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Cutaneous human papillomavirus infection, the *EVER2* gene and incidence of squamous cell carcinoma: A case-control study

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Abstract

The first evidence of an association between HPV and non-melanoma skin cancer comes from patients with epidermodysplasia verruciformis (EV). EV is a rare heritable disease characterized by cutaneous warts that display not only a high rate of progression to squamous cell carcinoma on sun-exposed sites, but also a strong predisposition to infection by β-HPVs, for which HPV 5 and 8 predominate. Two EV genes (*EVER1* and *EVER2*) have been identified, and we tested the hypothesis that variation in the *EVER2* gene (rs7208422) is related to seropositivity to HPV (of the genus β types) and risk of squamous cell carcinoma in a population-based case-control study of SCC (*n* = 239 cases and 432 controls). Among controls, variant genotype was associated with β-HPV seropositvity (OR = 2.3, 95%CI = 1.2–4.3), specifically HPV5 or 8 seropositivity (OR = 2.4, 95% CI = 1.1–5.1) and seropositivity for multiple β-HPV types (OR =2.7, 95%CI =1.1–6.6). Furthermore, variant genotype was also related to SCC risk [adjusted OR for homozygous variant *versus* homozygous wild type for the *EVER2* polymorphism 1.7, 95% CI 1.1–2.7]. These data provide evidence for a role of genetic variation in the *EVER2* gene in β-HPV infection and risk of SCC, shedding light on the link between HPVs and skin cancers.

Keywords

human papillomavirus; skin cancer; *EVER2*

The initial link between HPV and skin cancers was a rare autosomal inherited disease called Epidermodysplasia Verruciformis (EV) first described nearly a century ago.[1] The disease is characterized by cutaneous lesions that display a high rate of progression to squamous cell carcinomas (SCCs) beginning in the second decade of life. Furthermore, EV patients experience diminished cell-mediated immunity possibly resulting in an observed susceptibility to infection by specific HPV types falling into the genus β, with HPV5 and HPV8 the most prevalent infections in this patient population.[2]

Genome-wide linkage studies were performed to map two EV loci[3,4] and to identify the EVER genes (*EVER1* or *EVER2*, also referred to as *TMC6* and *TMC8*, respectively) in one of these

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loci.[5] The identification of *EVER1* and *EVER2* expressed sequence tags and cDNA from lymphoid tissue is consistent with a model in which EV mutations exert their direct effects in the immune system. It was recently discovered that 75% of EV patients studied have rare mutations in either of these adjacent, related, novel genes. To date, 10 truncating, loss of function mutations have been identified, 4 in *EVER1* and 6 in *EVER2* and were identified in EV patients from Algeria, Columbia, Poland and Japan.[5] More recently, 5 additional mutations have been detected in the *EVER* genes supporting the hypothesis that *EVER1* and *EVER2* are the molecular basis of EV.[6–8]

β-HPV DNA has been detected in up to 50% of SCC tumors from immunocompetent individuals and in up to 90% of lesions from immunocompromised individuals,[9,10] further emphasizing the role of host susceptibility in infection with these viruses. Recently, we reported that the presence of antibodies to viral proteins of HPV types in the genus β, particularly HPV5, was associated with risk of SCC.[11]

We hypothesize that a common nonsynonymous single nucleotide polymorphism in the *EVER2* gene also may be related to infection with β-HPVs. Here we report that this particular SNP (rs7208422), located in the most highly conserved region of the gene, is not only associated with SCC of the skin but also with the presence of antibodies to genus β-HPVs in control subjects.

Material and methods

Study population

All newly diagnosed cases of SCC in New Hampshire were identified using an incident survey previously described.[12] Controls, derived from the New Hampshire Department of Transportation and Medicare enrollment lists, were frequency matched to cases on gender and age. Inclusion criteria included residence in New Hampshire, age between 25 and 74, and English speaking with a working telephone number. For all participants, an interview administered questionnaire was used to gather information on sun-exposure histories and other demographic and lifestyle information, as previously described.[11] Overall, 696 Caucasian subjects were included in the current analysis (448 controls and 248 SCC cases).

Blood collection

From all participants, we requested a venous blood sample of 20–30 mL in heparinized tubes. Blood was separated by centrifugation at 2,500*g* for 20 min at 4°C. Plasma, red blood cells and the buffy coat were stored separately at −80°C until analysis. Each specimen was labeled with a type code (plasma, red blood cells or buffy coat) and a unique identifier so that the staff was masked to the disease status associated with all samples.

Multiplex serology

Frozen plasma samples were previously analyzed[11,13] for antibodies to the major capsid protein L1 of 16 types of human papillomavirus. These include types 2, 3, 6, 10, 16, 32, 57 belonging to the alpha genus; types 5, 8, 9, 15, 20, 24, 36, 38 belonging to the genus beta; and type 1 of the genus mu. We used an antibody detection method that is based on a glutathione *S*-transferase capture enzyme-linked immunosorbent assay, as previously described,[14,15] in combination with fluorescent bead technology.[13]

EVER2 *genotyping*

We collected data on a (A > T) coding SNP in exon 8, codon 306 of the *EVER*2 gene (rs7208422), resulting in a change from isoleucine to asparagine. DNA was extracted from buffy coat using Qiagen genomic DNA extraction kits. Genotyping of the *EVER2*

polymorphism was done using the Taqman® allelic discrimination technique (Applied Biosystems, Foster City, CA). For quality assessment, every tenth sample was an embedded duplicate.

Statistical analysis

We calculated crude and adjusted ORs and 95% CIs for the association of *EVER2* genotype and SCC using unconditional logistic regression, adjusted for age, sex and skin sensitivity (defined as the reaction to 1 hr of sun exposure the first time in the summer) using SAS v9.1. In addition to χ^2 calculations, the association between serology and *EVER2* genotype was also assessed using unconditional logistic regression.

Results and discussion

We collected data on a (A > T) coding SNP in exon 8, codon 306 of the *EVER*2 gene (rs7208422), resulting in a change from isoleucine to asparagine. Among controls, the T allele frequency was 0.45. There were no significant associations of *EVER2* genotype with age, sex, number of severe burns or skin type (data not shown). The prevalence of the TT genotype was 20% (89 out of 448) in controls and 25% (62 of 248) in SCC cases (Table I). The adjusted odds ratio (95% CI) for SCC for those with the AT genotype was 1.4 (0.9–2.1). This point estimate reached significance with the presence of a second T allele, with a 70% increase in SCC risk (OR = 1.7, 95% CI = 1.1–2.7), suggesting a dose effect with T alleles ($p_{trend} = 0.01$). Furthermore, we found that TT genotype was associated with positive β-HPV serology in controls. For example, those with the TT *EVER2* genotype were more than twice as likely to test positive for β-HPV antibodies (OR = 2.3, 95% CI of 1.2–4.3) and specifically β-HPV 5 or 8 (OR = 2.4, 95% CI = 1.1–5.1) as well as multiple β-HPV types (OR = 2.7, 95% CI = 1.1–6.6) (Table II). A similar trend was also apparent among SCC cases. The prevalence of any β-HPV ($p = 0.002$), β-HPV 5 or 8 ($p = 0.006$), and multiple β-HPVs ($p = 0.01$) were higher in those with the TT genotype, when compared to those cases with AA or AT genotype. These data provide evidence for a role of genetic variation in the *EVER2* gene in β-HPV infection.

Individuals with EV frequently manifest HPV-infected lesions that display a high rate of progression to squamous cell carcinoma. In addition to the 19 β-papillomavirus genotypes found in EV patients, those suffering from EV are usually infected with more than one genotype, sometimes as many as 10 (Ref. 2). We have shown here that a very common single nucleotide polymorphism increases the likelihood for HPV seropositivity. Although the precise role of *EVER2* is unknown, *EVER2* transcript has been isolated from spleen and thymus tissue, as well as from normal skin,[4,16] supporting its hypothesized role in the immunogenic response.

It further is known that *EVER1* and *EVER2* genes belong to a larger family of proteins referred to as the TMC family. The high amino acid conservation of 75–96% identity among human and mouse proteins[4,16] implies that mutations and polymorphisms that alter the coding sequence in the corresponding genes are subjected to significant selective pressure; thus advocating that TMC proteins have important cellular roles. The single nucleotide polymorphism studied here results in a change from the nonpolar isoleucine to the polar asparagine. This polymorphism is thought to be located in a putative transmembrane domain.[4]

We have found that the presence of a polymorphism in the *EVER2* gene is not only associated with the presence of HPV antibodies in control subjects but also an increased risk of squamous cell carcinoma of the skin. It is possible that *EVER* genes are involved in controlling the HPV pathogenesis in epidermal keratinocytes or that they directly affect the innate or adaptive immune responses which may control the clearance of EV-HPV-infected keratinocytes. Further investigations that incorporate additional genetic variation of the *EVER* genes as well

as other markers of HPV infections such as viral DNA in the tumors may help elucidate the function of these proteins in HPV infections and skin cancers.

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TABLE I

RELATION BETWEEN *EVER2* GENOTYPE AND SQUAMOUS CELL CARCINOMA OF THE SKIN

***EVER2* genotype**	**Controls, *n* = 448 [No. (%)]**	**SCC cases, *n* = 248 [No. (%)]**	**OR (95% CI)[1]**
AA	137 (31)	60 (24)	Reference
AT	222 (49)	126 (51)	1.4 (0.9–2.1)
TT	89 (20)	62 (25)	1.7 (1.1–2.7)

[1] Adjusted for gender, age, skin sensitivity to sun and HPV serology.

TABLE II

SEROPREVALENCE OF β-HPV TYPES IN CONTROL SUBJECTS (*N* = 448)

	***EVER2* genotype**					
	AA, *n* = 137 [No. (%)]	**AT, *n* = 222 [No. (%)]**	**TT, *n* = 89 [No. (%)]**	**Total**	**OR (95% CI)[1] AA versus AT**	**OR (95% CI)[1] AA versus TT**
Any β-HPV						
No[2]	114 (34)	163 (48)	61 (18)	338	Reference	Reference
Yes	23 (21)	59 (54)	28 (25)	110	1.7 (1.0–3.0)	2.3 (1.2–4.3)
β-HPV 5 and/or 8						
No[3]	123 (33)	185 (49)	70 (18)	378	Reference	Reference
Yes	14 (20)	37 (53)	19 (27)	70	1.6 (0.8–3.1)	2.4 (1.1–5.1)
Multiple β types						
No[4]	128 (33)	186 (48)	75 (19)	389	Reference	Reference
Yes	9 (15)	36 (61)	14 (24)	59	2.6 (1.2–5.6)	2.7 (1.1–6.6)

[1] ORs are adjusted for gender and age; referent group are those individuals with AA genotype.

[2] Defined as those with no HPV infection of any type.

[3] Defined as those with either no HPV infection of any type or with an HPV type other than 5 or 8.

[4] Defined as those with no HPV infection or a HPV infection with a single type.