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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 C/T, +49 A/G 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 C/T genotype of HPV in CSCC were determined by E6-, E7-based nested polymerase chain reaction. The results suggest that the −318 C/T 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). It counteracts the stimulation initiated by the CD 28 molecule (3).

Abbreviations: CSCC, cervical squamous cell carcinoma; CTLA-4, cytotoxic T-lymphocyte antigen-4; 95% CI, 95% confidence interval; HPV, human papillomavirus; HWE, Hardy–Weinberg equilibrium; OR, odds ratio; SNP, single-nucleotide polymorphism.

These authors contributed equally to this work.

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 subjects (mean age at sampling 40.2 ± 4.6 years) were randomly selected among the female population who sought gynecological examination 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.

DNA extraction

Formalin-fixed, paraffin-embedded tissue blocks from patients were sectioned and deparaffinized, 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 from 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- and E7-based nested polymerase chain reaction. A pair of degenerate primers, 

cated 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)n 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 C/T, +49 A/G and CT60 A/G) are associated with HPV-associated cervical squamous cell carcinoma (CSCC).

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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 Msel, BstEI and Ncol enzymes (New England BioLabs, Beverly, MA), respectively. The −318 C/T polymorphism was amplified with modified primers 5′-GTAGGGTGCC-CAGAAGAT-3′ and 5′-CTCAAAGGACAAACCAAGGC-3′ (40) resulting in a 172 bp product. The +49 A/G polymorphism was amplified with primers 5′-AAGGTCTAGTGAACCTGGT-3′ and 5′-CTGTGAAACAAATGGA-ACCC-3′ (41) resulting in a 153 bp product. The CT60 polymorphism was amplified with primers 5′-CACCACTATTTGGGATATACC-3′ and 5′-AGGTCTATTTTGGGACAGGGG-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 test (3 df). Weinberg equilibrium was developed at our hospital and is available on request (43).

Haplotype frequencies were estimated with an expectation–maximization algorithm of the Haploview program. Pairwise linkage disequilibrium between various haplotypes, alleles and phenotypes associated with the risk of all HPV-16-positive CSCC were estimated using the Haploview program (44). The linkage disequilibrium in the region was remarkable. For the haplotype study, frequencies of various SNPs in controls was also tested by the Haploview program. Statistical algorithm of the Haploview program. The genotype frequencies of the controls were in HWE (45). As with the −318 C/T polymorphism analysis, the Hardy–Weinberg equilibrium (HWE) was assessed for each SNP in the control group by chi-square test (3 df). The distribution of alleles and phenotypes did not. 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 genotype 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.

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**Fig. 1.** Polymerase chain reaction–restriction fragment length polymorphism analysis of CTLA-4 SNPs. (A) −318 C/T SNP: 100 bp ladder in lane M. Genotypes C/C (lane 1; 172 bp), C/T (lane 2; 172, 96 and 76 bp) and T/T (lane 3; 96 and 76 bp) are shown. Lane 4 indicates undigested polymerase chain reaction product. (B) +49 A/G SNP: 100 bp ladder in lane M. Genotypes A/A (lane 1; 131 bp), A/G (lane 2; 153 and 131 bp) and G/G (lane 3; 153 bp) are shown. Lane 4 indicates undigested polymerase chain reaction product. The expected 22 bp in genotypes A/A and A/G could not be visualized. (C) CT60 A/G SNP: 100 bp ladder in lane M. Genotypes A/A (lane 1; 196 bp), A/G (lane 2; 216 and 196 bp) and G/G (lane 3; 216 bp) are shown. Lane 4 indicates undigested polymerase chain reaction product. The expected 20 bp in genotypes A/A and A/G could not be visualized.
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>No. (%)</th>
<th>Controls (N = 378)</th>
<th>All CSCC (N = 144)</th>
<th>HPV-16-positive CSCC (N = 80)</th>
<th>All HPV-16-positive CSCC</th>
<th>P value (χ²)</th>
<th>OR (95% CI)</th>
<th>P value (χ²)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<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>
<td>0.03 (7.07)</td>
<td>0.55 (0.32–0.94)</td>
<td></td>
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</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>
<td>1.99 (1.16–3.42) *</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>T/T</td>
<td>5 (1.3)</td>
<td>1 (0.7)</td>
<td>0 (0.0)</td>
<td>0.09 (2.88)</td>
<td>0.70 (0.47–1.06)</td>
<td>0.08 (3.12)</td>
<td>0.64 (0.39–1.05)</td>
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<tr>
<td>Allele</td>
<td></td>
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<td></td>
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<tr>
<td>C</td>
<td>679 (89.8)</td>
<td>248 (86.1)</td>
<td>136 (85.0)</td>
<td>1.58 (1.00–2.47)</td>
<td>1.82 (1.06–3.12)</td>
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<tr>
<td>T</td>
<td>77 (10.2)</td>
<td>40 (13.9)</td>
<td>24 (15.0)</td>
<td>1.19 (0.70–1.99)</td>
<td>1.56 (0.95–2.54)</td>
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<tr>
<td>Phenotype</td>
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<td></td>
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<tr>
<td>C</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>
<td>0.10 (2.78)</td>
<td>2.37 (0.28–19.96)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</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>1.82 (1.06–3.12)</td>
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*P value for the C/T genotype versus the other genotypes: P value after correction (Pc) = 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, CGG 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 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 CSCC. 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 recently been supported experimentally (23–25). A number of investigators have also reported that immune responses mediated by T cells, including CD4+ T helper cells and CD8+ 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/

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Conflict of Interest Statement: None declared.