Evaluation of HLA Genotypes Associated with HPV-Related *in situ* and Invasive Vulvar Cancer

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Abstract

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HPV is related to ~70% of *in situ* and invasive vulvar cancers, ~80% of those HPV positive cases of associated with HPV16. Numerous studies in cervical cancer have shown that the major histocompatibility complex (MHC) genes influence the risk of disease, specifically human leukocyte antigen genes on chromosome 6. We examined associations between single and multiple co-occurring class I A, B, and C genes and class II DRB1 and DQB1 genes relative risk of HPV16-related *in situ* and invasive vulvar cancer (VSCC16). Cases and controls were self-identified white women residing in the greater Seattle, WA, area. We performed high-resolution HLA typing on peripheral blood samples from 238 VSCC16 women and 637 population-based control women, and HPV genotyping on FFPE biopsies from tumor tissue. Using logistic regression, we first assessed the relative risk of VSCC16 associated with HLA, then compared the results with our previously published associations between HPV16 cervical cancer cases (CXSCC16). Among 352 co-occurring allele combinations present in ≥ 5% in the case or control groups, 178 were significantly associated with VSCC16 risk. Three very strong trends stood out: 1) B*44:02 and B*44:03 were noted in various combinations to have significantly increased relative risk of VSCC16 results, particularly seen with B*44:02, DRB1*11:01, and DQB1*03:01 (OR 5.0, 95% CI 2.0-12.7). 2) When B*07:02, C*07:02 co-occur with DQB1*03:01 there was significantly increased risk of VSCC16, such as A*02:01, B*07:02, C*07:02, DRB1*11:01, DQB1*03:01 (OR 6.4, 95% CI 2.2-18.4). 3) A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01 showed significant decreased risk among VSCC16 samples (OR 0.6, 95% CI 0.4-1.0). Interestingly, there was a much larger number of significant associations with VSCC16 results than with CXSCC16, suggesting that the immune response is more important in the etiology of VSCC16. Overall, the data suggests that CD4 and CD8 T cell responses are important cofactors with HPV in HPV-related cancers. Significant co-occurring HLA alleles shared between vulvar and cervical case samples may be markers for disease risk and have future clinical value for targeted screening or new therapies.

INTRODUCTION:

Vulvar cancer is rare, currently ranked 28th among the most common cancer types within the USA [1] and 4th among gynecological cancers [2]. While rare, incidence rates of *in situ* and invasive vulvar cancer have been steadily rising with annual increases of ~2.3% and ~0.6% over the past three decades, respectively (Figure 1). Death rates have been rising ~1.2% every year during the same timeframe with no known reason for the increases [3]. Most vulvar cancers occur in the squamous cell epithelium, with epidemiologic and histopathologic parallels to squamous cell cancer of the cervix [4].

Approximately 80% of sexually active adults have had an oncogenic human papillomavirus (HPV) infection [5, 6]. HPV infections rarely progress to persistent infections, or low-grade neoplastic lesions, and even less progress from low-grade to high-grade neoplasia (HSIL). HSIL (VIN3) is the immediate precursor of HPV-related invasive squamous cell vulvar cancer and is characterized by disordered epithelium (enlarged nuclei, increased mitotic figures) throughout the epithelium. Research has shown that HPV is associated with greater than 80% of HSIL of the vulva and ~69% of warty/basaloid squamous cell carcinoma vulvar cancers [4, 7, 8].

Recent cervical cancer studies have investigated the role that human leukocyte antigen (HLA) class I and class II loci may play in the immune response to HPV infections. These studies have indicated that there are both increased and decreased relative risk of HPV-related *in situ* or invasive cancers associated with specific HLA alleles and haplotypes among different class I and class II molecules [9-16].

There are more than 200 human leukocyte antigen (HLA) genes in the human major histocompatibility complex (MHC) that regulate the immune response in humans. These genes are primarily located on the p arm of Chromosome 6 and are expressed as surface proteins. The genes are located relatively close to one another, and the genes in this region show evidence of extensive linkage disequilibrium [17-19]. HLA is a major determinant of viral infection and persistence [20]. Class I genes are presented on the surface of most nucleated cells with an α and β chain. The β-chain gene, beta$_2$-microglobulin, is encoded on chromosome 15 and is considered monomorphic. The α-chain consists of five domains: two peptide-binding domains (α_1 and α_2), one immunoglobulin-like domain (α_3), a trans-membrane anchor, and a cytoplasmic tail. Most of the polymorphisms of HLA-A, B, and C are due to differences in the α_1 and α_2 peptide-binding region, which are highly diverse between people. There are many other class I genes (E, F, and G) that are more limited in their diversity and are not seen as important in the regulation of viral immune responses. In our evaluation of class I HLA we focused on A, B, and C.

Class II genes are presented on the surface of antigen-presenting cells (APC), such as macrophages, B cells, activated T cells, and dendritic cells, with both an α and β chain: each chain contains two extracellular domains, a transmembrane region, and a cytoplasmic tail. Designation of class II genes consists of three letters and a number: the first letter indicates class (D); the second letter indicates family (M, O, P, Q, and R); the third letter indicates the chain (A or B for α and β, respectively); the number indicates the subcomponent of the α or β domain (1 or 2). For example, HLA-DRB1 indicates the class II HLA (D), R family, β chain and the β_1 domain. As with class I genes, there is a

high level of polymorphism among the class II genes, primarily seen in the B1 peptide-chains [19]. In our evaluation of class II HLA we are focused on DRB1 and DQB1.

Due to a great amount of diversity in HLA, standard nomenclature is followed allowing for identification of the type and subtype of a particular gene. For example, HLA-A*02:01, the first two digits describe the type, often corresponding to the serological antigen carried by the allotype (02); the third and fourth digits correspond to the subtype, assigned as identified (01).

For HLA class I molecules, small peptide chains from degrading proteins are trimmed by proteasomes in the cell, and bind to the peptide-binding groove of the molecules. Lysosomes with class II molecules fuse with an endosome containing external proteins, degrading the proteins, which then bind to the molecules. When the small peptide chains are bound to the molecules, the proteins complete folding and are transported to the cell surface. Once on the surface, the class I or II molecules present antigen fragments to the T cell receptor (TCR) of either CD8 or CD4 T cells, via class I or II molecules, respectively. If there is a viral or foreign infection, this is seen in the display of the small molecule peptides and will trigger a T cell immune response.

Another important component of HLA is the presence of linkage disequilibrium (LD). LD is the non-random association of alleles at different loci within a given population. This occurs when the frequency of association of different alleles is higher or lower than would be expected if the loci were independent or randomly associated. LD is influenced by several factors, including epistasis (gene-gene interaction), natural selection, rate of genetic recombination, and mutation rates. Complex and extensive LD is known to occur across HLA and contributes to the use of HLA genes as markers of population diversity.

The common view is HLA diversity is an evolutionary response to the wide variety of disease pathogens encountered by human populations during evolution [19, 20]. A combination of the rarity of vulvar cancer and the diversity of HLA make it imperative to design studies large enough to detect HLA gene or haplotype associations. To date, most research comparing HLA and HPV-related cancers has been associated with squamous cell cervical cancer (SCC). In this study, we achieved this goal by collecting *in situ* and invasive vulvar cases over a number of years.

The objective of this study is to evaluate the relative risk of HPV-related *in situ* and invasive vulvar cancer (hereafter referred to together as VSCC) associated with single and multiple class I and class II HLA loci. With an estimated 85% of HPV-related VSCC associated with HPV16 infection [21], the study will focus on HPV16 VSCC. Furthermore, comparison of the VSCC results will be made with HLA results from HPV16 cervical cancer (CXSCC16) to better assess similarities and differences between the two HPV-related disease types.

METHODS:

Study Population:

VSCC case subjects were women aged 18 -79 diagnosed with *in situ* or invasive vulvar cancer while residents of the 3-county metropolitan Seattle area (King, Snohomish, and Pierce counties) in Washington state between two time periods, January 1986 through June 1998 and January 2000 through December 2004 [4, 9]. Case subjects were identified from the National Cancer Surveillance System, a Surveillance and End Results (SEER) registry that is part of a nationwide National Cancer

Institute (NCI) system of cancer registries. The investigation was restricted to *in situ* (high-grade squamous intraepithelial lesions, HSIL) and invasive vulvar epithelial tumors coded by the registry with International Classification of Disease subjects for Oncology (ICDO) morphology codes 8010 - 8081.

Population-based control women were identified by the use of random-digit telephone dialing (RDD) and frequency matched to the age distribution of the case subjects in 5-year age intervals. At the reference date, the control subjects were residents of the same three-county area as cases in the Seattle area, had a working telephone, and were able to speak English. Only individuals with no history of HPV-related cancers were included as control participants.

For both cases and controls, extensive in-person interviews were performed covering demographic, lifestyle, reproductive, and sexual characteristics. For cases, questions in the interview referenced information prior to diagnosis date; for the control women, dates were assigned that match the distribution of case diagnosis dates, and all questions referred to the time before the assigned date. At the conclusion of the interview, all subjects were asked to provide a peripheral blood sample. For cases, the original formalin-fixed paraffin-embedded (FFPE) tissue was retrieved and analyzed for HPV typing.

The study population, HPV typing, and HLA typing were described in our prior paper [4, 9]. Briefly, this study included all cases with VSCC who were HPV16 DNA positive and had high-resolution HLA typing results available. The initial data set consisted of 510 women with squamous cell vulvar cancer available for analysis. Of those 277 women, those who met the acceptance criteria for continuing on with the study included 247 VSCC cases with *in situ* disease and 30 with invasive disease (54.3% of total cases). Furthermore, only 213 VSCC16 cases with *in situ* disease and 25 with invasive disease (46.7% of total cases) were positive for HPV16. For comparison to VSCC, we included 238 cases from our prior study with HPV16 positive squamous cell cervical cancer (CXSCC16) [9].

Additionally, we included women with a history of hysterectomy, who were removed from the prior study, as they were not at risk of cervical cancer. All study participants were restricted to self-identified white women, as they constituted the majority population in the parent study (reference) and HLA type differs importantly by race [20].

Data Collection

HLA Genotyping:

The peripheral blood was collected from both cases and controls, into an EDTA (ethylenediaminetetraacetic acid) vacutainer tubes, and peripheral blood mononuclear cells (PBMC) were prepared from the samples and suspended in RPMI 1640/DMSO before being stored at -80°C within 24 hours of being drawn. DNA was extracted from the stored PBMCs using a manual phenol:chloroform extraction method and stored in 0.5 mL aliquot tubes. Two methods were used to type HLA class I and II alleles, both using sequence-specific oligonucleotide probe (SSOP) reverse format assays (rSSOP) (as previously described [9]).

For DQB1*02, the difference between DQB1*02:01 and DQB1*02:02 is in exon 3. The genotyping reagents used to analyze the polymorphisms is in exon 2. The single allele assignments were made

based on common allele specifications in the presence of DRB1*03:01 or DRB1*07:01, associated with previous research [22]. Therefore, DQB1*02 became DQB1*02:01 if a subject carried DRB1*03:01 and DQB1*02:02 if a subject carried DRB1*07:01. If a person was typed with DQB1*02 and had neither DRB1*03:01 or DRB1*07:01, then the DQB1*02 allele could not be inferred and was kept missing for both allele level variables.

For DRB1*07, it was not possible to differentiate between the subtypes of the gene: DRB1*07:01, DRB1*07:03 and DRB1*07:04. The decision was made to label all DRB1*07 genes with the DRB1*07:01 subtype.

HPV Typing of Tumor Tissue

Tissue blocks from the case subjects were retrieved from local pathology laboratories. HPV typing was performed (as previously described [4, 9]) using PCR-RFLP (MY09/MY11 L1 consensus and HPV16 and 18 E6 type-specific primers, with co-amplification of 236 bp or 536 bp fragments of β-globin to assess for sample integrity). While not an optimal test method by current standards, the method was able to type 89% of cervical cancers with HPV oncogenic types from FFPE, suggesting that approximately 11% were not detected by this assay [23]. Newer assays would likely have found additional positive samples as well as additional HPV types. Human β-globin DNA was amplified as a positive control and was detected in 95% of the tumor tissue samples. HPV16 was detected in approximately 86% of all VSCC cases. Only HPV16 positive VSCC and CXSCC cases were included for analysis.

Data Analysis:

SEER Data Analysis

We evaluated trends in VSCC within the US over the past three decades using SEER*Stat 8.3.5. The dataset SEER 9 Regs Research Data, Nov 2017 Sub (1973-2015) <Katrina/Rita Population Adjustment> is publically available (https://seer.cancer.gov/seerstat/). Age-Adjusted incidence of vulvar *in situ* and invasive cancer was assessed for women between the ages of 20 to 84. The data presented is from 1986 to 2015, representing the beginning of the study to the most recent data available.

Single Locus Analysis

HLA class I (A, B, and C) and class II (DRB1 and DQB1) alleles present in ≥ 5% of the case or control women were included in the main single locus analysis. Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between individual alleles within a locus and VSCC16 and CXSCC16 risk were estimated by logistic regression.

Multilocus Analysis

To limit the number of possible comparisons, as each participant has 10 alleles identified, we restricted the number of co-occurring alleles to those that occurred in ≥ 5% of the case or control women. Odds ratios were used to estimate the relative risk of VSCC compared to general population controls using logistic regression. Polytomous regression was used to assess whether relative risk estimates differ between VSCC16 and CXSCC16 when compared to population controls.

RESULTS

Figure 1 illustrates the incidence of *in situ* and invasive vulvar cancer from 1986 to 2015. The graph indicates a steady rise in incidence over the past three decades among women within the USA. There is a dramatic increase of *in situ* incidence between the first decade (1986 to 1995) and the second (1996 to 2005) evaluated: incidence rate of 4.55 to 5.92 per 100,000 women. While incidence is still increasing in the third decade (2006 to 2015), the increase is more gradual with an incidence rate of 6.32 per 100,000 women. Invasive vulvar cancer rates indicate a more gradual rise in incidence rates, from 2.69 to 3.10 over the 3 decades evaluated.

In Table 1 from the case-control study, VSCC16 cases were older than CXSCC16 cases, with approximately 70% of women aged 40 years or older in VSCC16 compared to 52% for CXSCC16 and 62% for control women. Also, there are a noticeably higher percentage of current smokers for women with VSCC16, approximately 20% higher than CXSCC16, which in turn are approximately 20% higher than among the control women. It is also noted that control women generally have fewer sexual partners when compared with the case groups.

Table 2 shows the distribution of several loci that are associated with a significantly increased risk of VSCC16: C*07:02 (OR 1.4, 95% CI 1.0-2.0), B*07:02 (OR 1.5, 95% CI 1.1-2.1), C*06:02 (OR 1.5, 95% CI 1.0-2.2), C*16:01 (OR 1.9, 95% CI 1.2-3.1), B*44:03 (OR 2.1, 95% CI 1.3-3.2), and B*55:01 (OR 4.3, 95% CI 2.1-9.0). There were also decreased risks for VSCC16 associated with B*15:01 (OR 0.3, 95% CI 0.2-0.6) and B*35:01 (OR 0.4, 95% CI 0.2-0.8).

Using polytomous regression, we found significant differences between VSCC16 and CXSCC16 among the class I loci. B*35:01 had a significantly decreased risk associated with VSCC16 (OR 0.4, 95% CI 0.2-0.8), but not CXSCC16 (OR 1.0, 95% CI 0.6-1.7). A*29:02 had significantly increased risk associated with VSCC16 (OR 1.7, 95% CI 1.0-2.7), but no risk with CXSCC16 (OR 0.8, 95% CI 0.4-1.5). Conversely there is one allele that is significant for both case groups: B*15:01 had significantly decreased risk for both VSCC16 (OR 0.3, 95% CI 0.2-0.6) and CXSCC16 (OR 0.5, 95% CI 0.3-0.9). B*44:02 was associated with no risk for VSCC16 (OR 1.2, 95% CI 0.8-1.8), but was associated with an increased risk of CXSCC16 (OR 2.1, 95% CI 1.4-3.0).

Table 3 describes a number of class II HLA alleles which indicate increased risk, including: DRB1*15:01 (OR 1.5, 95% CI 1.1-2.1), DQB1*06:02 (OR 1.5, 95% CI 1.1-2.1), DQB1*03:01 (OR 1.6, 95% CI 1.2-2.2), DRB1*07:01 (OR 1.7, 95% CI 1.2-2.3), DQB1*02:02 (OR 1.8, 95% CI 1.2-2.5), and DRB1*11:01 (OR 3.3, 95% CI 2.1-5.1). Decreased risks of VSCC16 were noted with DQB1*02:01 (OR 0.6, 95% CI 0.4-0.9), DRB1*03:01 (OR 0.6, 95% CI 0.4-0.9), DQB1*05:01 (OR 0.5, 95% CI 0.3-0.7), DRB1*01:01 (OR 0.5, 95% CI 0.3-0.8), DQB1*06:03 (OR 0.2, 95% CI 0.1-0.5), and DRB1*13:01 (OR 0.2, 95% CI 0.1-0.5).

There were several class II alleles where significant differences between VSCC16 and CXSCC16 were found by polytomous regression. DQB1*05:01 had significantly decreased risk in VSCC16 (OR 0.5, 95% CI 0.3-0.7) with no risk difference associated with CXSCC16 (OR 1.1, 95% CI 0.8-1.6). Similarly DRB1*01:01 has decreased risk associated with VSCC16 (OR 0.5, 95% CI 0.3-0.8) and no risk associated with CXSCC16 (OR 1.1, CI 0.8-1.7). DQB1*03:03 was associated with no significant risk of VSCC16 (OR 1.0, 95% CI 0.6-1.7) but significantly increased risk of CXSCC16 (OR 1.9, 95% CI 1.2-3.0). Finally, DRB1*11:01 had significantly increased risk associated with both VSCC16 (OR 3.3, 95% CI 2.1-5.1) and CXSCC16 (OR 1.9, 95% CI 1.2-3.1).

VSCC16 and CXSCC16 shared similar associations with several class II alleles, including decreased risks of DQB1*02:01 (VSCC16 and CXSCC16: OR 0.6, 95% CI 0.4-0.9) and DRB1*03:01 (VSCC16 and CXSCC16: OR 0.6, 95% CI 0.4-0.9). Likewise, there were increased risks of VSCC16 and CXSCC16 associated with DRB1*07:01 (VSCC16: OR 1.7, 95% CI 1.2-2.3; CXSCC16: OR 1.4, 95% CI 1.0-2.0) and DQB1*03:01 (VSCC16: OR 1.6, 95% CI 1.2-2.2; CXSCC16: OR 1.4, 95% CI 1.0-1.9).

In Appendix 1, we examine the impact of all co-occurring HLA alleles may have on acquiring the disease, evaluated co-occurring alleles comprising of two to five loci. There were 352 total different combinations (175 instances with 2 loci, 126 instances with 3 loci, 43 instances with 4 loci, 8 instances with 5 loci). 178 different combinations were significant among VSCC16 cases: 73 instances with 2 loci, 70 instances with 3 loci, 30 instances with 4 loci, and 5 instances with 4 loci. Of those 131 were associated with increased risk and 47 were associated with decreased risk. CXSCC16 had 83 different co-occurring allele combinations that were significant: 37 instances with 2 loci, 32 instances with 3 loci, 12 instances with 4 loci, and 2 instances with 5 loci. Of those 58 were associated with increased risk and 25 were associated with decreased risk.

The majority of higher-order co-occurring alleles were associated with similar risks as lower-order sub-combinations. For example the 3 fold increased risk associated with the five-allele combination A*02:01, B*07:02, C*07:02, DRB1*15:01, DRQ1*03:01 (OR 3.2, 95% CI 1.4-7.4) was similar to several three- or four-allele combinations including: A*02:01, B*07:02, C*07:02, DRQ1*03:01 (OR 3.8, 95% CI 1.9-7.6) or B*07:02, C*07:02, DRB1*15:01, DRQ1*03:01 (OR 3.0, 95% CI 1.5-6.1), combination of 3 alleles B*07:02, C*07:01, DRQ1*03:01 (OR 3.2, 95%CI 1.9 -5.4) or B*07:02, DRB1*15:01, DQB1*03:01 (OR 3.0, 95% CI 1.5-6.1). Two of the two allele combinations also showed significantly increased risk B*07:02, DRQ1*03:01 (OR 3.1, 95% CI 1.5-5.2) or C*07:02, DRQ1*03:01 (OR 2.9, 95% CI 1.8-4.8).

The most strongly increased risk associated allele combinations for VSCC16 were B*44:02, DRB1*11:01 (OR 5.0, 95% CI 2.0-12.6) and A*02:01, B*07:02, C*07:02, DRB1*11:01, DQB1*03:01 (OR 6.4, 95% CI 2.2-18.4). CXSCC16 was also strongly associated with B*44:02, DRB1*11:01 (OR 8.8, 95% CI 3.6-21.1).

For VSCC16, the most strongly decreased risk associated allele combinations were various allele combinations of A*02:01, B*35:01, C*04:01, DRB1*01:01, and DRB1*05:01, including: C*04:01, DRB1*01:01 (OR 0.2, 95% CI 0.1-0.6), C*04:01, DRB1*01:01, DQB1*05:01 (OR 0.2, 95% CI 0.1-0.6), B*35:01, C*04:01, DRB1*01:01 (OR 0.2, 95% CI 0.1-0.7), B*35:01, DRB1*01:01, DQB1*05:01 (OR 0.2, 95% CI 0.1-0.7), B*35:01, C*04:01, DRB1*01:01, DQB1*05:01 (OR 0.2, 95% CI 0.1-0.7), C*04:01, DQB1*05:01 (OR 0.3, 95% CI 0.1-0.7), B*35:01, DQB1*05:01 (OR 0.3, 95% CI 0.1-0.8), A*02:01, DRQ1*05:01 (OR 0.4, 95% CI 0.1-0.8). VSCC16 and CXSCC16 are also strongly associated with A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01 (OR 0.6, 95% CI 0.4-1.0 and OR 0.6, 95% CI, 0.3-1.0 respectively).

There are 35 co-occurring alleles that were significantly different between VSCC16 and CXSCC16 as determined using polytomous regression. Of these the most significant differences are noted with various combinations of B*35:01, C*04:01, DRB1*01:01, DQB1*05:01; in VSCC16, these combinations are noted to lead to significantly decreased risk associated with disease (OR 0.2, 95%

CI 0.1-0.7), whereas in CXSCC16, there is no significant difference (OR 1.1, 95% CI 0.5-2.1). Additionally, B*07:02, C*07:02, DRB1*11:01, DQB1*03:01 alleles show significantly increased risk associated with disease in VSCC16 (OR 5.2, 95% CI 2.4-11.3), while CXSCC16 shows no significant among the alleles (OR 1.8, 95% CI 0.6-5.0).

In reviewing the data with co-occurring alleles associations for both VSCC16 and CXSCC16 three different combinations stand out within the evaluated population: 1) B*44:02 and B*44:03, 2) B*07:02, C*07:02, DQB1*03:01, and 3) A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01. Table 4 examines these associations across various multiples of co-occurring alleles.

Both B*44:02 and B*44:03 are noted to have increased OR when present. There are multiple co-occurring alleles that are of note, but those with the highest estimates are B*44:02, DRB1*11:01, DQB1*03:01 (OR 5.0, 95% CI 2.0-12.7), A*29:02, B*44:03, C*16:01, DRB1*07:01, DQB1*02:02 (OR 2.1, 95% CI 1.1-4.2), and A*02:01, B*44:02, C*05:01, DRB1*04:01, DQB1*03:01 (OR 2.1, 95% CI 1.2-3.8). In contrast, for CXSCC16, the allele of interest was B*44:02, not for B*44:03.

There appears to be a high level of LD between B*07:02 and C*07:02, and when combined with DQB1*03:01 there was a strong increased risk of disease. This is most evident with A*02:01, B*07:02, C*07:02, DRB1*11:01, DQB1*03:01 (OR 6.4, 95% CI 2.2-18.4) and A*02:01, B*07:02, C*07:02, DRB1*15:01, DQB1*03:01 (OR 3.2, 95% CI 1.4-7.4). Interestingly, while there is a significant increased risk with VSCC16, there are a limited number of co-occurring alleles with significant increased risk seen with CXSCC16.

A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01 co-occurring alleles show decreased risk between both VSCC16 (OR 0.6, 95% CI 0.4-1.0) and CXSCC16 (OR 0.6, 95% CI 0.3-1.0). There appears to be a high amount of LD associated with these co-occurring alleles. To assess LD, we examined the percentage of cases and controls over the co-occurring allele combinations. For a majority of the co-occurring combinations, the percentages of controls range from 14.6% to 19.8%. Similarly, the percentage of VSCC16 controls with the co-occurring alleles is fairly stable across most of the associations, from 9.2% to 15.4%.

DISCUSSION:

To our knowledge, this is the largest study undertaken to examine the association between carriage of HLA genes and HPV16-related squamous cell *in situ* and invasive vulvar cancer. We were able to review a large number of multilocus results, rarely evaluated before. Our study found several significant co-occurring allele combinations, with three groups standing out: B*44:02 and B*44:03 were found to be major risk-conferring alleles associated with increased risk when present, such as B*44:02, DRB1*11:01, DQB1*03:01 (OR 5.0, 95% CI 2.0-12.7); significantly increased risk was associated with multiple co-occurring allele combinations with the foundation of B*07:02, C*07:02, DQB1*03:01, such as with A*02:01, B*07:02, C*07:02, DRB1*11:01, DQB1*03:01 (OR 6.4, 95% CI 2.2-18.4); significantly decreased risk was associated with A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01 (OR 0.6, 95% CI 0.4-1.0). Many of the results were reproduced in CXSCC16: B*44:02, DRB1*11:01, DQB1*03:01 (OR 8.1, 95% CI 3.3-19.7) and significant decreased risk was associated with A*01:01, B*08:01, C*07:01, DRB1*03:01, DQB1*02:01 (OR 0.6, 95% CI 0.43-1.0). There were also several significant associations for vulvar

cancer which were not significant with CXSCC16, such as A*02:01, B*07:02, C*07:02, DQB1*03:01 (OR 1.2, 95% CI 0.2-6.0). These results suggest that while the immune response is important between both case groups, the immune response is more important in the etiology of vulvar cancer.

The incidence of *in situ* and invasive disease have increased over the past three decades. One hypothesis for this increase is associated with increases in HPV incidence [24]. It has been noted that there has been a decrease in the average age of women impacted by VSCC [25]. Younger women generally have disease associated with HPV infection than older women (> 65).

Our study indicated that there was a strong link between smoking and increased risk associated with *in situ* and invasive cancer. Other studies have shown a very high proportion of cases with *in situ* or invasive vulvar cancer that are currently smoking [2, 4, 26]. Recent cervical cancer research indicates that even a low level of smoking can lead to higher levels of HPV in infected cells [26-28]. With a higher percentage of women smoking and VSCC, it is likely this is a factor contributing to increased incidence.

In our evaluation of single HLA Class I alleles, HLA-A appears to be unassociated with risk, except for A*29:02. However, A*29:02 appears to be in LD with B*44:03, C*16:01, DRB1*07:01, DQB1*02:02, and therefore may not be a risk-conferring allele. There were several alleles which have variable estimates across different co-occurring combinations, including A*01:01, A*02:01, A*03:01, C*03:03, and C*04:01. These alleles seem to be a passenger associated with OR results.

We evaluated differences between the HLA subtype mutations that may explain some of the differences; we evaluated the known amino acid changes between the subtypes. For B*44:02 and 44:03, there is a single amino acid change at position 156, aspartic acid (B*44:02) to lysine (B*44:03). While both amino acids are polar, aspartic acid is positively charged while lysine is negatively charged. In our data, we saw instances of C*07:01 and C*07:02, which are noted to have two amino acid changes among the different subtypes: position 66 changes from asparagine (C*07:01) to lysine (C*07:02), position 99 changes from tyrosine (C*07:01) to serine (C*07:02). All the amino acids are noted to be polar, with lysine also containing a positively charged side-chain. C*07:01 is noted to mostly be associated with decreased OR and C*07:02 is associated with increased OR with VSCC16. In both instances the amino acid differences may underlie unmeasured immunologic differences by anatomic site.

There are a limited number of previous studies that have evaluated HLA alleles and HPV-related *in situ* and invasive vulvar cancer. Previous studies have been limited to examining single allele associations. Similar with the results in our study, previous results have indicated that there is increased risk associated with B*07, DRB1*11, and DQB1*03 and decreased risk associated with DQB1*05, DRB1*01, and DRB1*13 [29]. The study also saw increased risk associated with A*02, whereas our results showed no significance with most HLA-A alleles.

HLA significance with HPV-related cancers has been much more evaluated in cervical cancer cases. Studies evaluated HPV+ results but were not restricted to HPV16 results. As with vulvar cancer, previous studies have evaluated HLA on a single allele basis, not examining the potential impact of co-occurring alleles. A few studies have evaluated class I HLA results. Similar to our study, B*07 was

found to have significantly increased risk [12, 16]. As seen in our study class II HLA associations were noted to have significantly increased risk associated with DRB1*07, DRB1*11, DRB1*15, DQB1*03, and DQB1*06:02 [11]. Similar results were seen with decreased risks were associated with DRB1*03, DRB1*13:01, DQB1*06:03, and DQB1*05 [11, 12, 16]. Of note, there were several results which indicated different results including has been a prospective publication that illustrated decreased risk associated with DQB1*15:01 and DRB1*13 [30] and DQB1*03:02 [16].

The higher number of significant results associated with VSCC16 results suggests the important role that HLA has in HPV16 pathenogeneis. There is currently no screening test for *in situ* or vulvar cancer, relying on visual evaluation of the vulva as part of a pelvic examination at the time of cervical cancer screening to assess health, per the American College of Obstetrics and Gynecologists (ACOG) [31]. While prophylactic treatment of HPV, with the HPV vaccine [32] is suggested, only ~49% of eligible persons in the USA are receiving full dosage of the vaccine [33]. A screening test for *in situ* and invasive vulvar cancer, for better early diagnosis of disease is needed. This is especially important for high-risk subgroups such as immunosuppressed women who are known to be at higher risk of disease. Our study has shown that there are a large number of significant co-occurring HLA alleles shared between vulvar and cervical case samples. These may be markers for disease risk and may have future clinical value for targeted screening.

A limitation of this study is that not all genes in the HLA region were evaluated. The high LD seen between the HLA genes in our analysis may be markers of other genes in LD with those we find to be reduced or elevated in vulvar cancer. A strength of this work is the studies comparison with cervical cancer, which is pathogenically similar and therefore serves as a validation set for our analysis. The larger number of significant HLA associations found at both the single and multiple allele levels surprised us. This difference may suggest that vulvar lesions are more influenced by immune activity than cervical lesions.

While this is the largest dataset for evaluation of HLA with HPV related vulvar cancer, there are still limitations as there was low participation in the study for case and control subjects. It is not possible to know the potential bias associated with the HLA results when comparing the results. However, strong associations as was found with the study are not likely to be caused by this potential bias.

A strength of the paper is the restriction to HPV16 positive cases. Building on our prior study [12], which showed important differences between HPV16 and HPV18 cervical cancers, we focused here on the majority of vulvar cancers (~80%) that are the result of HPV16 infection. This allowed a more homogeneous population of cases to compare to controls. This restricted our CXSCC cases, of which ~50% were HPV16. However, the restriction allowed for direct comparisons between HLA and HPV16 results between the cases. A limitation is a lack of HPV testing of controls.

The data review is restricted to those women who self-define as white women only. This is important both because of the large diversity in HLA and because a large proportion of cases in the study were white women. This decision allowed the ability to evaluate more discrete trends in HLA, among white women. However, we are blinded from seeing different attributing factors that are present with women of different ethnicities.

In order to assess significance, we restricted results to HLA loci with prevalence ≥ 5% of either cases or controls. Even with this large study, the study number (n) may be too small due to the rarity of the cancer and the diversity of HLA. Conversely, the analysis could miss important risk estimates with small attributable proportions by excluding alleles with low prevalence (<5%).

FFPE tissue blocks were used to evaluate for HPV for *in situ* and invasive vulvar cancer cases. The formalin fixation and paraffin embedding process has been known to lead to sample degradation. The use of this sample type for viral testing could lead to an underestimation of the prevalence of HPV in the case samples tested, causing for a higher level of as false negatives in the case samples evaluated.

This study is the largest study to evaluate associations between co-occurring class I and class II HLA alleles and vulvar cancer. With a large dataset, we were able to evaluate statistical significance with a large number of different co-occurring HLA loci. We have made the study as specific as possible by restricting our investigation to VSCC16 and comparing those results to CXSCC16. Further research is needed to evaluate results with other HPV-related cancers. The data has shown that VSCC16 is largely similar to CXSCC with respect to HLA associations.

A recently published genome-wide association study (GWAS) found that only HLA region genes are highly associated with cervical cancer [34]. This agrees with previous work from our group [9, 34] and others [13, 15] indicate that risk of cancer may depend on specific HLA alleles or HLA-linked genes. Our current study found that there were a number of class I and II alleles with increased and decreased risk of vulvar cancer. This suggests that cytotoxic (CD8) and helper (CD4) T cells are important to pathogenesis of HPV16-related cancers. Development of future therapies, including potentially therapeutic vaccines, may benefit from these findings. Additionally, it may be that the reason some people have persistent infections necessary for HPV-related cancer development may be related to the ability of their HLA genes to recognize HPV antigens and present them to the immune system.

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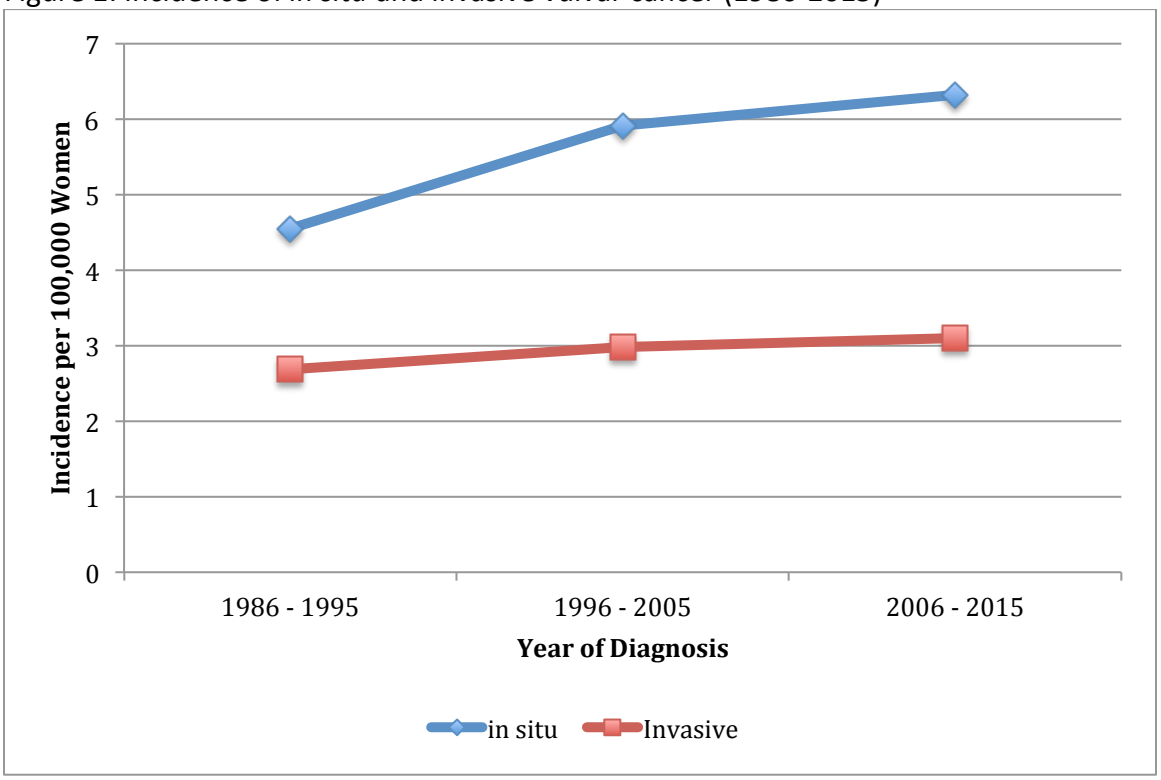
FIGURES:

Figure 1: Incidence of *in situ* and invasive vulvar cancer (1986-2015)



Age-adjusted incidence of *in situ* and invasive vulvar cancer per 100,000 women, US SEER data, ages 20-84, from 1986 to 2015.

TABLES:

Table 1: Distribution of Age, Smoking Status, Number of Partners, Education, Tumor Behavior, and HPV Type

	Controls (n=637)		**VSCC16 (n=238)**		**CXSCC16 (n=238)**	
	n	**%**	**n**	**%**	**n**	**%**
Age at reference						
<40	240	(37.7)	70	(29.4)	113	(47.5)
40-59	275	(43.2)	127	(53.4)	97	(40.8)
60+	122	(19.2)	41	(17.2)	28	(11.8)
Smoking						
Never	305	(47.9)	39	(16.4)	78	(32.8)
Former	187	(29.4)	55	(23.1)	61	(25.6)
Current	145	(22.8)	144	(60.5)	99	(41.6)
Total sex partners						
1	169	(26.7)	16	(6.8)	22	(9.2)
2-4	186	(29.4)	46	(19.6)	72	(30.3)
5-14	210	(33.2)	115	(48.9)	102	(42.9)
15+	67	(10.6)	58	(24.7)	42	(17.6)
Missing	5		3		0	
Education						
HS or Less	194	(30.5)	92	(38.7)	107	(45.0)
Post-HS	443	(69.5)	146	(61.3)	131	(55.0)
Behavior						
In situ			213	(89.5)	0	(0.0)
Invasive			25	(10.5)	238	(100.0)
HPV type						
16			206	(86.6)	195	(81.9)
16+			32	(13.4)	43	(18.1)

16+ indicates that HPV typing of the FFPE case samples included at least HPV16 and an additional HPV virus; the primary co-infections included HPV16 & 18 (2.5% of VSCC cases), HPV16 & 33 (3.8% of VSCC cases) and HPV6 & 16 (2.1% of VSCC cases).

Table 2: HLA class I A, B, and C allele OR results

	Control (n=637)		VSCC16 (n=238)				CXSCC16 (n=238)				Wald *p* value
	n	%	n	%	OR	95% CI	n	%	OR	95% CI	
B*15:01	83	13.9	11	4.7	0.3	(0.2-0.6)*	15	7.4	0.5	(0.3-0.9)*	0.240
B*35:01	65	10.9	12	5.1	0.4	(0.2-0.8)*	22	10.8	1.0	(0.6-1.7)	0.030
B*27:05	51	8.5	11	4.7	0.5	(0.3-1.0)	11	5.4	0.6	(0.3-1.2)	0.732
C*08:02	40	6.7	10	4.3	0.6	(0.3-1.3)	7	3.5	0.5	(0.2-1.1)	0.658
C*03:04	108	18.1	32	13.7	0.7	(0.5-1.1)	33	16.3	0.9	(0.6-1.4)	0.448
C*04:01	102	17.1	31	13.3	0.7	(0.5-1.1)	34	16.8	1.0	(0.6-1.5)	0.304
B*08:01	142	23.8	48	20.5	0.8	(0.6-1.2)	31	15.3	0.6	(0.4-0.9)*	0.157
C*07:01	175	29.4	58	24.9	0.8	(0.6-1.1)	44	21.8	0.7	(0.5-1.0)*	0.445
C*12:03	40	6.7	13	5.6	0.8	(0.4-1.6)	11	5.4	0.8	(0.4-1.6)	0.951
A*02:01	313	52.4	117	50.0	0.9	(0.7-1.2)	108	52.9	1.0	(0.7-1.4)	0.539
A*03:01	160	26.8	57	24.4	0.9	(0.6-1.2)	63	30.9	1.2	(0.9-1.7)	0.127
A*24:02	86	14.4	31	13.2	0.9	(0.6-1.4)	36	17.6	1.3	(0.8-2.0)	0.203
C*01:02	39	6.5	14	6.0	0.9	(0.5-1.7)	10	5.0	0.7	(0.4-1.5)	0.630
C*02:02	60	10.1	21	9.0	0.9	(0.5-1.5)	18	8.9	0.9	(0.5-1.5)	0.970
A*11:01	66	11.1	25	10.7	1.0	(0.6-1.6)	26	12.7	1.2	(0.7-1.9)	0.503
B*18:01	47	7.9	19	8.1	1.0	(0.6-1.8)	13	6.4	0.8	(0.4-1.5)	0.493
B*40:01	74	12.4	30	12.8	1.0	(0.7-1.6)	26	12.8	1.0	(0.6-1.7)	0.997
A*01:01	174	29.1	73	31.2	1.1	(0.8-1.5)	56	27.5	0.9	(0.6-1.3)	0.391
A*31:01	35	5.9	15	6.4	1.1	(0.6-2.1)	13	6.4	1.1	(0.6-2.1)	0.987
A*32:01	40	6.7	17	7.3	1.1	(0.6-2.0)	16	7.8	1.2	(0.6-2.2)	0.819
B*13:02	21	3.5	9	3.8	1.1	(0.5-2.4)	11	5.4	1.6	(0.7-3.3)	0.435
B*44:02	99	16.6	45	19.2	1.2	(0.8-1.8)	59	29.1	2.1	(1.4-3.0)*	0.017
B*51:01	56	9.4	25	10.7	1.2	(0.7-1.9)	20	9.9	1.1	(0.6-1.8)	0.776
C*03:03	60	10.1	27	11.6	1.2	(0.7-1.9)	17	8.4	0.8	(0.5-1.4)	0.276
C*05:01	100	16.8	45	19.3	1.2	(0.8-1.8)	50	24.8	1.6	(1.1-2.4)*	0.172
B*57:01	39	6.5	19	8.1	1.3	(0.7-2.2)	22	10.8	1.7	(1.0-3.0)*	0.333
C*07:04	16	2.7	8	3.4	1.3	(0.5-3.1)	14	6.9	2.7	(1.3-5.6)*	0.104
C*07:02	160	26.8	80	34.3	1.4	(1.0-2.0)*	67	33.2	1.4	(1.0-1.9)	0.798
B*07:02	150	25.1	79	33.8	1.5	(1.1-2.1)*	64	31.5	1.4	(1.0-1.9)	0.620
C*06:02	101	16.9	55	23.6	1.5	(1.0-2.2)*	43	21.3	1.3	(0.9-2.0)	0.564
A*29:02	45	7.5	28	12.0	1.7	(1.0-2.7)*	12	5.9	0.8	(0.4-1.5)	0.031
A*68:01	38	6.4	24	10.3	1.7	(1.0-2.9)	15	7.4	1.2	(0.6-2.2)	0.289
B*37:01	19	3.2	14	6.0	1.9	(1.0-3.9)	7	3.4	1.1	(0.4-2.6)	0.222
C*16:01	45	7.6	31	13.3	1.9	(1.2-3.1)*	15	7.4	1.0	(0.5-1.8)	0.050
B*44:03	54	9.0	40	17.1	2.1	(1.3-3.2)*	22	10.8	1.2	(0.7-2.1)	0.064
B*55:01	12	2.0	19	8.1	4.3	(2.1-9.0)*	7	3.4	1.7	(0.7-4.5)	0.046

ORs are arranged from lowest to highest for class I vulvar associations. ORs are not adjusted.

* indicates statistically significant associations, although rounding sometimes-caused confidence intervals to include 1.0.

The Wald *P* Value is shown for multiple comparisons and was based on all alleles at a locus.

Table 3: HLA class II DRB1 and DQB1 allele OR results

	Control (n=637)		VSCC16 (n=238)				CXSCC16 (n=238)				Wald *p* value
	n	%	n	%	OR	95%CI	n	%	OR	95%CI	
DQB1*06:03	75	11.8	7	3.0	0.2	(0.1-0.5)*	16	6.8	0.5	(0.3-0.9)*	0.064
DRB1*13:01	71	11.2	7	3.0	0.2	(0.1-0.5)*	15	6.4	0.5	(0.3-1.0)*	0.088
DQB1*05:01	128	20.1	25	10.6	0.5	(0.3-0.7)*	51	21.5	1.1	(0.8-1.6)	0.002
DRB1*01:01	100	15.8	19	8.1	0.5	(0.3-0.8)*	41	17.5	1.1	(0.8-1.7)	0.003
DRB1*13:02	62	9.8	13	5.6	0.5	(0.3-1.0)	11	4.7	0.5	(0.2-0.9)*	0.675
DRB1*03:01	157	24.8	41	17.5	0.6	(0.4-0.9)*	38	16.2	0.6	(0.4-0.9)*	0.711
DQB1*02:01	150	24.1	38	16.6	0.6	(0.4-0.9)*	38	16.2	0.6	(0.4-0.9)*	0.902
DQB1*06:04	47	7.4	10	4.3	0.6	(0.3-1.1)	10	4.2	0.6	(0.3-1.1)	0.985
DRB1*04:04	57	9.0	16	6.8	0.7	(0.4-1.3)	15	6.4	0.7	(0.4-1.2)	0.853
DQB1*03:02	151	23.7	51	21.7	0.9	(0.6-1.3)	46	19.4	0.8	(0.5-1.1)	0.538
DQB1*03:03	53	8.3	19	8.1	1.0	(0.6-1.7)	35	14.8	1.9	(1.2-3.0)*	0.024
DRB1*08:01	39	6.2	16	6.8	1.1	(0.6-2.0)	12	5.1	0.8	(0.4-1.6)	0.437
DRB1*04:01	128	20.2	56	23.9	1.2	(0.9-1.8)	64	27.4	1.5	(1.1-2.1)*	0.397
DQB1*05:03	26	4.1	12	5.1	1.3	(0.6-2.5)	16	6.8	1.7	(0.9-3.2)	0.451
DRB1*14:01	28	4.4	13	5.6	1.3	(0.6-2.5)	16	6.8	1.6	(0.8-3.0)	0.566
DQB1*06:02	164	25.8	81	34.5	1.5	(1.1-2.1)*	63	26.6	1.0	(0.7-1.5)	0.063
DRB1*15:01	164	25.9	80	34.2	1.5	(1.1-2.1)*	65	27.8	1.1	(0.8-1.5)	0.134
DQB1*03:01	200	31.4	99	42.1	1.6	(1.2-2.2)*	92	38.8	1.4	(1.0-1.9)*	0.464
DQB1*04:02	28	4.4	17	7.2	1.7	(0.9-3.2)	11	4.6	1.1	(0.5-2.2)	0.237
DRB1*07:01	149	23.5	79	33.8	1.7	(1.2-2.3)*	72	30.8	1.4	(1.0-2.0)*	0.489
DQB1*02:02	111	17.8	63	27.5	1.8	(1.2-2.5)*	48	20.4	1.2	(0.8-1.7)	0.075
DRB1*11:01	47	7.4	49	20.9	3.3	(2.1-5.1)*	31	13.2	1.9	(1.2-3.1)*	0.028

ORs are arranged from lowest to highest for class II vulvar associations. ORs are not adjusted.

* indicates statistically significant associations, although rounding sometimes-caused confidence intervals to include 1.0.

The Wald *P* Value is shown for multiple comparisons and was based on all alleles at a locus

DRB1*07 could not be discerned as *07:01, *07:03, or *07:04 by the assay (see Methods)

DQB1*02 could not be discerned as *02:01 or *02:02 by the assay (see Methods)