KIR gene content variation and risk of vulvar and cervical cancer: a population-based case-control study

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Abstract

KIR gene content variation and risk of vulvar and cervical cancer: a population-based case-control study

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The killer-cell immunoglobulin-like receptors (KIR) bind to MHC class I molecules that have been associated with risk of cervical and vulvar cancer. We investigated whether variation in KIR gene content was associated cervical and vulvar cancer in a population-based case-control study of women from Washington state. Study participants included 498 controls, 358 vulvar cancer cases, and 666 cervical cancer cases, including 227 squamous cell carcinomas, 419 adenocarcinomas, and 20 adenosquamous carcinomas. Controls were matched to cases on age and geographic region of residence and analyses were limited to whites to reduce the potential for bias due to population stratification. We found no associations between the presence of individual KIR genes and either vulvar cancer or all histologies of cervical cancer pooled. Three KIR genes were associated with cervical adenocarcinoma, including 2DS2 (OR 0.74, 95% CI 0.56-0.97), 2DL2 (OR 0.74, 95% CI 0.57-0.98), and 2DS3 (OR 0.72, 95% CI 0.53-0.98), though a permutation test provided no indication that these associations were more extreme than would be expected due to chance. These three KIR genes are among the centromeric haplotype B (CenB) KIR genes, though 2DS3 also occurs among the telomeric haplotype B KIR. The presence of CenB was associated with lower risk of cervical adenocarcinoma (OR 0.74, 95% CI 0.56-0.97). The presence of 2DS2 combined with one or more copies of its ligand, HLA-C group 1 alleles, defined by an asparagine residue at position 80, was associated with lower risk of cervical adenocarcinoma (OR 0.75, 95% CI 0.57-0.99). Tumor samples were tested for HPV DNA, and in polytomous logistic regression model we found that 3DS1 was associated with HPV 18-positive but not HPV 16-positive cervical adenocarcinoma. In conclusion, our study does not provide strong evidence that KIR gene content is

related to risk of either vulvar or cervical cancer, all histologies pooled. Subgroup analyses suggest that CenB KIRs may contribute to lower risk of cervical adenocarcinoma, especially for cancers caused by HPV 18. This study contributes to research that aims to clarify the role of immunogenetic variation in determining why some women exposed to HPV develop cervical or vulvar cancer.

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Chapter 1. Background

1.1 Cervical cancer burden

Cancer of the uterine cervix is the third most common type of cancer and the second most common cause of cancer death among women worldwide, with 530,000 diagnoses and 275,000 deaths in 2008. [1] Cervical cancer is caused by infection with high risk types of human papillomavirus (HPV), the most common sexually transmitted pathogen, infecting more than 50% of US women over their lifetime. [2-5] HPV also causes genital warts, as well as some vulvar, vaginal, most anal, penile, and most oropharyngeal cancers. [6]

Age-standardized incidence rates of cervical cancer vary at least 5-fold across regions of the world, from less than 6 per 100,000 in North America, 20-25 per 100,000 in parts of South America and central Asia, to greater than 30 per 100,000 in western Africa. [1] Women in many low resource countries have limited access to cervical cancer screening and treatment. Because of its high incidence and concentration in women of reproductive age, cervical cancer leads other cancers in many developing countries as a source of years of life lost (YLL), an important indicator of public health impact. [7]

In developed countries, preventive measures have reduced mortality due to cervical cancer, but at substantial economic cost. Routine Pap tests and treatment of HPV-related cervical lesions costs approximately $4.3 billion per year in the US, with another $350 million to treat cervical cancer. [8] These costs were calculated prior to the introduction of HPV vaccines in 2006 and exclude indirect costs due to lost economic activity. The Gardasil HPV vaccine costs $360 per person, which if administered to the approximately 2 million 12 year old girls in the US represents $720 million annually. Eventually, vaccines will reduce the frequency of Pap tests and the incidence of HPV-related cancer, but substantial mortality and economic cost will be incurred in the meantime.

1.2 Vulvar cancer burden

Vulvar cancer is less common than cervical cancer, but has a high rate of recurrence. Recent estimates of the age-standardized incidence of invasive and in situ vulvar cancer are, respectively, 2.5 cases and 5.0 cases per 100,000, and these estimates have been increasing steadily for the last 25 years in the US. [9] One study found that 23% of patients experienced local recurrence following surgery and treatment,

with most of these women experiencing multiple recurrences. [10] HPV is thought to be a causal agent in approximately 25% of invasive vulvar cancers and as much as 90% of in situ vulvar cancers, it is of interest whether other risk factors differ between vulvar cancers with and without HPV. [11, 12] HPV vaccine trials indicate that the vaccine reduces the risk of developing precursor lesions to vulvar cancer. [13, 14]

1.3 Natural history of HPV-related carcinogenesis

Natural history studies have delineated a progression from HPV infection to cervical cancer, and have generated insights into why only some infected women develop cervical cancer; most clear the virus without disease progression. [15-18] HPV type and viral sequence variation within types play a role. Of the over 100 types of HPV that infect human genital epithelia, only some are oncogenic, and of these, HPV-16 and HPV-18 account for 70-80% of all cervical cancers. [19] Recent and ongoing research investigating sequence variation among HPV-16 and HPV-18 and other high-risk oncogenic types that may have different associations with intermediates of disease, such as persistence of infection and higher viral copy number. [20, 21] Extensive research has identified oncogenic properties of HPV-16 and HPV-18 proteins. Most of this research, which is constrained by an inability to culture HPV *in vitro*, has focused on viral E6 and E7 proteins, which are expressed in cervical tumors. [22] The oncogenic properties of HPV-16/18 E6 and E7 proteins include cell cycle checkpoint subversion, the induction of genomic instability, and blocking apoptosis. [22]

1.4 Host genetics and HPV disease

Host genetic variation also plays a role in determining which HPV infections progress to cervical cancer. A recent nested case-control study identified single nucleotide polymorphisms (SNPs) in IFN-γ and EVER1/EVER2, where variants were associated with progression to CIN3/cancer. [23] Rare deletion mutations in EVER1/EVER2 underlie Epidermodisplasia vericruciforma, which is characterized by uncontrolled growth of HPV infected lesions. [24] The functions of EVER1 and EVER2 are not well understood, but there is some indication that these proteins are involved in intracellular zinc metabolism, a regulator of antiviral responses by immune cells including natural killer (NK) cells. [25] IFN-γ initiates a cascade of intracellular antiviral responses, including shutting down protein expression. Many viruses,

including HPV, have evolved strategies for disrupting IFN-γ signaling. For example, the E6 and E7 proteins of oncogenic HPV types block STAT-1, IRF-2 and IRF-3, intermediates in the IFN-γ response. [26] IFN-γ is released by NK cells in response to the detection of virus infected cells, further implicating these cells in the immune response to HPV infection.

Other studies have identified host genetic variation on chromosome 6 that is associated with cervical or vulvar cancer. For example, MHC class I alleles have been associated with both cervical and vulvar cancer. [27-29] MHC class I molecules play a central role in the immune response to viral infection. MHC class I molecules are expressed on normal cells of the cervix and present intracellular peptides to cytotoxic T lympocytes (CTLs), which selectively kill cells with MHC class I molecules presenting viral or otherwise unrecognized peptides. Many intracellular pathogens have evolved strategies for down-regulating MHC molecules, allowing them to avoid CTL detection. [30] MHC class I molecules also bind to the killer-cell immunoglobulin-like receptors (KIR), diverse receptors found on the surface of natural killer (NK) cells that regulate NK cell responses. Importantly, NK cells have the ability to kill virus-infected cells that have downregulated MHC class I expression. [31]

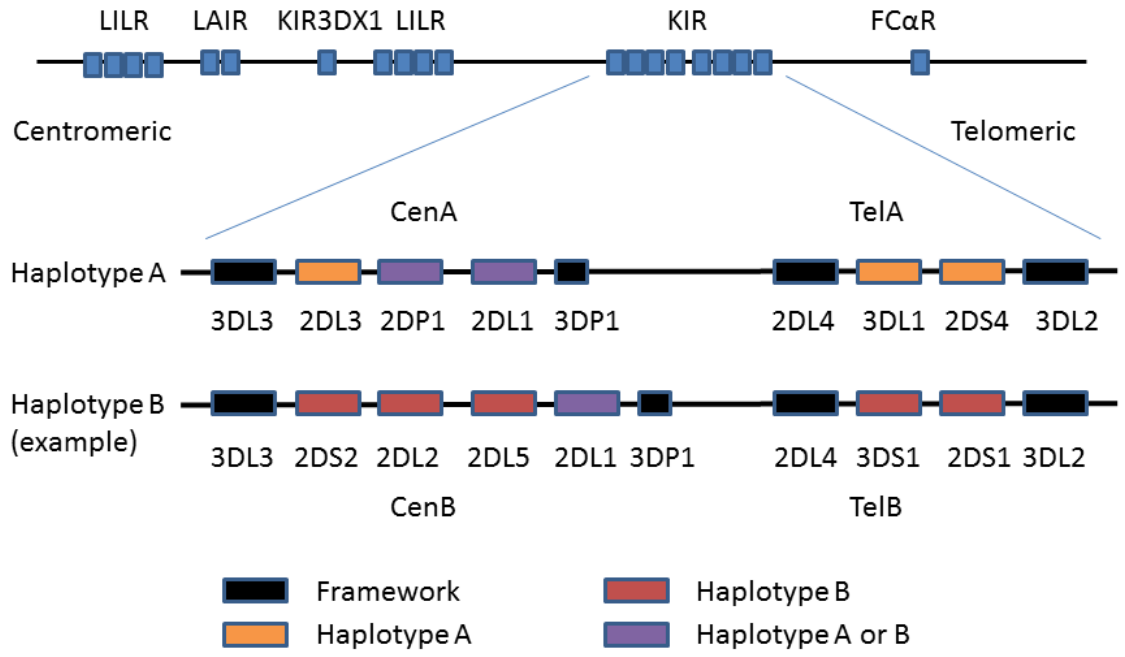
Besides MHC class I genes, other genes that regulate NK cell function have also been associated with cervical cancer risk, including MHC class I-like molecules MICA and MICB, which are in LD with the MHC class I loci. [32, 33] MICA and MICB molecules are expressed on the surface of cancer cells, signaling to NK cells and gamma-delta T cells the transformed state of the cell. MICB acts through binding to the NK cell receptor, NKG2D. Activation of NK cells following MICB-NKG2D binding is thought to require co-stimulatory binding of additional ligand-receptor pairs such as KIR-HLA. [34]

1.5 KIR structure, function, and variation

Genes encoding the KIR are arrayed in two clusters spanning 250 kb in the leukocyte receptor complex on chromosome 19 (**Figure 1**). A typical individual human genome carries only 7-14 of the 15 common KIR genes in the human population. Four of the KIR are found in nearly all individuals, and are termed “framework” KIR. The framework KIR genes bookend each of the clusters, with 3DL3 and 3DP1 enclosing the centromeric cluster and 2DL4 and 3DL2 enclosing the telomeric cluster. Each of the remaining KIR genes is either present or absent in each individual. In addition to framework KIR genes, the centromeric

cluster of haplotype A, referred to as CenA, consists of 2DL3, 2DP1, and 2DL1, and the telomeric cluster of haplotype A, termed TelA, consists of 3DL1 and 2DS4. Haplotype A has very little variation in KIR gene content. In contrast, haplotype B has extensive variation in KIR gene content. The centromeric cluster of haplotype B, CenB, includes framework KIR genes and 2DS2 and 2DL2 along with other KIR that may include 2DL5, 2DS3, 2DS5, 2DP1, and 2DL1. The telomeric cluster of haplotype B, TelB, includes the framework KIR and 3DS1 along with some subset of 2DL5, 2DS3, 2DS5, and/or 2DS1. [35] Both haplotypes A and B occur in all sampled human populations, and it is not unusual for an individual to have mixed haplotypes such as CenA and TelB on the same chromosome. [36]

Figure 1. Arrangement of KIR genes in the leukocyte receptor complex on chromosome 19



Adapted by the author from [35]

Despite broad homology among the KIR, structural variation underlies differences between them in NK cell regulation. Each KIR either activates or inhibits NK cell activity, determined by an intracellular tail that is either short (activating) or long (inhibitory). The KIR are displayed on the surface of NK cells, and regulate the response to target cells through interactions with MHC I molecules (HLA-A, -B, and -C)

on the surface of target cells. NK cells in contact with virus-infected and transformed cells release cytokines such as IFN-γ and tumor necrosis factor alpha, or kill cells directly.

Individual NK cells express only a subset of the KIR encoded in their genomes, in an apparently stochastic fashion. This means that subpopulations of NK cells with different receptor expression patterns exist within a single individual. Studies characterizing NK cell subsets from a small numbers of individuals have found that all KIR encoded by a genome are found on at least some NK cells. [37, 38]

The leading model for how KIR-HLA variation may regulate NK cell activity is that the threshold for NK cell activation is determined by the balance of activating and inhibitory KIR displayed at the cell surface. [35] Each inhibitory KIR that a cell displays on its surface raises the threshold of inhibition that needs to be overcome before the NK cell responds. Similarly, each activating receptor lowers the threshold, making NK cells more responsive. According to this model, having more activating KIR along with alleles for their HLA ligands would be associated with a higher risk of cancer if NK cell responses are carcinogenic. If the NK cell responses, on balance, fight cancer, then having more inhibitory ligand-receptor pairs would be associated with a higher risk of cancer because the cancer-fighting NK cell response would be muted in these individuals.

1.6 Prior studies of KIR-HLA variation and cancer risk

Two prior studies have investigated whether KIR genetic variation is associated with cervical cancer risk, and they provide conflicting support for the leading model for how KIR-regulated NK cell activation affects carcinogenesis. The first was a case-control study of 65 women with squamous cell cervical intraepithelial neoplasia and 150 controls in a Swedish population. [39] The study found that the presence of a KIR allelic variant, 2DL5B*002, was associated with lower risk of cervical cancer (OR 0.5, 95% CI 0.2-0.9). 2DL5B*002 does not code for a functional KIR and is almost always found with activating KIR 2DS3 in sampled human populations. [40] 2DS3 itself was not associated with cervical cancer risk at the $p<0.05$ threshold, though the OR was suggestive of a lower risk (OR = 0.6, 95% CI 0.3-1.1). In the same study, a common haplotype that included seven inhibitory KIRs: 2DL1, 2DL2, 2DL3, 2DL4, 3DL1, 3DL2, 3DL3, and one activating KIR: 2DS4, was associated with a greatly increased risk (OR 6.7, 95% CI 1.7-26.3). The suggestion that individuals with 2DS3 may be at a lower risk of cancer while those with an

abundance of inhibitory KIR may be at a higher risk is consistent with a protective role for NK cell activation in carcinogenesis.

In contrast, the second study to investigate KIR associations with cervical cancer risk suggested that NK cell activation may contribute to cancer risk. Carrington *et al.* included 196 cases and 330 controls and found that having activating KIR 3DS1 was associated with increased risk of CIN3/cervical cancer (OR 1.64, 95% CI 1.13-2.39). (In the Arnheim *et al.* study, having 3DS1 was not associated with risk (OR 1.0, 95% CI 0.5-1.7).) The Carrington *et al.* study included HLA genotypes, and ranked combined KIR-HLA genotypes according to degree of expected activation or inhibition of NK cells. Greater potential for NK cell inhibition was associated with lower risk of cervical cancer in a trend test.

Little is known about genetic risk factors for vulvar cancer, and it may be that genetic risk factors are shared with cervical cancer, at least for vulvar cancers that are due to oncogenic HPV infection. [41] Indeed, two studies have found that variation in the MHC class I genes is associated with vulvar cancer. [27, 29] No prior studies have reported on associations between KIR variation and vulvar cancer.

1.7 Rationale for this study

Despite impressive advances in our understanding of HPV-related carcinogenesis and the development of vaccines that prevent cervical cancer and other HPV-related anogenital disease, an important question about HPV-related anogenital cancers remains: why do only a small percentage of women infected with oncogenic HPV develop the persistent infections that lead to cervical cancer? Host immune variation likely plays a role, and clarifying how may enable therapeutic immune modulation to promote clearance of HPV infections and healing of HPV-related cancerous lesions. [42]

Given the mixed results to date and the potential importance of KIR variation in HPV-related cancer, we proposed to investigate KIR gene content variation in a population-based case-control study. Our study has several advantages over the two prior studies of KIR variation and cervical cancer risk. First, our sample size is larger, allowing more statistical power to detect associations. Second, our study included substantial numbers of adenocarcinomas, which differ etiologically in some ways from SCC, and we can measure associations in both histological groups. [43] Third, previously described models for how KIR and HLA variation may be related to cancer risk could be tested in a different population. For

example, is the activating versus inhibitory KIR dichotomy meaningful? What about KIR/HLA pairings? A consensus has not yet been reached on the best way to understand how KIR and HLA variation may affect disease risk, and the data generated in this study allowed for exploration of different categorizations of variation in these two gene regions.

This is the first study to investigate associations between KIR genetic variation and risk of vulvar cancer. We set out to perform exploratory analyses to investigate whether KIR variation is associated more strongly with HPV+ vulvar cancer compared to vulvar cancer cases with no HPV detected.

Another potential benefit of this study is that significant associations between KIR genes and cervical or vulvar cancer could help to identify persons at risk, enabling better targeting of cancer screening and HPV vaccination efforts in resource limited settings, if these findings are generalizable to other populations.

Chapter 2. Materials and Methods

2.1 Study population

This study utilized DNA specimens and subject demographic and health history data collected as part of a population-based case-control study of HPV-related anogenital cancers. The parent study has been described in detail elsewhere. [44-46] Briefly, cases were women diagnosed between 1986 and 2004 with squamous cell carcinoma (SCC) of the cervix, invasive or in situ adenocarcinoma of the cervix, adenosquamous carcinoma of the cervix, or invasive or in situ carcinoma of the vulva. Cases resided in a 13-county area of western Washington State and were identified through the Fred Hutchinson Cancer Research Center's Cancer Surveillance System, part of the National Cancer Institute's Surveillance, Epidemiology, and End Results Program. [47] Controls were recruited using random digit dialing of landline telephones and were frequency matched to cases in 5-year age groups and by geography. All study subjects were 18-79 years of age at the time of cancer diagnosis or a corresponding reference date for controls.

Study participants were interviewed in-person by professional interviewers using a structured instrument to collect demographic and health history information. Blood cell samples were collected from

consenting study participants, and DNA was extracted. Study participation proportions for the parent study have been reported previously. [48] Subjects included in this study were those for whom HLA-A, -B, and -C alleles had previously been typed, [48] and for whom sufficient DNA remained for additional genotyping assays.

2.2 KIR genotyping

Our goal was to ascertain the presence or absence of each of 15 common KIR genes in the genome of each study participant. The Carrington laboratory of the U.S. National Cancer Institute graciously provided us with validated PCR primer sequences (**Table 1**) and a PCR protocol (**Table 2**, personal communication).

PCR amplification

Each KIR gene had two primer pairs consisting of one forward and one reverse primer, allowing for two separate interrogations of each KIR gene, with the exception of 2DS1, which had two forward primers that differ in one nucleotide and one reverse primer sequence. Most primer pairs recognize nucleotide sequences in exons. Primers were chosen to perform well at a shared melting temperature (**Table 2**). This facilitates high throughput amplification, but has the drawback of making it difficult to resolve products on the basis of melting temperature. Successful amplification of a PCR product indicates that one or two copies of a KIR gene is present in the genome interrogated. Failure to amplify a PCR product indicates that a particular KIR gene is absent.

The PCR protocol we used was based on the Carrington laboratory protocol, adapted to replace agarose gel electrophoresis separation of PCR products with Sybr green determination of PCR product melt curves. [49] Sybr green is a dye with 1000x greater fluorescence when it is intercalated in double-stranded DNA than in the presence of single-stranded DNA. Thus, when a solution of double stranded PCR products and Sybr green is heated, fluorescence declines as the PCR products denature. Over a steady, linear increase in temperature, fluorescence tends to decline in a sigmoidal curve, rapidly decreasing close to a temperature indicative of the melting temperature for the PCR product. This rapid decline in fluorescence produces a spike in the absolute value of the derivative of fluorescence. Melt

curves that do not have a clean sigmoidal curve with a defined spike in the derivative of fluorescence generally indicate the absence of a PCR product.

Each PCR reaction was scaled to 5 µL, and included the following ingredients: PCR primer (1 µL), Sybr green (2.5 µL), water (1.4 µL), and DNA template at 50 ng/ µL. The PCR temperature cycle is given below in **Table 2**. PCR reaction mixtures were aliquoted onto 384 well plates using a Tecan Freedom EVO robot. PCR reactions were performed on Perkin Elmer 9600 desktop thermocyclers. Melt curve data were collected using an ABI 7900HT Sequence Detection System. Melt curve data were analyzed using customized algorithms coded in the R statistical language (version 12.2.1). These algorithms extracted the derivative of fluorescence curves, the average fluorescence, and the temperature at which the maximum derivative was reached.

Quality Control

PCR and genotyping quality control had several components. For each DNA sample from a case or control, a positive and negative control PCR reaction was included. The positive control reaction included PCR primers that amplify a fragment of HLA DRB1, a gene found in all humans. The negative control reaction included water in place of sample DNA. Another component of our quality control strategy was the inclusion on each genotyping plate of a DNA sample of known KIR genotype (African American Panel of 50) supplied by Corriell Cell Repositories (Corriell Institute for Medical Research, Camden, NJ). The full panel of KIR genes, as well as positive and negative controls, was genotyped for the Coriell sample on each 384 well plate (1 Coriell sample for every 11 test samples). Another component of our quality control strategy was the inclusion of blinded DNA sample duplicates from 10% of cases and controls. Blinded sample duplicates were distributed evenly among genotyping plates.

Genotype Assignment

Genotype assignment occurred in two stages. The first determined whether a PCR product was present or absent based on a computational analysis of melt curve fluorescence data. The second combined the results of the two PCR primer pairs for each KIR. Each genotyping stage is discussed in turn.

A PCR product was deemed present when the maximum derivative of fluorescence was >0.02, mean derivative was >0.015, and the temperature of the maximum derivative (*i.e.*, the melting

temperature) was >80C. The Carrington laboratory genotyping protocol recommended the first two components of the above algorithm for determining whether a KIR was present or not. A third component enhanced KIR genotype accuracy as determined using the 10% blinded sample duplicates. This determination was made by assigning genotypes to samples that had blinded duplicates using three different combinations of two or more of the algorithm components described above, and then having an independent study manager (LGJ) calculate the rates of agreement between genotypes of the original samples and the blinded duplicates in each of the three datasets, without unblinding sample duplicates for laboratory personnel or the analyst (SNM). Including all three components yielded the highest level of agreement, and so the genotype dataset based on that algorithm was used for all analyses.

The second stage of genotyping evaluated concordance between the two PCR primer pairs specific to each KIR. A negative result for both PCR reactions indicated that the KIR was absent. Two positive results indicated that the KIR was present. When only one PCR reaction yielded a product, the result was discordant. For discordant results, both PCR reactions for that KIR were repeated on a new genotyping plate. After discordant pairs of PCR reactions were repeated, the resulting data included one positive reaction from the first genotyping run and either zero, one, or two positive reactions for the repeat run.

A total of two or more positive PCR reactions between the initial and repeat runs for a KIR indicated that the KIR was present. We held to this rule regardless of whether the positive reactions occurred with the same primer pairs or with different primer pairs in the first and repeat runs. Our rationale was that, given our strict three component algorithm for genotype calling, two false positive results on the same sample would be an unlikely outcome.

Lastly, some PCR reactions were repeated for reasons other than KIR discordance. Any 384 well plate that had eight or more total discordant KIRs was repeated in its entirety, meaning that some concordant PCR reactions were repeated. Any sample for which either the positive control (HLA DRB1) or negative control (water) failed was repeated in its entirety. Likewise, any PCR reaction for which the corresponding KIR PCR reaction in the Coriell sample on the same plate yielded a negative result was also repeated.

Lab personnel (SNM, CR, KWM) and the data analyst (SNM) were blinded to case-control status until after final KIR genotypes had been assigned.

Table 1. KIR gene PCR primer sequences

Gene	Primer	Sequence
2DL1	F1	GTT GGT CAG ATG TCA TGT TTG AA
2DL1	R1	GGT CCC TGC CAG GTC TTG CG
2DL1	F2	TGG ACC AAG AGT CTG CAG GA
2DL1	R2	TGT TGT CTC CCT AGA AGA CG
2DL2	F1	CTG GCC CAC CCA GGT CG
2DL2	R1	GGA CCG ATG GAG AAG TTG GCT
2DL2	F2	GAG GGG GAG GCC CAT GAA T
2DL2	R2	TCG AGT TTG ACC ACT CGT AT
2DL3	F1	CTT CAT CGC TGG TGC TG
2DL3	R1	AGG CTC TTG GTC CAT TAC AA
2DL3	F2	TCC TTC ATC GCT GGT GCT G
2DL3	R2	GGC AGG AGA CAA CTT TGG ATC A
2DL4	F1	CAG GAC AAG CCC TTC TGC
2DL4	R1	CTG GGT GCC GAC CAC T
2DL4	F2	ACC TTC GCT TAC AGC CCG
2DL4	R2	CCT CAC CTG TGA CAG AAA CAG
2DS2	F1	TTC TGC ACA GAG AGG GGA AGT A
2DS2	R1	GGG TCA CTG GGA GCT GAC AA
2DS2	F2	CGG GCC CCA CGG TTT
2DS2	R2	GGT CAC TCG AGT TTG ACC ACT CA
2DS3	F1	TGG CCC ACC CAG GTC G
2DS3	R1	TGA AAA CTG ATA GGG GGA GTG AGG
2DS3	F2	CTA TGA CAT GTA CCA TCT ATC CAC
2DS3	R2	AAG CAG TGG GTC ACT TGA C
2DS4	F1	CTG GCC CTC CCA GGT CA
2DS4	R1	TCT GTA GGT TCC TGC AAG GAC AG
2DS4	F2	GTT CAG GCA GGA GAG AAT
2DS4	R2	GTT TGA CCA CTC GTA GGG AGC
2DS5	F1	TGA TGG GGT CTC CAA GGG
2DS5	R1	TCC AGA GGG TCA CTG GGC
2DS5	F2	ACAGAGAGGGGACGTTTAACC
2DS5	R2	ATGTCCAGAGGGTCACTGGG
2DS1	F1	CTT CTC CAT CAG TCG CAT GAA
2DS1	F2	CTT CTC CAT CAG TCG CAT GAG
2DS1	R3	AGA GGG TCA CTG GGA GCT GAC
3DL1	F1	CGC TGT GGT GCC TCG A
3DL1	R1	GGT GTG AAC CCC GAC ATG
3DL1	F2	CCC TGG TGA AAT CAG GAG AGA G

Gene	Primer	Sequence
3DL1	R2	TGT AGG TCC CTG CAA GGG CAA
3DL2	F1	CAA ACC CTT CCT GTC TGC CC
3DL2	R1	GTG CCG ACC ACC CAG TGA
3DL2	F2	CCC ATG AAC GTA GGC TCC G
3DL2	R2	CAC ACG CAG GGC AGG G
3DS1	F1	AGC CTG CAG GGA ACA GAA G
3DS1	R1	GCC TGA CTG TGG TGC TCG
3DS1	F2	CCT GGT GAA ATC AGG AGA GAG
3DS1	R2	GTC CCT GCA AGG GCA C
3DL3	F1	GTCAGGACAAGCCCTTCCTC
3DL3	R1	GAGTGTGGGTGTGAACTGCA
3DL3	F2	TTCTGCACAGAGAGGGGATCA
3DL3	R2	GAGCCGACAACTCATAGGGTA
2DL5	F1	GCG CTG TGG TGC CTC G
2DL5	R1	GAC CAC TCA ATG GGG GAG C
2DL5	F2	TGC AGC TCC AGG AGC TCA
2DL5	R2	GGG TCT GAC CAC TCA TAG GGT
2DP1	F1	GTC TGC CTG GCC CAG CT
2DP1	R1	GTG TGA ACC CCG ACA TCT GTA C
2DP1	F2	CCA TCG GTC CCA TGA TGG
2DP1	R2	CAC TGG GAG CTG ACA ACT GAT G
HLA DRB1	IC 5'/IC 3'	TGC CAA GTG GAG CAC CCA A
HLA DRB1		GCA TCT TGC TCT GTG CAG AT

Table 2. KIR gene PCR reaction conditions

Cycles	Temperature	Duration
	94◦C	3 minutes
	94◦C	15 seconds
x5 cycles	65◦C	15 seconds
	72◦C	30 seconds
	94◦C	15 seconds
x21 cycles	60◦C	15 seconds
	72◦C	30 seconds
	94◦C	15 seconds
x4 cycles	55◦C	1 minute
	72◦C	2 minutes
	72◦C	7 minutes
HOLD	4◦C	**HOLD**

2.3 Statistical analysis

Descriptive Statistics

We tabulated all KIR gene repertoires (combinations of the 15 KIR genes assayed) found in our study population. We also calculated Wn*, a measure of linkage disequilibrium appropriate for genes such as the KIR for which we have only binary (presence or absence) information. [50]

Classification of HLA alleles according to KIR affinity

HLA-A, -B, and –C alleles were grouped according to their putative KIR binding partners (**Table 3**). HLA-C alleles fall into two mutually exclusive groups based on the cytotoxicity of NK cell responses in cell culture assays, and these two groups are defined by a single amino acid position in the HLA-C heavy chain protein. [51, 52] HLA-C group 1 alleles have an asparagine (N) residue at position 80 and HLA-C group 2 alleles have a lysine (K) residue at position 80.[51, 52] These HLA-C group 1 and 2 differences are thought to affect NK cell responses through interactions with KIR, where the HLA-C group 1 alleles bind to 2DL2, 2DL3 and 2DS2 and HLA-C group 2 alleles bind to 2DL1 and 2DS2. [53] Amino acid sequences of HLA alleles were determined in the HLA database (http://www.ebi.ac.uk/cgi-bin/ipd/imgt/hla/align.cgi) and alleles found in our study population were assigned to HLA-C group 1 or group 2 accordingly.

Additionally, the HLA-B*46 and B*73 alleles were included in the HLA-C group 1 alleles due to amino acid sequence similarity with HLA-C group 1 at residues 66-76 of the alpha 1 helix and experimental observations that these alleles are recognized by 2DL2 and 2DL3. [54, 55]

Experimental evidence indicates that HLA-B alleles with the Bw4 epitope bind to 3DL1 and 3DS1. [56, 57] HLA-B Bw4 alleles were further subdivided into those that have an isoleucine residue at position 80 (Bw4-80I) which bind to 3DS1 and 3DL1, or those that have a threonine residue at position 80 (Bw4-80T) and bind with lower affinity to 3DL1 and do not bind to 3DS1. Certain HLA-A alleles with the Bw4 epitope also bind to 3DS1 and 3DL1. [35]

In vitro experiments of the specificity of NK cell lysis of target cells provide evidence that HLA-A3 and -A11 alleles bind to 3DL2. [58, 59]

Though there is experimental evidence for KIR specificity for only a handful of HLA-A, and -B alleles, we used amino acid sequence comparisons of the critical interaction domains to classify other HLA-A and –B alleles into HLA-A3/A11, and Bw4 groups for the purposes of defining KIR-HLA interacting pairs. [60]

Table 3. Classification of selected HLA-A, -B, and -C alleles by KIR specificity

KIR ligand groups	Key Amino Acid	HLA alleles[1]	KIR binding partner
HLA-C group 1	80N	C102, C302, C303, C304, C305, C701, C702, C703, C704, C705, C801, C802, C803, C1202, C1203, C1402, C1403, C1601, C1604	2DL2, 2DL3, 2DS2
HLA-C group 2	80K	C202, C401, C404, C501, C602, C1204, C1502, C1504, C1505, C1602, C1701, C1801, B7301	2DL1, 2DS1
Bw4 epitope	80I	B803, B1516, B1517, B2702, B3801 B4901, B5101, B5107, B5108, B5109, B5201, B5301, B5701, B5702, B5703, B5801, A2301, A2402, A2403, A2901, A2902	3DL1, 3DS1
Bw4 epitope	80T	B1301, B1302, B2705, B2707, B2709, B3701, B4403, B4405, B4701	3DL1
HLA-A3/A11	n/a	A3001, A301, A302, A305, A1101, A3101, A3301, A3303, A3402, A6601, A6801, A7401, A7402	3DL2

[1] Includes HLA-A, -B, and -C alleles observed in the parent study. [48]

Measurements of Association

Unconditional multivariable logistic regression was used to estimate odds ratios indicative of the relative risk of cancer associated with genotypes of interest, as well as corresponding 95% confidence intervals and Wald test p-values. All analyses were limited to self-identified whites to minimize the potential for confounding by race. All analyses were adjusted for age (as a continuous variable) because age is related to cervical and vulvar cancer risk as well as to case-control status in our study, and age was a matching factor. Statistical analyses were performed in STATA v.13 (StataCorp, LP, College Station, TX, USA). Our analysis plan included estimating associations of individual KIR genes (present or absent) with vulvar cancer and cervical cancer, as well as with cervical cancer subgroups, SCC and adenocarcinoma (combining invasive and in situ adenocarcinoma). We also estimated associations of cancer with known

KIR-HLA ligand-receptor pairs under a dominant model, as well as associations of cancer with the numbers of known activating and inhibitory KIR-HLA pairs. Additionally, we constructed variables that combined KIR gene presence or absence so as to reflect blocks of high linkage disequilibrium in centromeric and telomeric clusters KIR genes, termed CenA, CenB, TelA, and TelB, after Cooley *et al.*, and tested these variables' associations with cancer under a dominant model. [61] We also estimated individual KIR associations with oncogenic HPV-positive vulvar cancer.

We used polytomous logistic regression to test whether KIR associations differed for cervical adenocarcinoma and SCC. We also tested whether KIR associations differed between HPV 16-positive and HPV 18-positive cervical adenocarcinomas. In these analyses we compared two nested models, one holding associations constant between two groups and a second in which separate coefficients were estimated for each case group (all relative to controls), using a likelihood ratio test.

Another measure of the significance of associations with cancer risk was evaluated using a Monte-Carlo-based permutation test implemented in R version 3.0.2. [62] The permutation test provides a measure of the probability that observed associations between KIR genes and cancer risk are more extreme than the associations in the same data permuted randomly. The test takes into account linkage structure. Operationally, the permutation test produces a minimum p-value ("p-min") for a given set of KIR associations, indicating the probability that at least one KIR association in the set is significant. In the permutation test, case-control status was randomized, and associations with cancer risk were adjusting for age (continuously) among whites. The permutation test was run only once, to evaluate the significance of individual KIR associations with cervical adenocarcinoma risk.

2.4 Human subjects protections

This study conforms to institutional protections of human subjects. Study participants' consent is documented under FHCRC IRB file 452, and the overall study has been approved under FHCRC IRB file 6818.

Chapter 3. Results

3.1 KIR genotyping results

KIR genotyping was attempted on 1925 DNA samples, of which 196 were blinded quality control duplicates. The positive control (HLA-DBR1 gene) or negative control (water) reactions failed for 42 samples including 11 controls, 14 vulvar cancer cases, 7 SCC cases, and 10 adenocarcinoma cases, all of which were dropped from analyses. When the quality control duplicates were unblinded, we found disagreement in 0.7% of KIR genotype calls between test samples and blinded duplicates; no clear pattern was apparent. Specifically, we did not see a clustering of KIR genotype disagreements within a few individuals that might suggest that their DNA samples were problematic. Nor were the disagreements between samples and quality control duplicates clustered in a few KIR genes. Analysis of the genotyping results for Coriell samples suggested that 2DS3 had the highest level of PCR reaction failure at approximately 5%. The quality control duplicates were dropped from analyses, as were the approximately 10% of study participants who self-identified as a race or ethnic group other than white, leaving 1522 study participants included in analyses, including 498 controls, 358 vulvar cancer cases, and 666 cervical cancer cases.

3.2 Study participant characteristics

Selected characteristics of the study population, limited to whites successfully genotyped at the KIR loci, are given in **Table 4**. As observed in prior analyses of the parent study population, vulvar cancer cases tend to be older than controls and cervical cancer cases, and cervical adenocarcinoma cases tend to be younger. Current smoking is more common among vulvar cancer cases and cervical SCC cases, and a history of never having smoked is slightly more common among cervical adenocarcinoma cases compared to controls.

Table 4. Characteristics of vulvar cancer and cervical cancer cases and controls, Seattle-Puget Sound Region, 1986-2004

					Cervical Cancer Case[1]					
	Controls[1] (n=498)		Vulvar Cancer Cases[1] (n=358)		All Cervical Cancer[2] (n=666)		SCC (n=227)		Adeno-carcinoma (n=419)	
Characteristic	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Age (years)										
18-34	122	(24.5)	54	(15.1)	245	(36.8)	65	(28.6)	176	(42.0)
35-44	127	(25.5)	104	(29.1)	219	(32.9)	72	(31.7)	139	(33.2)
45-54	119	(23.9)	103	(28.8)	127	(19.1)	49	(21.6)	73	(17.4)
55-64	67	(13.5)	54	(15.1)	43	(6.5)	21	(9.3)	20	(4.8)
65-74	52	(10.4)	36	(10.1)	32	(4.8)	20	(8.8)	11	(2.6)
75+	11	(2.2)	7	(2.0)	0	0.0	0	0.0	0	0.0
Smoking behavior										
Never	241	(48.4)	73	(20.4)	320	(48.0)	84	(37.0)	228	(54.4)
Former	144	(28.9)	91	(25.4)	178	(26.7)	67	(29.5)	105	(25.1)
Current	112	(22.5)	194	(54.2)	168	(25.2)	76	(33.5)	86	(20.5)
Tumor HPV DNA status[3]										
No oncogenic HPV[4]			62	(17.3)	96	(14.4)	31	(13.7)	64	(15.3)
Oncogenic HPV[4]			228	(63.7)	347	(52.1)	105	(46.3)	226	(53.9)
Not tested			68	(19.0)	223	(33.5)	91	(40.1)	129	(30.8)
HLA allele groups										
HLA-A3/A11										
0 copies	248	(51.3)	192	(53.6)	322	(49.6)	102	(48.1)	209	(50.1)
1 copy	189	(39.1)	127	(35.5)	274	(42.2)	94	(44.3)	173	(41.5)
2 copies	46	(9.5)	39	(10.9)	53	(8.2)	16	(7.5)	35	(8.4)
Bw4-80I epitope										
0 copies	359	(74.5)	263	(73.5)	475	(73.1)	155	(73.1)	310	(74.2)
1 copy	116	(24.1)	85	(23.7)	159	(24.5)	51	(24.1)	98	(23.4)
2 copies	7	(1.5)	10	(2.8)	16	(2.5)	6	(2.8)	10	(2.4)
Bw4-80T epitope										
0 copies	369	(76.6)	261	(72.9)	503	(77.4)	160	(75.5)	325	(77.8)
1 copy	102	(21.2)	85	(23.7)	138	(21.2)	48	(22.6)	88	(21.1)
2 copies	11	(2.3)	12	(3.4)	9	(1.4)	4	(1.9)	5	(1.2)
HLA-C group 1										
0 copies	57	(11.9)	54	(15.1)	86	(13.3)	29	(13.7)	51	(12.2)
1 copy	240	(49.9)	164	(45.8)	305	(47.0)	98	(46.4)	199	(47.6)
2 copies	184	(38.3)	140	(39.1)	258	(39.8)	84	(39.8)	168	(40.2)

[1] Case and control groups limited to whites.
[2] All cervical cancer includes SCC, adenocarcinomas, and adenosquamous carcinomas (n=20).
[3] Controls were not tested for the presence of HPV DNA.
[4] Oncogenic HPV includes HPV types 16, 18, 31, and 45 alone or in combination.

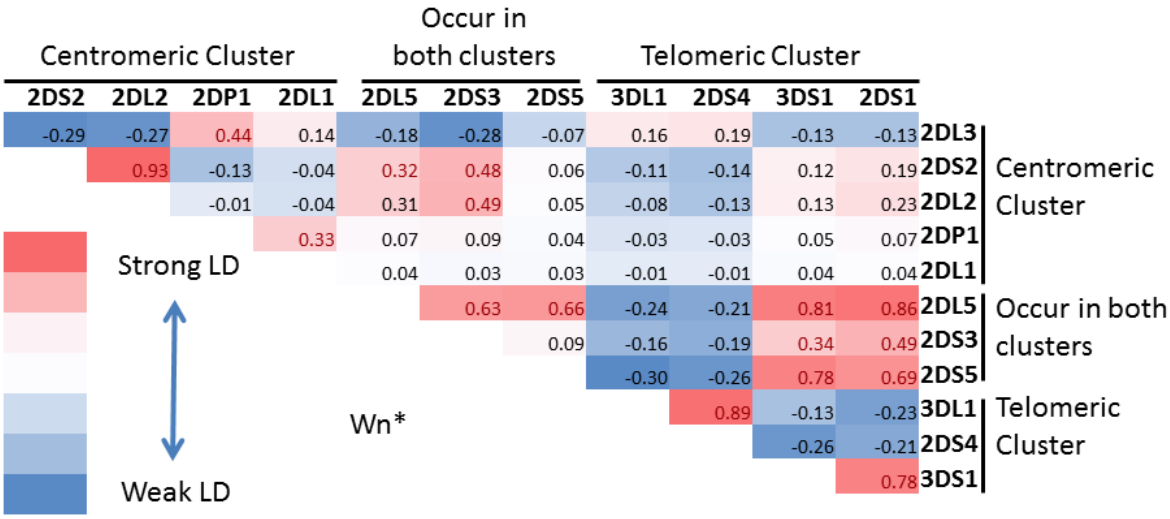
3.3 KIR linkage disequilibrium

The assayed framework KIR genes, 3DL3, 2DL4, and 3DL2, occurred in all subjects in our study, as expected, and thus were excluded from LD calculations. The pattern of LD shared by the non-framework KIR genes, shown in **Figure 2**, reflects the arrangement of KIR genes in centromeric and telomeric clusters of haplotype A and B. CenA KIR 2DL3 has low LD with CenB KIRs 2DL2 (Wn* = -0.27) and 2DS2 (Wn* = -0.29); yet 2DL2 and 2DS2 are in high LD with each other (Wn* = 0.96). In the telomeric cluster, TelA KIRs 2DS4 and 3DL1 are in high LD (Wn* = 0.86) with each other, as are TelB KIRs 3DS1 and 2DS1 (Wn* = 0.78), but TelA KIR 2DS4 is in low LD with TelB KIR 3DS1 (Wn* = -0.26). Between clusters but within haplotypes, CenA KIR 2DL3 is in modest LD with TelA KIR 2DS4 (Wn* = 0.19), as is CenB KIR 2DL2 and TelB KIR 3DS1 (Wn* = 0.13).

Certain KIR genes on haplotype B can occur in either the centromeric or telomeric cluster. 2DL5 is in high LD with TelB KIR 3DS1 (Wn* = 0.81) and in modest LD with CenB KIR 2DL2 (Wn* = 0.31), reflecting co-occurrence with and chromosomal proximity to each of these genes. Other haplotype B KIR genes that can occur in both centromeric and telomeric clusters include 2DS3 and 2DS5.

Some KIR genes can occur on either haplotype A or B. KIR 2DL1 and the pseudogene 2DP1 both occur more often on haplotype A, and are in modest LD with CenA KIR 2DL3 (Wn* = 0.14 and Wn* = 0.44, respectively) and in low LD with CenB KIR 2DL2 (Wn* = -0.04 and Wn* = -0.01, respectively).

Figure 2. Linkage Disequilibrium (Wn*) among controls, Seattle-Puget Sound Region, 1986-2004



Note: includes only white controls

3.4 Associations with individual KIR genes

Table 5 and **Table 6** show the frequency of cases and controls possessing one or more copy of each assayed KIR gene, as well as estimates of KIR associations with vulvar cancer (**Table 5**) and cervical cancer (**Table 6**). None of the associations of individual KIR genes with either vulvar cancer or cervical cancer were statistically significant in our study. As with the overall vulvar cancer results, analyses limited to oncogenic HPV-positive vulvar cancers compared to all controls, the presence of none of the individual KIR genes was associated with vulvar cancer (**Table 5**).

Table 7 shows estimates of associations between individual KIR genes and cervical cancer subtypes. None of the KIR was associated with SCC risk. The presence of 2DL2, 2DS2, and 2DS3 were each negatively associated with adenocarcinoma risk (2DL2 OR 0.74, 95% CI 0.57-0.98; 2DS2 OR 0.74, 95% CI 0.56-0.97; and 2DS3 OR 0.72, 95% CI 0.53-0.98) at the $p<0.05$ threshold for significance. 2DL2 and 2DS2, inhibitory and activating, respectively, are adjacent and in high LD (Wn* = 0.929) with each other, and in modest LD with 2DS3 (Wn* = 0.48-0.49). A permutation test indicated that the observed associations between individual KIR and adenocarcinoma were no more significant than would be expected by chance alone. In a polytomous logistic regression model, associations with 2DL2 differed between cervical SCC and adenocarcinoma risk (likelihood ratio test p-value = 0.008).

Table 5. Associations between individual KIR genes and risk of vulvar cancer, Seattle-Puget Sound Region, 1986-2004

		Controls[1] (n=498)		All Vulvar Cancer Cases[1] (n=358)			Oncogenic HPV-Positive Vulvar Cancer Cases[1,3] (n=228)		
KIR	Haplotype	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)
Centromeric Cluster									
2DL3	A	454	(91.2)	335	(93.6)	1.34 (0.79-2.27)	209	(91.7)	1.01 (0.57-1.78)
2DS2	B	256	(51.4)	169	(47.2)	0.86 (0.65-1.13)	105	(46.1)	0.82 (0.60-1.13)
2DL2	B	272	(54.6)	188	(52.5)	0.93 (0.71-1.23)	119	(52.2)	0.92 (0.67-1.27)
2DP1	A or B	489	(98.2)	354	(98.9)	1.64 (0.50-5.37)	225	(98.7)	1.36 (0.36-5.08)
2DL1	A or B	497	(99.8)	356	(99.4)	0.38 (0.03-4.20)	227	(99.6)	0.44 (0.03-7.09)
Centromeric or Telomeric Cluster									
2DL5	B	244	(49.0)	177	(49.4)	1.03 (0.78-1.35)	113	(49.6)	1.04 (0.76-1.42)
2DS3	B	141	(28.3)	94	(26.3)	0.94 (0.69-1.27)	61	(26.8)	0.96 (0.67-1.37)
2DS5	B	172	(34.5)	120	(33.5)	0.95 (0.71-1.26)	77	(33.8)	0.96 (0.69-1.34)
Telomeric Cluster									
3DL1	A	473	(95.0)	342	(95.5)	1.13 (0.60-2.16)	216	(94.7)	0.96 (0.47-1.96)
2DS4	A	478	(96.0)	342	(95.5)	0.89 (0.46-1.75)	216	(94.7)	0.76 (0.36-1.59)
3DS1	B	192	(38.6)	138	(38.5)	1.00 (0.76-1.33)	92	(40.4)	1.08 (0.79-1.49)
2DS1	B	247	(49.6)	175	(48.9)	0.98 (0.75-1.29)	110	(48.2)	0.95 (0.70-1.31)

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] Oncogenic HPV includes HPV types 16, 18, 31, and 45 alone or in combination.

Table 6. Associations between individual KIR genes and risk of cervical cancer, Seattle-Puget Sound Region, 1986-2004

		Controls[1] (n=498)		Cervical Cancer Cases[1,2] (n=666)		
KIR	Haplotype	n	(%)	n	(%)	OR[3] (95% CI)
Centromeric Cluster						
2DL3	A	454	(91.2)	615	(92.3)	1.17 (0.76-1.81)
2DS2	B	256	(51.4)	323	(48.5)	0.87 (0.68-1.10)
2DL2	B	272	(54.6)	356	(53.5)	0.92 (0.73-1.17)
2DP1	A or B	489	(98.2)	654	(98.2)	0.89 (0.36-2.19)
2DL1	A or B	497	(99.8)	665	(99.8)	1.51 (0.09-25.0)
Centromeric or Telomeric Cluster						
2DL5	B	244	(49.0)	326	(48.9)	0.96 (0.76-1.22)
2DS3	B	141	(28.3)	175	(26.3)	0.84 (0.64-1.10)
2DS5	B	172	(34.5)	229	(34.4)	0.97 (0.75-1.25)
Telomeric Cluster						
3DL1	A	473	(95.0)	639	(95.9)	1.28 (0.72-2.28)
2DS4	A	478	(96.0)	640	(96.1)	1.06 (0.58-1.96)
3DS1	B	192	(38.6)	255	(38.3)	0.99 (0.77-1.26)
2DS1	B	247	(49.6)	332	(49.8)	0.98 (0.78-1.25)

[1] Case and control groups limited to whites.
[2] Cervical cancer cases include SCC (n=227), adenocarcinomas (n=419), and adenosquamous carcinomas (n=20).
[3] ORs adjusted for age.

Table 7. Associations between individual KIR genes and risk of cervical SCC and adenocarcinoma, Seattle-Puget Sound Region, 1986-2004

KIR	Haplotype	Controls[1] (n=498) n	(%)	SCC Cases[1] (n=227) n	(%)	OR (95% CI)	Adenocarcinoma Cases[1] (n=419) n	(%)	OR (95% CI)
Centromeric Cluster									
2DL3	A	454	(91.2)	205	(90.3)	0.90 (0.52-1.54)	393	(93.8)	1.52 (0.90-2.58)
2DS2	B	256	(51.4)	120	(52.9)	1.05 (0.76-1.44)	191	(45.6)	**0.74 (0.56-0.97)**
2DL2	B	272	(54.6)	136	(59.9)	1.23 (0.89-1.69)	207	(49.4)	**0.74 (0.57-0.98)**
2DP1	A or B	489	(98.2)	221	(97.4)	0.65 (0.23-1.86)	413	(98.6)	1.13 (0.38-3.38)
2DL1	A or B	497	(99.8)	227	(100.0)	-	418	(99.8)	0.94 (0.06-16.1)
Centromeric or Telomeric Cluster									
2DL5	B	244	(49.0)	115	(50.7)	1.06 (0.78-1.46)	198	(47.3)	0.88 (0.67-1.15)
2DS3	B	141	(28.3)	65	(28.6)	0.99 (0.70-1.41)	102	(24.3)	**0.72 (0.53-0.98)**
2DS5	B	172	(34.5)	80	(35.2)	1.03 (0.74-1.43)	142	(33.9)	0.95 (0.71-1.26)
Telomeric Cluster									
3DL1	A	473	(95.0)	215	(94.7)	0.95 (0.47-1.93)	404	(96.4)	1.42 (0.71-2.82)
2DS4	A	478	(96.0)	216	(95.2)	0.82 (0.39-1.75)	404	(96.4)	1.13 (0.55-2.33)
3DS1	B	192	(38.6)	89	(39.2)	1.04 (0.75-1.44)	156	(37.2)	0.95 (0.72-1.25)
2DS1	B	247	(49.6)	115	(50.7)	1.05 (0.76-1.43)	204	(48.7)	0.93 (0.71-1.22)

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.

In a polytomous logistic regression model comparing risks of HPV 16-positive and HPV-18 positive cervical adenocarcinomas, we found that associations with 3DS1 differed between the two cervical adenocarcinoma groups (likelihood ratio test p-value, 0.027) (**Table 8**). 3DS1 was significantly associated only with HPV 18-positive cervical adenocarcinomas (OR 0.52, 95% CI 0.30-0.89). KIR associated only with HPV 16-positive adenocarcinoma in these exploratory analyses included 2DS2 (OR 0.63, 95% CI 0.41-0.97) and 2DL2 (OR 0.61, 95% CI 0.40-0.94). KIR 2DL5 was associated with HPV 18-positive adenocarcinoma (OR 0.60, 95% CI 0.37-0.98).

Table 8. Associations between individual KIR genes and risk of HPV 16-positive versus HPV 18-positive cervical adenocarcinoma, Seattle-Puget Sound Region, 1986-2004

		Controls[1] (n=498)		HPV16-Positive Adenocarcinoma Cases[1] (n=113)			HPV 18-Positive Adenocarcinoma Cases[1] (n=83)			LRT[3]
KIR	Hap.	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)	p-value
Centromeric Cluster										
2DL3	A	454	(91.2)	108	(95.6)	2.27 (0.86-5.97)	78	(94.0)	1.59 (0.61-4.18)	0.565
2DS2	B	256	(51.4)	48	(42.5)	**0.63 (0.41-0.97)**	41	(49.4)	0.86 (0.54-1.38)	0.259
2DL2	B	272	(54.6)	51	(45.1)	**0.61 (0.40-0.94)**	45	(54.2)	0.92 (0.57-1.48)	0.157
2DP1	A or B	489	(98.2)	112	(99.1)	1.68 (0.20-14.2)	81	(97.6)	0.71 (0.15-3.42)	0.420
2DL1	A or B	497	(99.8)	113	(100.0)	1.00	82	(98.8)	0.19 (0.01-3.07)	0.186
Centromeric or Telomeric Cluster										
2DL5	B	244	(49.0)	54	(47.8)	0.90 (0.59-1.37)	31	(37.3)	**0.60 (0.37-0.98)**	0.169
2DS3	B	141	(28.3)	29	(25.7)	0.76 (0.47-1.23)	18	(21.7)	0.64 (0.36-1.13)	0.606
2DS5	B	172	(34.5)	35	(31.0)	0.85 (0.54-1.34)	22	(26.5)	0.69 (0.41-1.17)	0.512
Telomeric Cluster										
3DL1	A	473	(95.0)	108	(95.6)	1.05 (0.38-2.91)	81	(97.6)	2.04 (0.47-8.88)	0.438
2DS4	A	478	(96.0)	108	(95.6)	0.82 (0.29-2.34)	81	(97.6)	1.61 (0.36-7.12)	0.434
3DS1	B	192	(38.6)	44	(38.9)	1.04 (0.67-1.60)	20	(24.1)	**0.52 (0.30-0.89)**	**0.027**
2DS1	B	247	(49.6)	52	(46.0)	0.85 (0.56-1.29)	34	(41.0)	0.71 (0.44-1.14)	0.506

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] Likelihood ratio test compares a nested polytomous logistic regression models, one allowing ORs to differ between case groups.

3.3 Associations with CenA, CenB, TelA, and TelB KIR groups

CenB, the centromeric haplotype defined by the presence of 2DS2 and 2DL2, was associated with a lower risk of adenocarcinoma (OR 0.74, 95% CI 0.56-0.97) (**Table 9**). No other centromeric or telomeric haplotype was associated with risk of cervical cancer or vulvar cancer.

Table 9. Associations between centromeric and telomeric KIR haplotypes A and B and cervical and vulvar cancer risk, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Vulvar Cancer Cases[1] (n=358)			All Cervical Cancer Cases[1] (n=666)		
KIR group	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)
CenA	454	(91.2)	335	(93.6)	1.34 (0.79-2.27)	615	(92.3)	1.17 (0.76-1.81)
CenB	273	(54.8)	188	(52.5)	0.93 (0.71-1.22)	356	(53.5)	0.91 (0.72-1.16)
TelA	473	(95.0)	342	(95.5)	1.13 (0.60-2.16)	638	(95.8)	1.24 (0.70-2.20)
TelB	20	(4.0)	16	(4.5)	1.12 (0.57-2.19)	25	(3.8)	0.91 (0.49-1.69)
	Controls[1] (n=498)		Cervical SCC Cases[1] (n=227)			Cervical Adenocarcinoma Cases[1] (n=419)		
KIR group	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)
CenA	454	(91.2)	205	(90.3)	0.90 (0.52-1.54)	393	(93.8)	1.52 (0.90-2.58)
CenB	273	(54.8)	136	(59.9)	1.21 (0.88-1.67)	207	(49.4)	**0.74 (0.56-0.97)**
TelA	473	(95.0)	215	(94.7)	0.95 (0.47-1.93)	403	(96.2)	1.33 (0.68-2.62)
TelB	20	(4.0)	11	(4.8)	1.22 (0.57-2.59)	14	(3.3)	0.83 (0.40-1.73)

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.

3.4 KIR gene combinations

We identified 61 distinct KIR genotypes that occurred in one or more study participant in our study population (**Table 10** and **Table 11**). (The term KIR repertoire refers to the set of KIR genes in a person's genome, and alternatively to the set of KIR expressed by a population of NK cells. To avoid confusion between these meanings, we avoid the term KIR repertoire and instead use the term KIR genotype to indicate the subset of the 15 assayed KIR genes that are present in a person's genome.) These tables show those KIR genotypes that occur in 1% or more of controls. The most common KIR genotype (genotype 1 in **Table 10** and **Table 11**), defined by the presence of 2DL1, 2DL3, 3DL1, 2DS4, and 2DP1 and the absence of other non-framework genes, is two copies of haplotype A, a haplotype common among Caucasians. In visual comparisons of KIR genotype frequencies across case and control groups, the higher frequency of genotypes 1 and 3 in adenocarcinoma cases compared to controls stands out. Genotype 1 represents two copies of haplotype A and genotype 3 is two copies of CenA, the centromeric side of haplotype A, and one copy each of TelA and TelB, the telomeric portions of haplotypes A and B, respectively.

Table 10. Counts and frequencies of KIR genotypes in vulvar cancer cases and controls, Seattle-Puget Sound Region, 1986-2004

Geno-	KIR genes[2]												Controls[1] (n=498)		Vulvar Cancer Cases[1] (n=358)	
	Centromeric Cluster					Centromeric or Telomeric			Telomeric Cluster							
type	2DL3	2DS2	2DL2	2DP1	2DL1	2DL5	2DS3	2DS5	3DL1	2DS4	3DS1	2DS1	n	(%)	n	(%)
1	+	-	-	+	+	-	-	-	+	+	-	-	141	(28.3)	103	(28.8)
2	+	+	+	+	+	-	-	-	+	+	-	-	77	(15.5)	52	(14.5)
3	+	-	-	+	+	+	-	+	+	+	+	+	56	(11.2)	46	(12.8)
4	+	+	+	+	+	+	-	+	+	+	+	+	30	(6.0)	19	(5.3)
5	+	+	+	+	+	+	+	-	+	+	-	+	30	(6.0)	22	(6.1)
6	+	+	+	+	+	+	+	+	+	+	+	+	28	(5.6)	14	(3.9)
7	+	+	+	+	+	+	+	-	+	+	+	+	16	(3.2)	15	(4.2)
8	+	+	+	+	+	+	+	-	+	+	-	-	11	(2.2)	9	(2.5)
9	-	+	+	+	+	+	+	-	+	+	+	+	8	(1.6)	6	(1.7)
10	-	+	+	+	+	+	+	+	+	+	+	+	8	(1.6)	2	(0.6)
11	+	-	-	+	+	-	-	-	+	+	-	+	7	(1.4)	6	(1.7)
12	+	-	-	+	+	+	+	-	+	+	+	+	6	(1.2)	2	(0.6)
13	-	+	+	-	+	-	-	-	+	+	-	-	6	(1.2)	2	(0.6)
14	-	+	+	+	+	+	+	+	-	-	+	+	6	(1.2)	3	(0.8)
15	+	+	+	+	+	+	+	+	-	-	+	+	6	(1.2)	6	(1.7)
16	+	-	-	+	+	-	-	+	+	+	-	+	5	(1.0)	1	(0.3)

[1] Case and control groups limited to whites.
[2] + indicates the presence of one or more copy of the KIR gene, and – indicates its absence.

Table 11. Counts and frequencies of KIR genotypes in cervical cancer cases and controls, Seattle-Puget Sound Region, 1986-2004

Geno-	KIR genes[2]												Controls[1] (n=498)		Cervical Cancer Cases[1] (n=666)	
	Centromeric Cluster					Centromeric or Telomeric Cluster			Telomeric Cluster							
type	2DL3	2DS2	2DL2	2DP1	2DL1	2DL5	2DS3	2DS5	3DL1	2DS4	3DS1	2DS1	n	(%)	n	(%)
1	+	-	-	+	+	-	-	-	+	+	-	-	141	(28.3)	191	(28.7)
2	+	+	+	+	+	-	-	-	+	+	-	-	77	(15.5)	97	(14.6)
3	+	-	-	+	+	+	-	+	+	+	+	+	56	(11.2)	92	(13.8)
4	+	+	+	+	+	+	-	+	+	+	+	+	30	(6.0)	37	(5.6)
5	+	+	+	+	+	+	+	-	+	+	-	+	30	(6.0)	41	(6.2)
6	+	+	+	+	+	+	+	+	+	+	+	+	28	(5.6)	28	(4.2)
7	+	+	+	+	+	+	+	-	+	+	+	+	16	(3.2)	28	(4.2)
8	+	+	+	+	+	+	+	-	+	+	-	-	11	(2.2)	10	(1.5)
9	-	+	+	+	+	+	+	-	+	+	+	+	8	(1.6)	11	(1.7)
10	-	+	+	+	+	+	+	+	+	+	+	+	8	(1.6)	10	(1.5)
11	+	-	-	+	+	-	-	-	+	+	-	+	7	(1.4)	7	(1.1)
12	+	-	-	+	+	+	+	-	+	+	+	+	6	(1.2)	3	(0.5)
13	-	+	+	-	+	-	-	-	+	+	-	-	6	(1.2)	9	(1.4)
14	-	+	+	+	+	+	+	+	-	-	+	+	6	(1.2)	3	(0.5)
15	+	+	+	+	+	+	+	+	-	-	+	+	6	(1.2)	7	(1.1)
16	+	-	-	+	+	-	-	+	+	+	-	+	5	(1.0)	2	(0.3)

[1] Case and control groups limited to whites.
[2] + indicates the presence of one or more copy of the KIR gene, and – indicates its absence.

3.5 HLA-KIR ligand receptor pairs and cervical and vulvar cancer risk

For all the known HLA-KIR ligand-receptor pairs, having the HLA alleles and matching KIR was not significantly associated with either vulvar cancer or cervical cancer (**Table 12**). In cervical cancer subgroup analyses (**Table 13**), having at least one copy of KIR 2DS2 and at least one allele belonging to HLA-C group 1 was associated with lower risk of adenocarcinoma (OR=0.75, 95% CI 0.57-0.99). No other joint KIR-HLA genotype was significantly associated with altered risk of a cervical cancer subtype.

Table 12. Associations between HLA-KIR ligand-receptor pairs and risk of cervical and vulvar cancer, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Vulvar Cancer Cases[1] (n=358)			Cervical Cancer Cases[1] (n=666)		
HLA-KIR pairs	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)
HLA-C1- and/or KIR 2DL2/3-	58	(12.1)	54	(15.1)	ref	86	(13.3)	ref
HLA-C1+ and KIR 2DL2/3+	423	(87.9)	304	(84.9)	0.77 (0.52-1.15)	563	(86.7)	0.88 (0.61-1.27)
HLA-C1- and/or KIR 2DS2-	263	(54.7)	216	(60.3)	ref	380	(58.6)	ref
HLA-C1+ and KIR 2DS2+	218	(45.3)	142	(39.7)	0.80 (0.61-1.06)	269	(41.4)	0.83 (0.65-1.06)
HLA-C2- and/or KIR 2DL1-	185	(38.5)	142	(39.7)	ref	259	(39.9)	ref
HLA-C2+ and KIR 2DL1+	296	(61.5)	216	(60.3)	0.95 (0.72-1.26)	390	(60.1)	0.93 (0.72-1.19)
HLA-Bw4- and/or KIR 3DS1-	429	(88.1)	320	(89.4)	ref	586	(88.7)	ref
HLA-Bw4+ and KIR 3DS1+	58	(11.9)	38	(10.6)	0.84 (0.57-1.25)	75	(11.3)	1.00 (0.72-1.39)
HLA-Bw4- and/or KIR 3DL1-	277	(57.5)	186	(52.0)	ref	362	(55.7)	ref
HLA-Bw4+ and KIR 3DL1+	205	(42.5)	172	(48.0)	1.25 (0.94-1.67)	288	(44.3)	1.08 (0.84-1.39)
HLA-A3,A11- and/or KIR 3DL2-	248	(51.3)	192	(53.6)	ref	322	(49.6)	ref
HLA-A3,A11+ and KIR 3DL2+	235	(48.7)	166	(46.4)	0.78 (0.58-1.06)	327	(50.4)	1.24 (0.95-1.61)

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.

Table 13. Associations between HLA-KIR ligand-receptor pairs and risk of cervical SCC and adenocarcinoma, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		SCC Cases[1] (n=227)			Adenocarcinoma Cases[1] (n=419)		
HLA-KIR pairs	n	(%)	n	(%)	OR[2] (95% CI)	n	(%)	OR[2] (95% CI)
HLA-C1- and/or KIR 2DL2/3-	58	(12.1)	29	(13.7)	ref	51	(12.2)	ref
HLA-C1+ and KIR 2DL2/3+	423	(87.9)	182	(86.3)	0.88 (0.54-1.42)	367	(87.8)	0.92 (0.61-1.41)
HLA-C1- and/or KIR 2DS2-	263	(54.7)	115	(54.5)	ref	251	(60.0)	ref
HLA-C1+ and KIR 2DS2+	218	(45.3)	96	(45.5)	0.99 (0.72-1.38)	167	(40.0)	**0.75 (0.57-0.99)**
HLA-C2- and/or KIR 2DL1-	185	(38.5)	84	(39.8)	ref	169	(40.4)	ref
HLA-C2+ and KIR 2DL1+	296	(61.5)	127	(60.2)	0.93 (0.67-1.30)	249	(59.6)	0.94 (0.71-1.25)
HLA-Bw4- and/or KIR 3DS1-	429	(88.1)	198	(88.8)	ref	374	(89.5)	ref
HLA-Bw4+ and KIR 3DS1+	58	(11.9)	25	(11.2)	0.89 (0.57-1.40)	44	(10.5)	0.97 (0.67-1.42)
HLA-Bw4- and/or KIR 3DL1-	277	(57.5)	116	(54.7)	ref	238	(56.9)	ref
HLA-Bw4+ and KIR 3DL1+	205	(42.5)	96	(45.3)	1.05 (0.75-1.47)	180	(43.1)	1.05 (0.79-1.39)
HLA-A3,A11- and/or KIR 3DL2-	248	(51.3)	102	(48.1)	ref	209	(50.1)	ref
HLA-A3,A11+ and KIR 3DL2+	235	(48.7)	110	(51.9)	1.32 (0.93-1.86)	208	(49.9)	1.25 (0.93-1.68)

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.

3.6 Number of activating and inhibitory KIR-HLA pairs and cancer risk

The number of activating or inhibitory KIR-HLA receptor ligand pairs was not significantly associated with vulvar cancer (**Table 14**), cervical cancer overall (**Table 15**), or SCC (**Table 16**). Each additional activating KIR-HLA receptor-ligand pair was associated with a lower risk of adenocarcinoma (OR=0.84, 95% CI 0.71-0.99, p-trend 0.038) (**Table 17**).

Table 14. Number of activating and inhibitory KIR-HLA pairs and risk of vulvar cancer, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Vulvar Cancer Cases[1] (n=358)			
KIR-HLA pairs	n	(%)	n	(%)	OR[2] (95% CI)	P-trend[3]
Activating						
0	180	(37.5)	143	(39.9)	ref.	
1	178	(37.1)	139	(38.8)	0.99 (0.72-1.35)	0.277
2	103	(21.5)	63	(17.6)	0.78 (0.53-1.14)	
3	19	(4.0)	13	(3.6)	0.89 (0.43-1.88)	
Inhibitory						
1	68	(14.2)	50	(14.0)	ref.	
2	163	(34.0)	105	(29.3)	0.88 (0.57-1.37)	0.422
3	150	(31.3)	129	(36.0)	1.18 (0.77-1.83)	
4	83	(17.3)	60	(16.8)	1.00 (0.61-1.63)	
5	16	(3.3)	14	(3.9)	1.18 (0.53-2.64)	

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] P-trend is the Wald p-value on a log additive model.

Table 15. Number of activating and inhibitory KIR-HLA pairs and cervical cancer risk, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Cervical Cancer Cases[1] (n=666)			
KIR-HLA pairs	n	(%)	n	(%)	OR[2] (95% CI)	P-trend[3]
Activating						
0	180	(37.5)	259	(39.9)	ref.	
1	178	(37.1)	236	(36.4)	0.88 (0.67-1.17)	0.375
2	103	(21.5)	120	(18.5)	0.78 (0.56-1.08)	
3	19	(4.0)	34	(5.2)	1.14 (0.62-2.08)	
Inhibitory						
1	68	(14.2)	99	(15.3)	ref.	
2	163	(34.0)	199	(30.7)	0.79 (0.54-1.16)	0.720
3	150	(31.3)	200	(30.8)	0.86 (0.58-1.26)	
4	83	(17.3)	125	(19.3)	0.93 (0.61-1.43)	
5	16	(3.3)	26	(4.0)	1.12 (0.55-2.30)	

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] P-trend is the Wald p-value on a log additive model.

Table 16. Number of activating and inhibitory KIR-HLA pairs and risk of cervical SCC, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Cervical SCC Cases[1] (n=227)			
KIR-HLA pairs	n	(%)	n	(%)	OR[2] (95% CI)	P-trend[3]
Activating						
0	180	(37.5)	75	(35.5)	ref.	
1	178	(37.1)	87	(41.2)	1.10 (0.76-1.60)	0.905
2	103	(21.5)	44	(20.9)	1.19 (0.75-1.86)	
3	19	(4.0)	5	(2.4)	0.60 (0.22-1.69)	
Inhibitory						
1	68	(14.2)	35	(8.4)	ref.	
2	163	(34.0)	65	(15.6)	0.90 (0.55-1.47)	0.458
3	150	(31.3)	68	(16.3)	1.03 (0.63-1.69)	
4	83	(17.3)	39	(9.3)	1.23 (0.70-2.15)	
5	16	(3.3)	4	(1.0)	0.80 (0.24-2.71)	

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] P-trend is the Wald p-value on a log additive model.

Table 17. Number of activating and inhibitory KIR-HLA pairs and risk of cervical adenocarcinoma, Seattle-Puget Sound Region, 1986-2004

	Controls[1] (n=498)		Cervical Adenocarcinoma Cases[1] (n=419)			
KIR-HLA pairs	n	(%)	n	(%)	OR[2] (95% CI)	P-trend[3]
Activating						
0	180	(37.5)	186	(44.5)	ref.	
1	178	(37.1)	150	(35.9)	0.74 (0.54-1.00)	**0.038**
2	103	(21.5)	68	(16.3)	0.72 (0.49-1.07)	
3	19	(4.0)	14	(3.3)	0.62 (0.29-.32)	
Inhibitory						
1	68	(14.2)	77	(18.4)	ref.	
2	163	(34.0)	153	(36.6)	0.87 (0.58-.30)	0.304
3	150	(31.3)	111	(26.6)	0.69 (0.46-.05)	
4	83	(17.3)	72	(17.2)	0.97 (0.61-.56)	
5	16	(3.3)	5	(1.2)	0.42 (0.13-.33)	

[1] Case and control groups limited to whites.
[2] ORs adjusted for age.
[3] P-trend is the Wald p-value on a log additive model.

Chapter 4. Discussion

4.1 Summary of key findings

We observed individual KIR frequencies similar to those of other Caucasian populations. [63] Also, the complete KIR genotypes (ie, the set of the 15 common KIR genes present in an individual genome) are the same common KIR genotypes reported in other Caucasian study populations. [63] In analyses of vulvar cancer and all-types of cervical cancer, we found no associations between the presence or absence of any individual KIR gene and cancer risk. Similarly, we found no association between the joint presence of a KIR gene and its HLA ligand and vulvar or cervical cancer. Broadly, we interpret this to mean that the KIR are not likely to have a large effect on the risk of cervical or vulvar cancer. In cervical cancer subgroup analyses, carriers of three KIR genes, 2DS2, 2DL2, and 2DS3 were at lower risk of adenocarcinoma. Two of these, 2DS2 and 2DL2, are markers for the centromeric portion of the B haplotype, CenB. The third, 2DS3, also occurs on CenB but can sometimes be found on TelB. Among KIR-HLA pairings, having both 2DS2 and one or more copy of a HLA-C group 1 (80N) allele was associated with lower risk of cervical adenocarcinoma. We also found that having more activating KIR

was associated with lower risk of adenocarcinoma. These associations with adenocarcinoma hint that the KIR and NK cells may play a larger role in adenocarcinoma risk than in risk of cervical SCC or vulvar cancer.

4.2 Limitations of the study

One of the limitations of our study was that we may have been underpowered to detect small effects of individual KIR gene presence or absence on cancer risk. Our *a priori* power calculations gave us 80% power to detect associations as low as an OR of 1.3 for all-cervical cancer combined. Our final study population had smaller than anticipated numbers due to limitations in available DNA from study participants in the parent study and occasional genotyping failure. In calculations using our final sample size, we still had 80% powered to detect a minimum OR of 1.4 at individual KIR gene frequencies relevant to most of the variable KIR genes (ie, 0.2-0.5). Other studies have reported ORs with individual KIR genes and/or individual HLA alleles and cervical cancer of 0.6-2.1, [48] 0.6-2.7, [39] OR 0.4-2.1. [64] Our study had sufficient power to detect associations at the upper and lower ends of the ranges previously reported.

Another limitation of our study is that we considered only dominant genetic effects through which the KIR may affect cancer risk because our genotyping strategy captured only the presence or absence of each KIR gene. A dominant genetic model is consistent with analyses in the literature of how KIR-HLA mediated inhibition or activation of NK cells affects disease risk. [35, 64] The flexibility to consider recessive and log-additive genetic effects might have allowed us to detect associations that we may have missed.

Our study did not measure allelic variation at HLA-G, which binds to 2DL4. [65, 66] It is not clear whether there are any allelic variants of HLA-G that do not bind to 2DL4; if there are then our study has an incomplete picture of KIR-HLA variation. Also, natural ligands for the KIR may exist that have not been identified, in which case our counts of activating KIR-HLA pairs would be incomplete.

Another limitation of this study is that HPV exposure data were not available for some study participants. Some prior studies have restricted analyses to subjects who were exposed to HPV, with exposure determined by blood antibodies to HPV, or by PCR detection of HPV DNA in tumor cells or

normal cervical cells (among controls). The absence of HPV exposure history for controls could lead to confounding of associations between germline KIR variation and cancer risk only if HPV exposure history is associated with germline KIR variation. We can think of no plausible mechanism whereby this might occur.

Many of the statistical tests performed in this study are not independent; they represent different models for relating KIR and HLA frequency variation with cancer risk. This multiplicity of models reflects the field of KIR epidemiology. [67] We included several models in our study to allow direct comparison with other studies of KIR variation and cervical cancer risk, [68, 69] the principal motivation of this study. There are several consequences of our decision to test multiple parameterizations of how KIR variation (alone and with HLA binding partners) may affect cervical and vulvar cancer risk. One is that all of the statistically significant findings in this study may reflect the same "signal." The three KIR individually associated with adenocarcinoma risk are in high LD with each other. These three KIR are all found on CenB, which was itself significantly associated with adenocarcinoma risk. The KIR-HLA pairing that was associated with adenocarcinoma risk involved one of these three KIR, 2DS2, paired with HLA-C group 1 alleles. Our finding that having more activating KIR was inversely associated with adenocarcinoma risk likely reflects that two of the individually inversely associated KIR were activating KIR and so having another activating KIR often equates to having one or both of these KIR genes.

By using multiple parameterizations of KIR and HLA variation in our study, we had hoped to evaluate whether certain models were better than others at explaining a possible relationship between KIR variation and cervical or vulvar cancer risk. For example, is grouping KIR genes as CenA, CenB, TelA, and TelB, more meaningful than testing associations with KIR genes individually? A significant p-value was our primary indicator that a model was valuable. As such, our study supports testing for associations with the presence or absence of individual KIR genes, KIR genes grouped as CenA, CenB, TelA, and TelB, the presence of one or more copies of HLA-KIR pairs, and the number of activating KIR genes present in the genome. In short, we found support in our analyses of cervical adenocarcinoma risk for all of the models we tested except the number of inhibitory KIR genes present in the genome. These other models for relating KIR and HLA variation with disease risk have been useful in identifying associations with other diseases. [35] For example, counting activating and inhibitory KIR allowed Butsch

Kovacic *et al.* to identify associations with nasopharyngeal carcinoma. [70] Khakoo *et al.* identified a potential role for KIR-HLA variation in the resolution of hepatitis C virus infection by measuring associations with KIR-HLA ligand-receptor pairs. [71]

4.3 Strengths of the study

Any pair of PCR reactions that resulted in a discordant call for the presence or absence of a KIR was repeated in our study. Additionally, PCR reactions were repeated for other reasons, including the 10% blinded quality control duplicates, any failures in positive control or negative control PCR reactions, and failure of positive control Coriell samples. This multi-layered approach to quality control gives us confidence that KIR genotype assignments approach the technical limitations of our method. Though we have high confidence in our KIR genotype calls, we found 0.7% discordance between study sample genotypes and blinded replicates. Error is typically not reported in other KIR population genotyping studies. We believe that this residual uncertainty likely reflects biological reality, including KIR allelic variants that are poorly recognized by PCR primers not tailored to those variants as well as larger structural variation at the KIR locus such as gene and segmental duplications. Thus, our reporting of KIR genotype discordance between blinded replicate samples and study samples represents a step forward for the field. Uncertainty in KIR genotype using our and similar methods also provides motivation to deploy advanced genotyping technology, such as deep sequencing, to measure variation in this complex region.

Though a larger sample size is always desirable, especially when expected effect sizes are likely to be small, it is important to note that our study is the largest study of KIR variation and cervical cancer risk to date.

4.4 Cervical cancer significance

Two prior studies reported associations between KIR variation and cervical cancer risk. Carrington *et al.* found that the activating KIR 3DS1 was associated with increased risk of SCC. [64] The effect was more pronounced in joint KIR-HLA genotypes that involved HLA alleles that bind to KIR. We did not replicate these results, as we discuss below. In contrast, Arnheim *et al.* found that having a non-transcribed allele of inhibitory KIR 2DL5 (2DL5B) was associated with lower risk of SCC in a Swedish population. [39] 2DL5

can occur in either the centromeric or telomeric clusters. In the centromeric cluster it is often the allelic variant 2DL5B*002, and it is in high LD with 2DS3. [72] Our study did not distinguish 2DL5B from other 2DL5 alleles, so we were not able to test whether this result replicates in our study population. Nor did we find an association between 2DS3 and SCC risk in our study.

Our study failed to replicate the main findings of Carrington *et al.* We did not observe even the suggestion of an association between 3DS1 and risk of SCC. (The Carrington *et al.* study did not include cervical adenocarcinomas). Three key differences between the Carrington *et al.* study and our study may explain the lack of consistency in results. First, the Carrington *et al.* study pooled subjects from Costa Rica and the United States, and the association between cervical cancer risk and 3DS1 was much stronger in the Costa Rican study population. It is possible that an association between 3DS1 and cervical cancer risk is weaker or even non-existent in US Caucasians. Second, Carrington *et al.* included many pre-cancerous lesions, and it is possible that the role of KIR variation in cervical carcinogenesis differs between early and late disease progression. Thirdly, the Carrington *et al.* study was smaller, and the associations they report may have been due to chance.

Our study does not provide strong support for the model that KIR variation affects cancer risk through regulation of NK cell activation. We did not replicate prior findings that supported this model, and in our population found that neither individual KIR nor the number of inhibitory or activating KIR was associated with either SCC or all cervical cancer pooled. Any possible support for a role of NK cell activation in cancer risk comes from our findings with cervical adenocarcinoma. Having more activating KIR was associated with a lower risk of adenocarcinoma in our study, suggesting that NK cell activation may protect against cancer.

The associations we observed between KIR variation and cervical adenocarcinoma, if true, provide evidence for a stronger innate immune contribution to adenocarcinoma relative to SCC development. Other associations also provide evidence for an innate immune role in adenocarcinoma development. In our study population and others, current cigarette smoking is less common among adenocarcinoma cases compared to controls, but is associated with excess risk among SCC cases. [44] Cigarette smoking suppresses immune function, including NK cell responses. [73, 74] Also in our study, cervical adenocarcinoma cases tended to be younger than SCC cases as well as controls in our study.

Younger women tend to have more robust immune function compared to older women, though this relationship does not always apply to natural killer cell populations. [75] Innate immune cells, such as NK cells, can contribute to cancer through the release of angiogenic cytokines and the creation of reactive oxygen species that cause DNA damage. [76] Innate immune responses may also contribute to carcinogenesis by suppressing or activating an adaptive immune response.

We explored difference between HPV 16-positive and HPV 18-positive cervical adenocarcinomas, and found that 3DS1 was associated with a lower risk of HPV 18-positive but not HPV 16-positive cervical adenocarcinoma. Molecular explanations for how the presence or absence of 3DS1 could affect risk differentially based on HPV genotype may involve HLA-Bw4 molecules, which present peptides derived from HPV. 3DS1 binding to HLA-Bw4 molecules presenting HPV 18 peptides could result in more carcinogenic NK cell activation than binding to HLA-Bw4 molecules presenting HPV 16 peptides. Alternatively, perhaps T-cell receptors bind poorly to HLA-Bw4 molecules presenting HPV 18 peptides compared to HPV 16 peptides, and the engagement of 3DS1 as a backup defense has carcinogenic results. We were not able to find statistical support for a role of HLA-Bw4 alleles mediating a 3DS1 association, but this could easily be due to limited statistical power.

4.5 Vulvar cancer significance

Our study is the first to measure associations between KIR variation and vulvar cancer risk. That we found no associations suggests that variation in KIR repertoire may not play a large role in the development of vulvar cancer. Important questions related to vulvar cancer remain, however. For example, does KIR variation play a role in vulvar cancer recurrence? The high frequency of recurrence of vulvar cancer is what often makes this type of cancer so insidious, and to be able to identify contributing factors could help identify recurrences sooner through enhanced monitoring or could lead to the development of new therapies that prevent recurrence.

Recently, a randomized trial found that Imiquimod was effective at inducing the clearance of vulvar intraepithelial lesions. [77] The mechanism of action of Imiquimod is not entirely clear, but it is thought to act to enhance immune recognition of subclinical lesions, and it is possible that this involves NK cells. [78] That KIR variation was not associated with vulvar cancer risk makes it less plausible that

NK cells are involved in the response to HPV infected vulvar lesions, but it does not rule out this possibility.

Not all vulvar cancers are caused by HPV infection, and given the variety of NK cell functions, KIR variation may play a different role in vulvar cancers not attributable to HPV. However, we found no evidence for such a role in analyses of all vulvar cancer cases or limited to oncogenic HPV-positive vulvar cancers. A recent study supported prior work describing that HPV involvement was much more common in in situ vulvar lesions than in invasive vulvar cancers. [12] Our study population includes both in situ and invasive vulvar cancer cases, though insufficient numbers of the latter to allow robust statistical testing.

4.5 Future directions

Our study suggests several future directions for research into KIR variation and risk of vulvar and cervical cancer. First, a pooling of available study populations would enable greater statistical power. As noted previously, prior KIR association studies of cervical cancer risk reported modest effect sizes. [39, 64]

Another future direction suggested by our study is to take an agnostic approach to genetic variation in the KIR region. The KIR region has extensive variation not captured in our study, and using a tagSNP approach could provide a less expensive way to capture more variation than our study did. Indeed, a method has been developed for inferring KIR genotypes from SNP genotypes. [79] Similarly, in measuring the presence or absence of common KIR genes, our study captured only a small portion of the total KIR genetic variation that includes gene content variation, allelic variation, noncoding single nucleotide polymorphisms, and structural variation including gene and segmental duplications. A future study could measure other aspects of KIR genetic variation, focusing on one or more types of variation mentioned, or could attempt to capture all germline KIR variation through deep sequencing.

Alternatively, non-genetic variation, such as epigenetic regulation of KIR expression [80] and age or behavior-related changes in NK cell subsets, may be related to cancer phenotypes [81] and could provide an avenue for future research.

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