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Genetic variations of the *EVER* genes, cutaneous human papillomavirus (HPV) infection, and squamous cell carcinoma of the skin

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Keywords

EVER polymorphisms; squamous cell carcinoma; skin cancer; human papillomavirus

Seropositivity to cutaneous human papillomavirus (HPV) β types and β-HPV DNA in eyebrow (EB) hairs have been associated with an increased risk of cutaneous squamous cell carcinoma (SCC) [1,2]. While immunocompromised individuals[3] have an increased susceptibility to cutaneous HPV infection, factors predisposing to β-HPV infection among immunocompetent individuals are largely unknown. We examined genetic factors associated with cutaneous β-HPV infection in cancer-free, immunocompetent individuals and assessed if the same genetic factors were associated with SCC.

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Single nucleotide polymorphisms (SNPs) in *EVER* genes, regulating anti-HPV barrier [4], have been associated with β2-HPV seropositivity, among immunocompromised patients [5]. Only one previous study evaluated the association of a single SNP in *EVER*2 with β-HPV infection and SCC among immunocompetent individuals [6]. We examined the associations of SNPs in *EVER*1/*EVER*2 with β-HPV infection in EB and β-HPV seropositivity among cancer-free immunocompetent individuals, as well as the association between *EVER* SNPs and β-HPV DNA in SCC tumors among SCC patients.

Histologically confirmed SCC cases (n=185) and controls (n=281) were enrolled in a previously conducted clinic-based case-control study designed to evaluate the association between cutaneous HPV infection and SCC [1]. HPV serology and EB DNA data were available for 168 cases and 290 controls from the previous study [2]. In the current study, additional data on *EVER* genotypes was obtained from 142 cases and 265 controls. Of the 142 cases, both tumor DNA and *EVER* genotype data were available for 119 cases. All participants provided written informed consent and the study protocol was approved by the institutional review board.

HPV DNA was extracted from EB and SCC tumor tissue with the QIAGEN EZ1 DNA Tissue Kit, and HPV genotyping was performed using a type-specific multiplex genotyping assay to detect DNA from 25 genus-β HPV types (5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93 and 96) [7]. Serum antibodies to the major capsid protein L1 for 16 cutaneous β-HPV types (5, 8,9,15,17,20, 23, 24, 36, 38, 49, 75, 76, 92, 96, 107) were measured using an enzyme linked immunosorbent assay and multiplex serology[8]. Genomic DNA was extracted from EB and genotyping of 21 TagSNPs in *EVER*, previously associated with cervical cancer[9] or SC [6], was performed by multiplex PCR and Luminex hybridization[9].

Associations between SNPs and ‘any’ (>=1β-HPV type versus none), ‘single’ (1 β-HPV type versus none) and ‘multiple’ (>1 β-HPV types versus =<1 types) infections were examined. This approach was used for defining both HPV status in EB and HPV seropositivity. Due to the ubiquitous prevalence of β-HPV[10], single HPV infection in EB or by serology may not be clinically relevant ; hence, results for multiple infections among controls are presented. In contrast, SCC tumors have lower viral DNA load compared to EB [2], therefore, tumor DNA positivity was defined by the presence of a single HPV type.

Among controls, SNPs were evaluated for associations with multiple β-HPV infection overall and by species, (β1 [HPV types 5,8,12,14,19,20,21,24,25,36,47,93], β2 [HPV types 9,15,17,22,23,37,38,80,107], separately for EB DNA positivity and seropositivity. Among SCC cases, associations were examined between SNPs and β-HPV DNA in SCC tumor tissue (>=1 β-HPV vs. none). All SNP associations were examined using a log-additive model to estimate odds ratios (OR) and 95% confidence intervals (CI), adjusting for confounders. False discovery rates were calculated to account for multiple comparisons. Analyses were conducted using R (version 3.0.2), SAS 9.1.3 (SAS Institute Inc., Cary, North Carolina) and PLINK (version 1.07) softwares[11], as appropriate .

Compared to controls, SCC cases were significantly older and more likely to be male (Table 1). Among controls, none of the SNPs were associated with seropositivity to multiple β-HPV infections (Table 2). Two SNPs, rs16970829 and rs1048591, were significantly associated with increased risks of multiple β-HPV infections in EB (Table 2), after correcting for multiple testing, particularly with multiple β1-HPV infections [rs16970829 (OR=3.1, 95% CI=1.3 -7.2), rs1048591 (OR=3.8, 95% CI=1.6 -9.1)]. The SNP rs16970829 was also positively associated with 'any' and 'single'' β1 HPV and with 'any' and 'single' β2 HPV, while rs2290907 was associated with 'any' β-HPV infection in EB, among controls (data not shown). Among SCC cases, rs16970829 and rs1048591, were significantly and inversely associated with β-HPV DNA in SCC tumor tissue (OR= 0.3, 95% CI=0.1 -0.9 for both SNPs, Table 2). No SNPs were associated with SCC overall, after adjusting for confounders (data not shown).

In this clinic-based study, two SNPs (rs16970829 and rs1048591), in perfect linkage disequilibrium (R^2=1.0), were positively associated with β-HPV infection in EB among immunocompetent individuals. Interestingly, the same SNPs were inversely associated with β-HPV DNA in SCC tumor tissues, among cases. The SNPs rs16970829 and rs104859, located in TNRC6C gene, have been shown to bind to transcription factors [12], and regulate gene expression and protein synthesis[13], respectively.

While our findings do not support a direct role for *EVER* SNPs in HPV-associated SCC, they may be involved in the development of premalignant skin lesions that harbor β-HPV, perhaps giving rise to SCC tumors that have lost β-HPV gene expression during progression[14].

Unlike the positive associations reported previously[6], we did not observe any associations between the variant genotype at rs7208422 and either SCC or seropositivity to HPV using genotypic model (data not shown). In another study of immunocompromised patients, SNPs in *EVER* were associated with seropositivity to β2-HPV, among controls[5], in contrast to our null association between *EVER* SNPs and seropositivity to HPV in immunocompetent individuals. However, similar to our study, SNPs in *EVER* were not associated with SCC[5].

Our findings are limited by small sample and inadequate statistical power to detect modest SNP associations and may be biased if the true associations are affected by unknown or unexamined HPV types. Although the TagSNPs captured >80% genetic variation in *EVER* [9], rare genetic variants may have been missed. Despite these limitations, our results were based on the evaluation of multiple β-HPV markers and provide critical evidence in support of genetic regulation of cutaneous β-HPV infection and its possible indirect role in SCC.

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Table 1

Characteristics of the cutaneous squamous cell carcinoma (SCC) cases and healthy controls

Variable	Controls n=265 n(%)	SCC cases n=142 n(%)	p-value
Age[1]	55.43 (11.9)	64.93 (9.5)	<0.001
Gender			
Female	164 (61.9)	45 (31.7)	<0.001
Male	101 (38.1)	97 (68.3)	
Tanning ability			
Unable to tan	20 (7.7)	22 (17.5)	<0.001
Can tan if you work at it	87 (33.3)	57 (45.2)	
Tans easily	154 (59.0)	47 (37.3)	
History of blistering sunburns			
No	83 (31.6)	32 (25.2)	0.20
Yes	180 (68.4)	95 (74.8)	
Smoking status[2]			
Never	130 (49.1)	37 (29.1)	<0.001
Ever	135 (50.9)	90 (70.9)	
Multiple p-HPV infection in eyebrow hairs*			
No	130(49.1)	45(31.7)	<0.001
Yes	135(50.9)	97(68.3)	
Multiple β1-HPV infections in eyebrow hairs			
No	127(64.1)	65(54.6)	0.09
yes	71(35.9)	54(45.4)	
Multiple β2-HPV infections in eyebrow hairs			
No	146(66.4)	56(48.3)	0.001
Yes	74(33.6)	60(51.7)	
Seropositivity to multiple β-HPV infections*			
No	143(54.0)	62(43.7)	0.05
Yes	122(46.0)	80(56.3)	
Seropositivity to multiple β1-HPV infections			
No	130(67.0)	65(58.6)	0.14
Yes	64(33.0)	46(41.4)	
Seropositivity to multiple β2-HPV infections			
No	152(65.2)	67(57.3)	0.15
Yes	81(34.8)	50(42.7)	

** no seropositivuty to any β-HPV type or seropositivity to one β-HPV type used as reference

[1] mean(standard deviation),

[2] based on ever smoked 100 cigarettes during entire lifetime

* no β-HPV DNA in eyebrow hairs or single β-HPV type DNA used as reference

Table 2

Associations between SNPs in *EVER* genes with multiple cutaneous HPV infections in eyebrow hairs and serum, among controls and SCC tumors, among cases

			HPV DNA in eyebrow hairs			HPV antibodies			HPV DNA in SCC tumor tissue
			Overall n=265	β1 species n=198	β2 species n=220	Overall n=265	β1 species n=194	β2 species n=233	Overall N=119
SNP	**Gene name**	**MA (MAF %)**	**OR(95%CI)[a]**	**OR(95%CI)[b]**	**OR(95%CI)[c]**	**OR(95%CI)[a]**	**OR(95%CI)[b]**	**OR(95%CI)[c]**	**OR(95%CI)[d]**
rs16970811[1]	TNRC6C	C (6.9)	0.7 (0.3 -1.4)	0.9 (0.4 -2.1)	1 (0.4 -2.2)	1.4 (0.7 -2.7)	1.1 (0.4 -2.6)	1.5 (0.7 -3.2)	1.8 (0.6 -5.7)
rs2311001[1]	TNRC6C	C (34.5)	1.4 (1.0 -2.1)	1.7 (1.1 -2.6)	1.2 (0.8 -1.8)	1.4 (1.0 -2.0)	1.2 (0.7 -1.8)	1.4 (0.9 -2.1)	0.8 (0.5 -1.4)
rs2290907[1]	TNRC6C	G (15.2)	1.7 (1.0 -2.9)	1.8 (1.0 -3.3)	1.4 (0.8 -2.5)	1.2 (0.7 -2.0)	0.7 (0.4 -1.4)	1.5 (0.9 -2.5)	0.6 (0.3 -1.3)
rs16970829[1]	TNRC6C	**G (6.9)**	**3.9 (1.6 -9.4)***	**3.1 (1.3 -7.2)**	1.6 (0.7 -3.8)	0.9 (0.5 -1.9)	0.6 (0.3 -1.6)	1.3 (0.6 -2.8)	**0.3 (0.1 -0.9)**
rs1048591[2]	TNRC6C	**G (6.9)**	**4.9 (1.9 -12.3)****	**3.8 (1.6 -9.1)**	1.8 (0.8 -4.2)	0.9 (0.5 -1.9)	0.7 (0.3 -1.6)	1.4 (0.6 -3)	**0.3 (0.1 -0.9)**
rs9807014[2]	TMC6/*EVER1*	T (16.7)	1.2 (0.8 -2.0)	1.3 (0.8 -2.3)	1 (0.6 -1.7)	1.4 (0.9 -2.3)	1.4 (0.8 -2.4)	1.3 (0.8 -2.2)	0.9 (0.4 -1.8)
rs3813026[1]	TMC6/*EVER1*	C (5.5)	1.7 (0.8 -3.7)	1.5 (0.7 -3.2)	1.3 (0.6 -3)	1.2 (0.6 -2.5)	1.0 (0.4 -2.5)	1.1 (0.5 -2.5)	0.7 (0.2 -2.5)
rs11658760[1]	TMC6/*EVER1*	T (48.2)	0.7 (0.5 -1.0)	1.0 (0.7 -1.5)	0.7 (0.5 -1.1)	1.1 (0.8 -1.5)	1.1 (0.7 -1.6)	0.9 (0.6 -1.2)	1.2 (0.7 -2)
rs383603[1]	TMC6/*EVER1*	G (23.3)	1.3 (0.9 -2)	0.8 (0.5 -1.4)	1.3 (0.8 -2.1)	0.9 (0.6 -1.4)	1.1 (0.7 -1.8)	1.2 (0.8 -1.9)	0.7 (0.4 -1.3)
rs450474[1]	TMC8/*EVER2*	C (12.4)	0.8 (0.5 -1.4)	0.8 (0.4 -1.5)	0.8 (0.5 -1.5)	0.7 (0.5 -1.2)	0.7 (0.4 -1.4)	1 (0.6 -1.7)	2.5 (0.7 -9.0)
rs7208422[3]	TMC8/*EVER2*	T (47.0)	0.8 (0.6 -1.2)	1.2 (0.8 -1.9)	0.7 (0.5 -1.0)	0.9 (0.6 -1.3)	0.9 (0.6 -1.4)	0.9 (0.6 -1.3)	0.9 (0.6 -1.6)
rs412611[1]	TMC8/*EVER2*	A (6.9)	0.7 (0.3 -1.5)	0.7 (0.2 -1.9)	0.7 (0.3 -1.9)	0.6 (0.3 -1.3)	0.6 (0.3 -1.6)	1.0 (0.4 -2.2)	4.3 (0.9 -19.6)
rs8068430[1]	TMC8/*EVER2*	C (18.1)	1.2 (0.8 -1.9)	1.3 (0.8 -2.2)	1.5 (0.9 -2.4)	0.9 (0.6 -1.4)	1 (0.6 -1.7)	0.8 (0.5 -1.3)	1.2 (0.6 -2.6)
rs16970849[1]	TMC8/*EVER2*	A (3.4)	1.0 (0.4 -2.5)	0.5 (0.1 -1.7)	1.6 (0.6 -4.5)	0.8 (0.3 -1.9)	1.8 (0.5 -6)	1.4 (0.5 -3.8)	0.3 (0.1 -1.3)
rs17773842[1]	TMC8/*EVER2*	T (45.8)	0.8 (0.6 -1.2)	0.9 (0.6 -1.4)	0.7 (0.5 -1.0)	1.1 (0.8 -1.5)	0.9 (0.6 -1.4)	1.1 (0.8 -1.6)	1.2 (0.7 -2.0)
rs17773854[1]	TMC8/*EVER2*	A (31.6)	0.8 (0.5 -1.1)	1.0 (0.6 -1.5)	0.5 (0.3 -0.8)	1.2 (0.8 -1.7)	1.0 (0.6 -1.5)	1.2 (0.8 -1.8)	1.2 (0.7 -2.2)
rs4789015[2]	TMC8/*EVER2*	A (47.4)	1.1 (0.8 -1.6)	1.0 (0.7 -1.5)	1.3 (0.9 -2.0)	1.0 (0.7 -1.4)	1.0 (0.7 -1.5)	0.9 (0.6 -1.3)	0.9 (0.5 -1.4)
rs9915090[4]	C17orf99	T (43.2)	1.3 (0.9 -1.7)	1.0 (0.7 -1.6)	1.3 (0.9 -1.9)	1.0 (0.8 -1.4)	1.1 (0.7 -1.6)	1.0 (0.7 -1.4)	0.7 (0.4 -1.1)

			HPV DNA in eyebrow hairs			HPV antibodies			HPV DNA in SCC tumor tissue
			Overall n=265	β1 species n=198	β2 species n=220	Overall n=265	β1 species n=194	β2 species n=233	Overall N=119
SNP	**Gene name**	**MA (MAF %)**	**OR(95%CI)[a]**	**OR(95%CI)[b]**	**OR(95%CI)[c]**	**OR(95%CI)[a]**	**OR(95%CI)[b]**	**OR(95%CI)[c]**	**OR(95%CI)[d]**
rs748708[1]	C17orf99	T (11.5)	0.9 (0.6 -1.5)	0.7 (0.4 -1.4)	1.2 (0.7 -2.0)	0.8 (0.5 -1.3)	0.8 (0.4 -1.6)	0.8 (0.4 -1.4)	1.1 (0.4 -3.2)
rs7217374[1]	C17orf99	A (46.2)	0.8 (0.6 -1.1)	1.0 (0.7 -1.5)	0.7 (0.4 -1.0)	1.0 (0.7 -1.4)	0.9 (0.6 -1.4)	1.0 (0.7 -1.4)	1.6 (0.9 -2.7)
rs11656744[1]	C17orf99	A (14.6)	1.2 (0.7 -1.9)	1.2 (0.7 -2.0)	1.4(0.8 -2.3)	0.8 (0.5 -1.2)	0.9 (0.5 -1.6)	0.7 (0.4 -1.2)	1.6 (0.6 -4.0)

SNPs were

[1] Intronic

[2] 3 prime UTR

[3] Non-synonymous coding or

[4] upstream of gene. All analysis using log additive regression model, adjusted for age.

[a] No HPV infection or single HPV infection as reference group.

* P value= 0.02 FDR

** P value =0.01 FDR.

[b] No HPV infection with any type or single β1 HPV infection vs. multiple β1 HPV infection.

[c] No HPV infection with any type or single β2 HPV infection vs. multiple β2 HPV infection.

[d] No HPV DNA in SCC tumors as reference group. SNP location based on information from Database of Single Nucleotide Polymorphisms(dbSNP), Bethseda (MD): National Center for Biotechnology Information, National Library of Medicine[15].