CTLA-4 gene and susceptibility to human papillomavirus-16-associated cervical squamous cell carcinoma in Taiwanese women

Tsung-Hsien Su1,4,1, Tzu-Yang Chang2,1, Yann-Jinn Lee3,5,6, Chih-Kai Chen2, Hsin-Fu Liu2, Chen-Chung Chu2, Marie Lin2, Pu-Tsui Wang1, Wen-Chu Huang1, Tze-Chien Chen1 and Yuh-Chang Yang1,2,4,5,6

1Department of Gynecology and Obstetrics, 2Department of Medical Research and 3Department of Pediatrics, Mackay Memorial Hospital, 45, Min-Sheng Road, Tamshui 25115, Taipei, Taiwan, 5Department of Medicine, Nursing and Management College, Taipei 11260, Taiwan, 6Department of Gynecology and Obstetrics and 7Department of Pediatrics, Taipei Medical University, Taipei 11031, Taiwan

Human papillomavirus (HPV) is considered to be a necessary but not sufficient cause for cervical cancer. The host immunogenetic background plays an important role in the persistence of HPV infection and subsequent development of cervical cancer. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a molecule expressed mainly on activated T cells and is important in the down-regulation of T-cell activation. The aim of this study was to determine if polymorphisms of the CTLA-4 gene are associated with HPV-induced cervical cancer in Taiwanese women. Polymerase chain reaction–restriction fragment length polymorphism was used to genotype –318 CT, +49 AG and CT60 A/G polymorphisms in 144 women with cervical squamous cell carcinoma (CSCC) and 378 ethnicity-matched healthy control women. The presence and genotypes of HPV in CSCC were determined by E6-, E7-based nested polymerase chain reaction. The frequency of CT7 genotype of –318 CT polymorphism was significantly higher in patients with HPV-16-positive CSCC compared with controls (odds ratio = 1.99, 95% confidence interval = 1.16–3.42, P = 0.03). No significant associations were found for +49 AG and CT60 A/G polymorphisms. Analysis of haplotypes, computationally inferred from genotype data, also revealed no significant differences in distribution among all subjects with CSCC, those with HPV-16-positive CSCC and controls. Our results suggest that the –318 CT7 variant in the promoter region of the CTLA-4 gene is associated with HPV-16-associated CSCC in Taiwanese women.

Introduction

Carcinoma of the cervix is a major cause of mortality for women in developing countries. It is a serious health problem in Taiwan, with 2700 women developing this disease each year, second in incidence only to breast cancer (1). It is widely accepted that specific oncogenic human papillomaviruses (HPVs) are primary etiologic factors in malignancies of the uterine cervix (2). However, the majority of infected women do not develop the cancer. Other environmental and host factors must also play a role in the persistence of HPV infection and the subsequent development of cervical cancer, but these factors have not received nearly the same amount of research attention as has the virus itself.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4), encoded by a gene on chromosome 2q33, is a receptor expressed by activated T lymphocytes. It counteracts the stimulation initiated by the CD 28 molecule (3). Several reports indicate that CTLA-4 gene polymorphisms are implicated in both autoimmune diseases (Graves disease, Hashimoto thyroiditis, Addison disease, type 1 diabetes, celiac disease and rheumatoid arthritis) (4–7) and malignancy susceptibility (8–11). Mice deficient in CTLA-4 develop a severe lymphoproliferative disorder, autoimmune disease and die early (12,13). Non-obese diabetic mice, an animal model of autoimmune diabetes, have lymphocytes with reduced expression of CTLA-4 (14). Additionally, CTLA-4 blockade leads to enhancement of the immune response (15), rejection of tumors (16) or even cure of tumors in mice when used in combination with tumor vaccines (17).

The CTLA-4 gene consists of four exons. The most frequently studied polymorphisms are a C/T transition in the –318 position of the promoter sequence, an A/G transition in exon 1 at position +49, a dinucleotide (AT) repeat in the 3′-untranslated region and more recently an A/G transition in the 3′-untranslated region at +6230 (CT60) (18). Significantly increased expression of CTLA-4 mRNA and protein have been shown in individuals carrying thymine at position –318 of the CTLA-4 promoter and those homozygous for adenine at position 49 in exon 1 (19). Ueda et al. (18) reported that the G allele at CT60 position was associated with a 50% decrease in the soluble CTLA-4 isoform. Several lines of evidence have shown that variant single-nucleotide polymorphisms (SNPs) in the CTLA-4 gene exert differential effects on gene expression and on T-cell activity (19–22), including the vigor of the T-cell response to viral infection (23–25). Furthermore, immune responses mediated by T cells, especially cytotoxic T lymphocytes, are important in controlling both HPV infections and HPV-associated cancers (26). A number of studies of cervical cancer have focused on genetic polymorphisms of the human leukocyte antigen (HLA) (27–29), tumor necrosis factor-α (TNF-α) (30–32), matrix metalloproteinase-1 (MMP-1) (33,34), Fas (35,36), p53 (37) and interferon-γ (IFN-γ) genes (38). Most of these studies have demonstrated that development of cervical cancer is associated with these genetic polymorphisms. However, CTLA-4 gene polymorphisms have not yet been examined with respect to cervical cancer.

Given the pivotal role of CTLA-4 in regulating the immune response, we designed this study to test the hypothesis that specific CTLA-4 SNPs (i.e. –318 CT7, +49 AG and CT60 AG) are associated with HPV-associated cervical squamous cell carcinoma (CSCC).

Materials and methods

Study subjects

A hospital-based case–control study was conducted including 144 unrelated women with CSCC (mean age at diagnosis 48.3 ± 10.8 years) residing in northern Taiwan. The diagnosis of CSCC was confirmed in all cases by histological examination of tissue from biopsy or resected specimens. Three hundred and seventy-eight control women (mean age at sampling 40.2 ± 4.6 years) were randomly selected among the female population who sought pa
ternity testing service in Mackay Memorial Hospital. All patients and controls provided written informed consent according to the procedures approved by the Institutional Review Board of Mackay Memorial Hospital, Taiwan.

DNA extraction

Formalin-fixed, paraffin-embedded tissue blocks from patients were sectioned and dewaxed, and genomic DNA was then extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol for HPV typing. Genomic DNA was extracted as previously described from peripheral blood leukocytes from patients and controls for CTLA-4 genotyping (39).

HPV detection and typing

Tissue blocks for HPV DNA genotyping were obtained for 137 (95%) of the 144 patients. No tissue samples were available in seven patients because they had had a hysterectomy in other hospitals before they were enrolled in this study. HPV DNA was detected and typed in cervical tissue samples by using E6-, E7-based nested polymerase chain reaction. 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 polymerase chain reaction 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 378 control subjects.

**CTLA-4 genotyping**

Using DNA extracted from peripheral blood leukocytes, \( -318 \, C/T \), \( +49 \, A/G \) and \( CT60 \) polymorphisms were examined by polymerase chain reaction–restriction fragment length polymorphism using MseI, BstEII and Ncol enzymes (New England BioLabs, Beverly, MA), respectively. The \( -318 \, C/T \) polymorphism was amplified with modified primers 5'-GTAGGGATGCC-CAGAAGAT-3' and 5'-CTCAACTGAAACAAACAAGC-3' (40) resulting in a 172 bp product. The \( +49 \, A/G \) polymorphism was amplified with primers 5'-AAGGCTAGCTGGAACTGTTG-3' and 5'-CTGTGAAACAATGGA-ACCC-3' (41) resulting in a 153 bp product. The \( CT60 \) polymorphism was amplified with primers 5'-CACCACTATTGGGATATACC-3' and 5'-AGGCTCTATTTCCAGGAAAGGC-3' (42) resulting in a 216 bp product. The amplified products were digested overnight and analyzed on 3.5% agarose gel.

The \( -318 \, C/T \) polymorphisms were as follows—C/C: indicated by one 172 bp band; C/T: a 172, 96 and 76 bp band and T/T: a 96 and 76 bp band (Figure 1A). The \( +49 \, A/G \) polymorphisms were A/A: a 131 and 22 bp band; A/G: a 153, 131 and 22 bp band and G/G: a 153 bp band (Figure 1B). The \( CT60 \) polymorphisms were A/A: a 196 and 20 bp band; A/G: a 216, 196 and 20 bp band and G/G: a 216 bp band (Figure 1C). The 22 and 20 bp enzyme-generated bands indicating \( +49 \, A/G \) and \( CT60 \) A/A and A/G polymorphisms were too small to be seen in the gels.

**Statistical analysis**

Genotype, allele and phenotype frequencies of the \( -318 \, C/T \), \( +49 \, A/G \) and \( CT60 \) polymorphisms were determined by direct counting. The Hardy–Weinberg equilibrium (HWE) was assessed for each SNP in the control group by chi-square analysis. The genotype, allele and phenotype distributions of controls and all patients with CSCC or of controls and patients with HPV-16-positive CSCC were also compared by a chi-square test (3 \( \times \) 2 or 2 \( \times \) 2 contingency tables) with Yates' correction where appropriate (one expected number < 5). Odds ratios (ORs) and 95% confidence intervals (95% CIs) for particular genotypes, alleles and phenotypes associated with the risk of all CSCC or HPV-16-positive CSCC were calculated. The statistical software used was developed at our hospital and is available on request (43).

A haplotype is a combination of SNPs in a gene or a locus that occurs more often than expected by chance, indicating linkage disequilibrium in the region. For the haplotype study, frequencies of \( CTLA-4 \) haplotypes (\( -318 \, C/T \), \( +49 \, A/G \) and \( CT60 \)) in controls and in all patients with CSCC as well as those with HPV-16-positive CSCC were estimated using the Haploscope program (44). The haplotype frequencies were estimated with an expectation–maximization algorithm of the Haploscope program. Pairwise linkage disequilibrium between various SNPs was also tested by the Haploscope program, with statistical differences, ORs and 95% CIs for the association of various haplotypes with all CSCC or HPV-16-positive CSCC were determined. The Bonferroni correction was used to correct for multiple comparisons where appropriate. \( P \) values of <0.05 (two tailed) were considered to be statistically significant.

**Results**

HPV DNA was detected in 88.3% of the 137 tumors tested, with 58.4% positive for HPV type 16, 9.5% for HPV type 18 and 20.4% for other HPV types (including 2.9% positive for HPV type 31, 4.4% for HPV type 33, 0.7% for HPV type 35, 2.2% for HPV type 45, 2.2% for HPV type 52, 7.3% for HPV type 38 and 0.7% for HPV type 69). The \( -318 \, C/T \) SNP in the \( CTLA-4 \) gene was successfully genotyped in 378 controls, 144 women with CSCC and 80 women with HPV-16-positive CSCC (Table 1). The genotype frequencies of the controls were in HWE (\( P = 0.83 \)). The distribution of genotypes, alleles and phenotypes of \( -318 \, C/T \) SNP was not significantly different between controls and all patients with CSCC except for the C/T genotype, which was more frequent in all CSCC patients as compared with controls (26.4 versus 17.7%, \( OR = 1.66, 95\% \, CI = 1.06–2.62, P_c = 0.09 \)). The genotypes differed significantly between women with HPV-16-positive CSCC and controls (\( P = 0.03, \chi^2 = 7.07, 2 \) df), but the distribution of alleles and phenotypes did not differ. In particular, the frequency of the C/T genotype was significantly greater (\( OR = 1.99, 95\% \, CI = 1.16–3.42, P_c = 0.03 \)) and the C/C genotype was less prevalent (\( OR = 0.55, 95\% \, CI = 0.32–0.94, P_c = 0.09 \)) among those patients as compared with controls. The T-allele phenotype occurred more frequently in HPV-16-positive CSCC as compared with controls (\( OR = 1.82, 95\% \, CI = 1.06–3.12, P_c = 0.06 \)).

The \( +49 \, A/G \) SNP was successfully genotyped in 375 controls, 139 patients with CSCC and 75 patients with HPV-16-positive CSCC (supplementary Table SII is available at Carcinogenesis Online). The genotypes frequencies of the controls were in HWE (\( P = 0.65 \)). There were no significant differences in the frequencies of \( +49 \, A/G \) genotypes, alleles and phenotypes between all women with CSCC and controls or between women with HPV-16-positive CSCC and controls.

The \( CT60 \) SNP was successfully genotyped in 378 controls, 139 women with CSCC and 76 patients with HPV-16-positive CSCC (supplementary Table SIII is available at Carcinogenesis Online). The genotypes in the controls did not significantly deviate from the HWE (\( P = 0.98 \)). As with the \( +49 \, A/G \) SNP, genotype, allele and phenotype frequencies did not differ significantly between any of the groups tested. Linkage disequilibrium analysis among these SNPs revealed remarkable disequilibrium between \( -318 \, C/T \) and \( +49 \, A/G \) (\( D' = 0.95 \)), \( +49 \, A/G \) and \( CT60 \) (\( D' = 0.90 \)) and \( -318 \, C/T \) and \( CT60 \) (\( D' = 0.70 \)) in controls.
**CTLA-4 gene and susceptibility to HPV-16-associated cervical squamous cell carcinoma**

**Table I.** Genotype, allele and phenotype frequencies of the −318 C/T polymorphism in controls and in women with all CSCC and those with HPV-16-positive CSCC

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (N = 378)</th>
<th>All CSCC (N = 144)</th>
<th>HPV-16-positive CSCC (N = 80)</th>
<th>All CSCC</th>
<th>HPV-16-positive CSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>P value (χ²)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>C/C</td>
<td>306 (81.0)</td>
<td>105 (72.9)</td>
<td>56 (70.0)</td>
<td>0.08 (5.10)</td>
<td>0.63 (0.41–1.00)</td>
</tr>
<tr>
<td>C/T</td>
<td>67 (17.7)</td>
<td>38 (26.4)</td>
<td>24 (30.0)</td>
<td>1.66 (1.06–2.62)</td>
<td>0.52 (0.08–3.41)</td>
</tr>
<tr>
<td>T/T</td>
<td>5 (1.3)</td>
<td>1 (0.7)</td>
<td>0 (0.0)</td>
<td>1.82 (1.06–3.12)</td>
<td>0.42 (0.05–3.55)</td>
</tr>
<tr>
<td>Allele C</td>
<td>679 (89.8)</td>
<td>248 (86.1)</td>
<td>136 (85.0)</td>
<td>0.09 (2.88)</td>
<td>0.70 (0.47–1.06)</td>
</tr>
<tr>
<td></td>
<td>77 (10.2)</td>
<td>40 (13.9)</td>
<td>24 (15.0)</td>
<td>1.42 (0.95–2.14)</td>
<td>1.56 (0.95–2.54)</td>
</tr>
<tr>
<td>Allele T</td>
<td>373 (98.7)</td>
<td>143 (99.3)</td>
<td>80 (100.0)</td>
<td>0.12 (2.44)</td>
<td>1.92 (0.29–12.46)</td>
</tr>
<tr>
<td></td>
<td>72 (19.0)</td>
<td>39 (27.1)</td>
<td>24 (30.0)</td>
<td>1.58 (1.00–2.47)</td>
<td>0.55 (0.32–0.94)</td>
</tr>
</tbody>
</table>

\*P value for the C/T genotype versus the other genotypes: P value after correction (P<sub>c</sub> = 0.03.

We also analyzed the possible haplotypes constructed by −318 C/T, +49 A/G and CT60 SNPs in controls, all women with CSCC and those with HPV-16-positive CSCC. Of the seven observed haplotypes, CCG was the most prevalent, but the difference was not statistically significant (supplementary Table SIV is available at Carcinogenesis Online). No significant differences were found in any haplotypes between any of the groups tested.

**Discussion**

In this study, we investigated a specific SNP or haplotype of the CTLA-4 gene in Taiwanese women with CSCC. We found that women with HPV-16-positive tumors had a significantly higher frequency of the C/T genotype at position −318 in the CTLA-4 promoter region as compared with healthy controls. No other significant associations were found between genotypes, alleles and phenotypes of +49 A/G and CT60 SNPs among women with all CSCC, those with HPV-16-positive CSCC and healthy control women. Analysis of haplotype distribution also demonstrated a lack of differences among the groups tested. However, we cannot exclude the possibility that a (as yet unidentified) true disease-causing haplotype exists in the CTLA-4 gene. It is interesting to speculate that −318 C/T is a part of both associated and non-associated haplotypes, and a combination of functional variants located in the promoter region, rather than other single variants outside this region, is required to predispose to HPV-16-positive CSCC.

Since the CTLA-4 gene product has inhibitory effects on the immune system, any variation in its expression or function may lead to the breakdown of the delicate homeostasis of this system. Various alleles of this gene contribute to susceptibility to a wide variety of disorders such as Graves disease, Hashimoto thyroiditis, Addison disease, type 1 diabetes, celiac disease and rheumatoid arthritis (4–7). Abnormalities of CTLA-4 may also contribute to susceptibility to certain malignancies (8–11). Understanding the genetic basis of the resulting disordered immune responses may allow for the development of new management strategies for both autoimmune diseases and cancer.

Considering CTLA-4 promoter region is the binding site for transcription factors and regulates the expression level of the gene, it is conceivable that the C/T transition in the −318 position of the promoter site may affect the expression of CTLA-4. In fact, some studies of functional role of CTLA-4 promoter polymorphism in gene expression have been reported (19,22). The association between CTLA-4 promoter polymorphism and various malignancies has also been investigated (9,11). The higher frequency of the heterozygous variant allele (C/T) in our patients with HPV-16-positive tumors leads us to infer that T cells from these patients would have higher levels of CTLA-4 following T-cell activation than would those from controls. The increased expression of CTLA-4 and the resulting down-regulation of the immune system might then contribute to the progression of HPV-16-associated CSCC.

The immune system normally plays a pivotal role in the outcome of infections with exogenous agents like HPV. The immune response raised against HPV determines whether the virus will be cleared or whether it will persist and, in some individuals, eventually result in CSCC. Patients with defects in cellular immune competence are more likely to remain chronically infected rather than to clear the virus, supporting the role of cellular immune molecules like CTLA-4 in the pathogenesis of HPV-related cancers. Differences in cellular immunity regulated by CTLA-4 might explain variations in the outcomes of viral persistence or clearance. The hypothesis that polymorphisms of the CTLA-4 gene might be involved in the clearance or persistence of hepatitis B and hepatitis C viruses has been supported experimentally (23–25). A number of investigators have also reported that immune responses mediated by T cells, including CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T lymphocytes, are important in controlling HPV-associated neoplasms (45,46). These studies as well as our own results support the inference that specific polymorphisms of the CTLA-4 gene may increase susceptibility to persistent HPV infections, thus increasing the risk of CSCC.

Epidemiologic studies based on comparisons between case and control groups do not, of course, prove a cause-and-effect relationship even when a statistically significant association is present. Additionally, our study may have been underpowered to examine −318 T homozygosity. However, a study such as ours may indicate particular polymorphisms that can be evaluated as risk markers for HPV-associated CSCC. If further investigation bears out the utility of such a marker, it could identify high-risk women who require more frequent cervical cancer screening or who are priority candidates for HPV vaccine prophylaxis.

In summary, the present study demonstrates that C/T genotype in the CTLA-4 −318 promoter region is more frequent in Taiwanese women with HPV-16-positive CSCC. In theory, this genotype might render an individual more susceptible to persistent HPV-16 infection and the development of CSCC.

**Supplementary material**

Supplementary Tables SII–SIV can be found at http://carcin.oxfordjournals.org/

**Acknowledgements**

We thank Dr Mary Jeanne Buttery for her review and criticism of this paper. This research was supported by the Mackay Memorial Hospital, Taiwan (grants MMH 9480 and MMH-E 94007).

**Conflict of Interest Statement:** None declared.
References


Received January 5, 2007; revised February 13, 2007; accepted February 16, 2007