88 (0.68-1.36)	1.14 (0.79-1.65)
rs3829078						
A[d]	617	167	186	0.896	1.00	1.00
G	31	9	8		0.90 (0.39-2.09)	0.87 (0.35-2.14)
rs1048638						
C[d]	611	164	172	0.026[a]	1.00	1.00
A	37	12	22		1.29 (0.65-2.54)	1.93 (1.02-3.64)
376deletion393						
Insertion[d]	568	156	171	0.934	1.00	1.00
Deletion	80	20	23		0.90 (0.53-1.54)	0.96 (0.54-1.70)

Statistical analysis: chi-square and multiple logistic regression models for the AOR with its 95% CI after controlling for age; [a]$p < 0.05$. [b]Comparison between patients with cervical preinvasive squamous lesions and normal women. [c]Comparison between patients with cervical invasive saquamous cell carcinoma and normal women. [d]used as a reference to evaluate the odds ratio of another subtype. AOR, adjusted odds ratio; 95% CI, 95% confidence interval.

Table 4. Haplotypes distribution of carbonic anhydrase 9 (CA9) gene in patients with invasive cancer or preinvasive lesions of uterine cervix and control women.

CA9 haplotypes[a]	Control women	Patients with preinvasive lesions	Patients with invasive cancer	AOR and 95% CI[b]	AOR and 95% CI[c]
G1AC	215	61	55	1.00 (reference)	1.00 (reference)
A1AC	286	74	86	0.87 (0.59-1.29)	1.09 (0.71-1.67)
A1AA	34	12	21	1.30 (0.63-2.67)	2.01 (0.99-4.07)
A0AC	1	0	0	u.a.	u.a.
A0AA	1	0	0	u.a.	u.a.
G1AA	2	0	1	u.a.	u.a.
G1GC	31	9	8	0.84 (0.35-2.01)	0.97 (0.38-2.49)
G0AC	78	20	23	0.86 (0.38-1.55)	1.11 (0.59-2.09)

Statistical analysis: the adjusted OR with its 95% CI was estimated by multiple logistic regression models after controlling for age. [a]Single nucleotide polymorphisms and 376deletion393 of CA9 gene in order: rs2071676, 376del393, rs3829078 and rs1048638. 1, insertion; 0, deletion; G1AC was used as a reference. [b]Comparison between patients with cervical preinvasive squamous lesions and normal women.[c]Comparison between patients with cervical invasive squamous cell carcinoma and normal women. AOR, adjusted odds ratio; 95% CI, 95% confidence interval; u.a., unavailable.

Table 5. Diplotypes distribution of carbonic anhydrase (CA9) genetic polymorphisms in patients with invasive cancer or preinvasive lesions of uterine cervix and control women.

CA9 diplotypes[a]	Control women	Patients with preinvasive lesions	Patients with invasive cancer	AOR and 95% CI[b]	AOR and 95% CI[c]
Others/others	292	76	76	1.00 (reference)	1.00 (reference)
A1AA/others	32	12	21	1.58 (0.77-3.23)	2.10 (1.05-4.20)

Statistical analysis: the adjusted OR with its 95% CI was estimated by multiple logistic regression models after controlling for age. [a]Single nucleotide polymorphisms and 376deletion393 of CA9 gene in order: rs2071676, 376del393, rs3829078 and rs1048638. 1, insertion. [b]Comparison between patients with cervical preinvasive squamous lesions and normal women. [c]Comparison between patients with cervical invasive squamous cell carcinoma and normal women. AOR, adjusted odds ratio; 95% CI, 95% confidence interval.

Discussion

This study revealed that genotypes CA/AA increase the susceptibility of Taiwanese women to cervical squamous neoplasia, assigning wild homozygote CC as a reference in CA9 SNP rs1048638. The increased risk to cervical neoplasia relied on the significant increase to the development of cervical invasive squamous cell carcinoma but not preinvasive squamous lesions because no significant difference of genotype distribution was found in SNP rs1048638 between patients with preinvasive lesions and control women. Even though controlling for age, the risk still existed. However, mutant homozygote AA tended to increase the susceptibility of Taiwanese women to cervical invasive cancer using GG/GA as a reference in rs2071676. The dominant promoting risk effect of invasive cancer from CA/AA in CA9 rs1048638 was supported by the analysis of allelic frequency. Only one mutant allele A was strong enough to increase the risk of cervical invasive cancer in CA9 SNP rs1048638. In contrast, paired alleles mutation (mutant homozygote AA) may be needed to have the tendency of developing cervical invasive cancer using GG/GA as a reference in CA9 SNP rs2071676.

CA9 was found to be overexpressed in many cancers and suggested as a common feature of cancer cells [30, 33-42]. It indicated that CA9 was required for tumor progression. This enzyme may result in intracellular alkalization for cancer cells survival and proliferation, as well as produce and maintain an extracellular acidic tumor microenvironment for cancer cell invasiveness in hypoxic condition [12, 14]. Although moderate/strong CA9 expression was associated with squamous cell carcinoma of uterine cervix [43], no study relates the CA9 genetic polymorphisms to cervical invasive cancer. In agreement with our finding for cancer, the CA9 SNP rs1048638 was demonstrated to be able to predict the susceptibility of urothelial cell carcinoma in Taiwan [31]. The CA9 gene, which is located on chromosome 9p13-p12, comprises 11 exons and encodes for a 459 amino acid protein [24]. The CA9 SNP rs1048638 (+1584, C/A) is in the 3′-UTR of CA9 gene exon 11. A SNP in a 3′-UTR of a gene probably exerts an influence on biological processes [25, 32]. Bioinformatics analysis reveals that microRNAs may bind the 3′-UTR where the CA9 SNP rs1048638 locates based on the TargetScanHuman prediction server. This binding may exhibit an impact on the expression of CA9 protein [26, 30]. A nucleotide from cytosine to mutant adenine may affect the mircoRNA/target duplexes interaction and further exerts an impact on the expression of CA9 protein. In addition to CA9 SNP rs1048638, Chien et al. also found that rs2071676 has potential to predict oral carcinogenesis significantly in Taiwan [29].

Similar to our finding, the guanine replaced by adenine in CA9 SNP rs2071676 was found in 59% of tumors in patients who had renal cell carcinoma with distant metastasis [32]. SNPs may exert the nonsynonymous function by changing the encoded amino acids, in addition to occurring in noncoding region and being silent, i.e. synonymous [25]. They may affect gene expression and mRNA conformation and thereafter have an impact on disease. The CA9

SNP rs2071676 (+201, G/A) is located on exon 1 in chromosome 9p13.3 and changing the encoded amino acids from valine to methionine may lead to a nonsynonymous function via a nucleotide from G to mutant A in a coding sequence [25, 32]. The region, where the C A9 SNP rs2071676 is located, is concerned with the signal peptide of CA9 and probably affects its function [32].

Haplotype association mapping has been reported to provide a method that identifies susceptibility genes and molecular pathways, underlying a given trait [44]. Moreover, It has been showed that haplotypes, containing each genetic polymorphism, may have a strong statistical power to demonstrate the susceptibility of disease and are even better than individual SNP analysis for the association of alleles with disease phenotypes [45]. Haplotypes have important and clinically relevant associations with diseases such as Parkinson's disease and schizophrenia [46, 47]. We found that haplotype A1AA tends to increase the risk of cervical invasive cancer. Taiwanese women with ditplotypes, which carried at least one A1AA, were significantly susceptible to cervical invasive cancer.

This study has two important features. Firstly, to our knowledge, this is the first study to clarify that the CA9 genetic polymorphisms are not related to the development of cervical preinvasive squamous cell lesions for Taiwanese women. Secondly, we utilize the CA9 haplotypes and diplotypes, in addition to CA9 SNPs, to strengthen the roles of CA9 genetic polymorphisms in the formation of cervical squamous cell carcinoma. However, there are two limitations in this study. Firstly, the sample sizes of patients with cervical preinvasive lesions or invasive cancer were small. More sample sizes are needed to strengthen our results in the future. Secondly, the detection rates of human papillomavirus (HPV) in studied subgroups were lacking. This was partially attributed to the too conservative attitude for the control women to accept the HPV test. The HPV test was still no generalized in Taiwan. To our knowledge, this study is however the first report to associate the CA9 genetic polymorphisms with the development of uterine cervical cancer.

In conclusion, Taiwanese women with genotypes CA or CA/AA in CA9 SNP rs1048638 display a more risk in developing invasive squamous cell carcinoma of uterine cervix, using wild genotype CC as a reference. Importantly, when they have the diplotypes, carrying at least one A1AA, they are significantly susceptible to cervical invasive squamous cell carcinoma. However, CA9 genetic polymorphisms are not associated with the development of cervical preinvasive squamous lesions.

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Competing Interests

The authors have declared that no competing interest exists.

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