Short Communication

Variation of the Killer Cell Immunoglobulin-Like Receptors and *HLA-C* Genes in Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is an Epstein-Barrvirus (EBV)-associated malignancy. Previous studies have shown that NPC is associated with specific human leukocyte antigen (HLA) alleles which function in adaptive immunity to present viral and other antigens to the immune system. The role of innate immunity in NPC development is unknown. To determine whether innate immunity is associated with NPC, a case-control study was conducted among 295 Taiwanese NPC cases (99% EBV seropositive) and 252 community controls (29% EBV seropositive). Using high-resolution genotyping, we evaluated the variation of HLA class I alleles and killer cell immunoglobulin-like receptor (KIR) alleles. Located on the surface of natural killer (NK) cells and a subset of T cells, inhibitory KIRs diminish NK cytolysis of target cells upon binding to their HLA class I ligands and activating KIRs are thought to stimulate NK destruction of target cells. Our results suggest that an increasing number of activating KIRs may be associated with increasing NPC risk, particularly in individuals seropositive for anti-EBV antibodies known to be linked to NPC susceptibility (P_{trend} = 0.07). Among EBV-seropositive individuals, carriers of ≥5 activating KIRs had a 3.4-fold increased risk of disease (95% confidence interval, 0.74-15.7) compared with individuals with no functional activating KIRs. In contrast, there was no clear evidence of risk associated with increasing numbers of inhibitory KIRs. When evaluating HLA-C*w* alleles, we observed that carriers of HLA-C*w0401 alleles were at a significantly reduced NPC risk (odds ratio, 0.46; 95% confidence intervals, 0.23-0.92), an effect that could not be explained by linkage disequilibrium with other NPC-associated HLA alleles. Our results suggest that KIR-mediated activation may be associated with NPC risk. As this finding is consistent with a recent report examining cervical cancer, a malignancy caused by human papillomavirus, the data raises the possibility that KIRs, and more generally innate immunity, may be involved in the pathogenesis of viral-associated cancers.** (Cancer Epidemiol Biomarkers Prev 2005;14(11):2673–7)

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy that is linked to the ubiquitous Epstein-Barr virus (EBV). In addition to EBV, several host susceptibility factors have been shown to be associated with NPC development, including polymorphisms in human leukocyte antigen (*HLA*) genes. Human leukocyte antigen (HLA) class I molecules (i.e., HLA-A, -B, -C*w*) function in adaptive immunity by presenting specific foreign antigens to cytotoxic CD8+ T cells which subsequently recognize and lyse infected cells (1). The innate immune response the adaptive response, facilitates early cytokine production, and directs nonspecific cytotoxicity against invading virus (1). Previous studies have convincingly shown the association of HLA alleles involved in adaptive immunity with NPC (2-6), and these findings are consistent with studies showing HLA associations with other virally induced cancers, such as cervical and liver cancers (7-12). However, no studies to date have examined NPC risk in the context of innate immunity, and little is known about the association between NPC and HLA-C*w* alleles, HLA class I molecules involved in both the adaptive and innate immune responses.

Natural killer (NK) cells are necessary elements of innate immunity and through activating and inhibitory receptors, modulate NK cell activity (13). Among the NK cell (and other effector cell) receptors, the killer cell immunoglobulin-like receptors (KIR) are by far the most polymorphic. This family of receptors consists of both activating and inhibitory allotypes. Inhibitory KIRs interact with specific HLA class I molecules to impede NK cytolysis and protect ''self'' cells from spontaneous destruction, whereas activating receptors, presumably including the activating KIR, stimulate NK cell–directed destruction of target cells, including transformed tumor cells, virus-infected cells, and cells undergoing other types of ''stress'' (14, 15). HLA-C*w* is a predominant ligand for inhibitory KIRs (specifically KIR2DL1 and KIR2DL2/3) and as the extracellular domains of the activating KIR2DS1 and KIR2DS2 have striking homology with their inhibitory counterparts, these receptors are likely to bind HLA-C*w* as well, albeit with lower affinity (16-18). HLA-C*w* molecules are classified as either group 1 (HLA-C*w* group 1) or group 2 (HLA-C*w* group 2) based on dimorphisms that determine their specificity for particular KIR (19, 20). HLA-C*w* group 1 alleles are ligands for the inhibitory KIR2DL2 and KIR2DL3, and HLA-C*w* group 2 allotypes are

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Note: M. Martin with M. Butsch Kovacic contributed equally to this work.

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ligands for KIR2DL1. HLA-B alleles can also serve as ligands for KIRs; in particular, Bw4 allotypes function as ligands for KIR3DL1 (21). KIR binding specificity for HLA molecules raises the possibility that HLA-NPC associations reported previously reflect the involvement of both the adaptive and innate immune responses in NPC pathogenesis.

To evaluate the possibility that the innate immune response may influence susceptibility to NPC through HLA-KIR interactions, we have applied high-resolution HLA and KIR genotyping methods to a case-control study in Taiwan. As an extension of our previous NPC-HLA report, we also examined specific HLA-C*w* alleles and their association with NPC.

Materials and Methods

Study Population. Details of our case-control study methods have been described previously (5, 22-25). Briefly, both cases and controls had to have been county or city residents of Taipei, Taiwan (primarily a Fujianese population) for at least 6 months. Incident NPC cases (n = 378 eligible) <75 years of age and having histologically confirmed NPC were identified through two tertiary care hospitals between July 15, 1991 and December 31, 1994, and were matched to controls at a 1:1 ratio on age (within 5 years), sex, and geographic residence. Controls (n = 374; four cases went unmatched) were selected using the National Household Registration System. A total of 375 cases (99%) and 327 controls (88%) provided informed consent.

Risk Factor Questionnaire. A detailed in-person risk factor questionnaire was administered by a trained nurse interviewer. For cases, interviews were conducted at the time of biopsy for histologic confirmation of NPC and before treatment. The questionnaire obtained information on sociodemographic factors, cigarette smoking, diet, and occupational history (22-24).

Biological Specimen Collection and Testing. Peripheral blood was collected from 367 cases and 321 controls. Serum was tested for viral capsid antigen IgA, Epstein-Barr nuclear antigen 1 IgA, and anti-DNase as previously described (23). Although >90% of adults worldwide are infected with EBV, only a small fraction of individuals test positive for the three EBV markers listed above, and these markers have been shown to be predictive of NPC risk (26). DNA was tested for cytochrome P450 2E1 (CYP2E1) genotypes, for HLA-A, HLA-B, and HLA class II antigens as previously described (25, 27).

Residual DNA was used for HLA-C*w* PCR-based typing using locus-specific primers flanking exons 2 and 3 of each locus. The PCR products were blotted on nylon membranes and hybridized with a panel of sequence-specific oligonucleotide probes developed by the 13th International Histocompatibility Workshop (see http://www.ihwg.org/protocols/protocol.htm). Reaction patterns of the sequence-specific oligonucleotide probes were ascertained and alleles were assigned. Ambiguous results were sequenced and subsequently assigned. KIR genotyping was completed using PCR amplification with primers specific for each locus for the presence or absence of the following activating *KIR* genes: 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1; and inhibitory *KIR* genes: 2DL1, 2DL2/2DL3, 2DL5, and 3DL1 as previously described (28). Internal control primers for a 796 bp fragment of the third intron of *DRB1* were also included in each PCR.

KIR genotyping results were available for the present analysis on 295 cases (79% of consenting cases) and 252 controls (77% of consenting controls). Successful HLA-C*w* genotyping results were available on 213 NPC cases (57% of consenting cases) and 200 controls (61% of consenting controls). KIR and HLA-C*w* results are not available on 121 individuals who consented to the study because their DNA was unavailable for testing largely due to the fact that DNA aliquots at the laboratory were exhausted by earlier DNA-based studies conducted as part of our NPC case-control study. In addition, KIR and HLA-C*w* genotyping was unsuccessful despite the availability of DNA for testing of 20 individuals (KIR) and 154 individuals (HLA-C*w*), respectively. The relatively high rate of unsuccessful HLA-C*w* genotyping attempts is explained for the most part by amplification failures resulting from the need to amplify a relatively large fragment of the HLA-C*w* gene (~1.2 kb). We cannot rule out the possibility that the success of HLA-C*w* genotyping is related to specific genotypes. However, comparing subjects that were successfully KIR- and HLA-C*w*-genotyped to those that were not genotyped or had unsuccessful genotyping, we found no statistically significant differences with respect to case-control status, sex, ethnicity, and education (data not shown). Subjects without HLA-C*w* genotyping data were slightly older (mean age 47 versus 45 years) than those with genotyping data (P = 0.01). The distribution of HLA-C*w* alleles among our controls is consistent with that reported from Taiwan in the literature (29), providing further reassurance that our results are not biased.

Statistical Analysis. Allele frequencies were computed and compared between cases and controls with Pearson's χ^2 test or Fisher's exact test (when there were less than five subjects in a cell). Odds ratios (OR) were determined and statistical significance was assessed with 95% confidence intervals (95% CI). Unconditional logistic regression was used to determine the association between NPC and exposures of interest. Conditional logistic regression was not used in order to avoid loss of information from cases and controls without a matched pair. The correlation between previously identified ''high-risk'' alleles and haplotypes and HLA-C*w* alleles were assessed by Pearson correlation coefficients. Adjustment in logistic models for age, sex, ethnicity, duration of smoking, duration of formaldehyde exposure, duration of wood dust exposure, dietary nitrosamine and nitrite exposure, and presence of the CYP2E1 polymorphism did not affect the risk estimates for NPC by >7%. Crude distribution estimates are shown in the text and tables. The effects of EBV seropositivity on the associations examined were evaluated through stratification. In these stratified analyses, all cases (because all but four NPC cases were EBV+) were compared with EBV+ controls. The statistical significance of logistic regression terms representing multiplicative interaction between carriers of HLA-B*4601 and KIR2DS2 in relation to NPC risk was assessed through the Wald χ^2 test.

Results

Activating and inhibitory *KIR* genes were evaluated separately. When activating KIRs were examined, we observed a tendency for risk to increase with increasing number of activating KIRs (Fig. 1A). Compared with individuals who carried no activating KIRs, those with 1, 2 to 4, and 5+ activating KIRs had ORs of 1.7 (95% CI, 0.78-3.6), 1.6 (95% CI, 0.74-3.4), and 2.6 (95% CI, 0.92-7.5), respectively (P_{trend} = 0.26). The effect was more pronounced in analysis restricted to EBV-seropositive controls, with OR estimates for individuals with 1, 2 to 4, and 5+ activating KIRs being 2.0 (95% CI, 0.73-5.3), 2.5 (95% CI, 0.92-6.9), and 3.4 (95% CI, 0.74-15.7), respectively (P_{trend} = 0.07). Activating KIRs were also evaluated independently. Although none of the individual activating KIR were found to be significantly associated with NPC, we observed modest increases in NPC risk associated with KIR2DS2, KIR2DS3, and KIR3DS1 (OR, 1.5; 95% CI, 0.81-2.8; OR, 1.4; 95% CI, 0.71-2.7; and OR, 1.4; 95% CI, 0.81-2.5, respectively) in EBV-seropositive individuals only.

In contrast to activating KIRs, there was no overall suggestion that risk of disease was amplified with increasing number of inhibitory KIRs (Fig. 1B). Compared with individuals who carried six or fewer inhibitory KIRs (this baseline was chosen because KIR2DL1, KIR2DL2/3, KIR2DL4, KIR3DL1, KIR3DL2, and KIR3DL3 are nearly ubiquitous in all study participants), those with seven and eight inhibitory KIRs had ORs of 1.1 (95% CI, 0.74-1.6) and 1.0 (95% CI, 0.60-1.6), respectively (P_{trend} = 0.91). In EBV-restricted analysis, there was some evidence of increasing risk of disease with increasing number of inhibitory KIRs, with OR estimates for individuals with seven and eight inhibitory KIRs being 1.5 (95% CI, 0.85-2.7) and 1.8 (95% CI, 0.75-4.2), respectively (P_{trend} = 0.09). However, this effect may reflect the association with disease seen for activating KIRs, because the two KIRs that define individuals with seven or eight inhibitory KIRs (KIR2DL2 and KIR2DL5) are in complete linkage disequilibrium with activating *KIR* genes KIR2DS2 and KIR2DS3/KIR2DS5. To evaluate possible risk of disease associated with nearly ubiquitous inhibitory KIRs, KIR3DL1, KIR2DL2/3 and KIR2DL1, we examined the presence or absence of their respective HLA ligands (HLA-Bw4, HLA-C*w* group 1, and HLA-C*w* group 2) under the premise that presence of the ligand is required for KIR function. Although none of the individual inhibitory KIRs were found to be significantly associated with NPC, we observed a modest reduction in NPC risk associated with HLA-C group 2 and Bw4 carriers (OR, 0.76; 95% CI, 0.48-1.2 and OR, 0.89; 95% CI, 0.61-1.3, respectively, in the overall analysis; OR, 0.78; 95% CI, 0.38-1.6 and OR, 0.68; 95% CI, 0.37-1.3, respectively, in the EBV+ analysis).

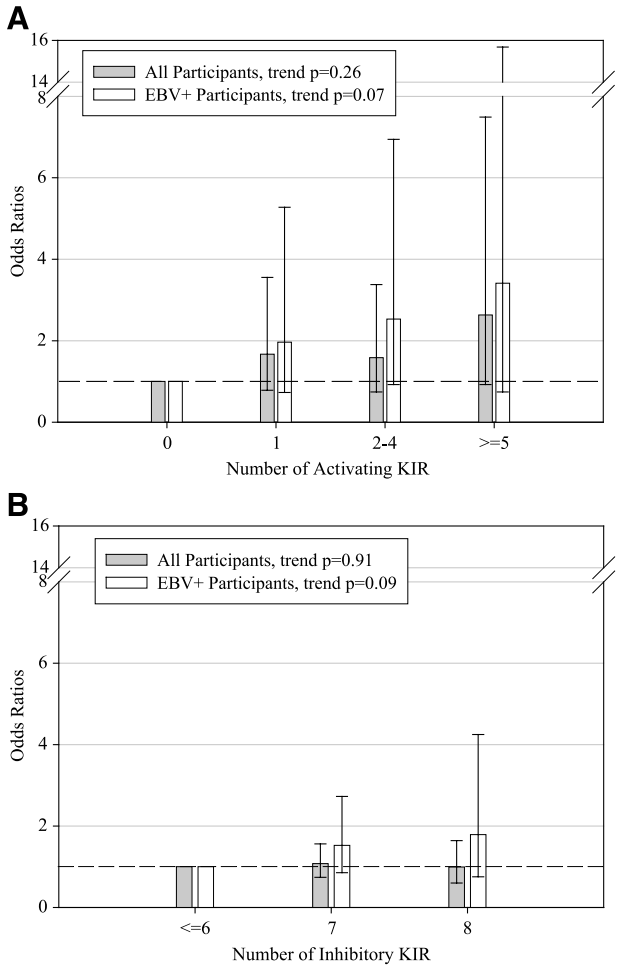

Figure 1. Association of NPC with number of activating KIRs. There are 547 study participants (295 cases and 252 controls) with KIR data including 370 EBV+ individuals (295 cases and 75 controls). Individuals are considered EBV+ if they are positive for viral capsid antigen IgA, Epstein-Barr nuclear antigen 1 IgA, or anti-DNase. *Bars,* 95% CI. values for two-sided linear trend test. **A.** Considers only individuals with full-length KIR2DS4 and not the nonfunctional/mutant form of KIR2DS4 (having a 22 bp deletion), as having an activating KIR. For all study participants, there were 13 cases and 18 controls with no activating KIR, 135 cases and 112 controls with a single activating KIR, 127 cases and 111 controls with two to four activating KIR, and 19 cases and 10 controls with five or more activating KIRs (only five subjects had six activating KIRs total). For EBV+ participants, there were 13 cases and 7 controls with no activating KIR, 135 cases and 37 controls with a single activating KIR, 127 cases and 27 controls with two to four activating KIR, and 18 cases and 3 controls with five or more activating KIRs. **B.** For all study participants, there were 288 subjects (154 cases and 134 controls) with six or less inhibitory KIRs with none of these subjects having four or less inhibitory KIRs and only four subjects having five inhibitory KIRs. In addition, there were 181 subjects (100 cases and 81 controls) with seven inhibitory KIRs and 77 subjects (41 cases and 36 controls) with eight inhibitory KIRs. For EBV+ participants, there were 201 subjects (47 controls) with six or less inhibitory KIRs, 120 subjects (20 controls) with seven inhibitory KIRs, and 48 subjects (7 controls) with eight inhibitory KIRs.

We next evaluated NPC risk of specific combinations of KIRs and their known/presumed HLA class I ligands. Our analyses found no evidence to suggest that joint effects of KIR/HLA pairs are notably or significantly different from the main effects seen for HLA alleles or KIRs alone (data not shown), with the possible exception of the HLA-B*4601/KIR2DS2 pair (Fig. 2). Overall, carriers of both HLA-B*4601 and KIR2DS2 had an OR of 2.9 (95% CI, 1.3-6.4), carriers of HLA-B*4601 alone had an OR of 1.6 (95% CI, 1.0-2.5) and carriers of KIR2DS2 alone had an OR of 0.94 (95% CI, 0.60-1.5), compared with carriers of neither allele (P = 0.23 when comparing carriers of HLA-B*4601/KIR2DS2 to carriers of B*4601 alone). This effect was more pronounced in analysis restricted to EBV-seropositive participants, with OR estimates for individuals with HLA-B*4601/KIR2DS2, B*4601 alone, and KIR2DS2 alone being 9.4 (95% CI, 1.2-72.6), 2.7 (95% CI, 1.2-6.0), and 1.6 (95% CI, 0.66-3.8), respectively (P = 0.29 when comparing HLA-B*4601/KIR2DS2 carriers to carriers of HLA-B*4601 alone). We found no statistically significant interaction between carriers of HLA-B*4601 and KIR2DS2 regardless of whether we examined all participants (P = 0.16) or stratified on EBV+ participants (P = 0.40).

Finally, the effect of individual HLA-C*w* alleles on NPC risk was investigated (Table 1). When the HLA-C*w* allele frequencies observed among cases were compared with those observed among controls, a significant elevation was seen for HLA-C*w**0302 and a significant reduction was seen for HLA-C*w**0401. Similar differences were observed in analysis restricted to EBV-seropositive controls. HLA-C*w**0302 is known to be in tight linkage disequilibrium with HLA-B*5801 in Asian populations (for this study, σ = 0.93), and HLA-B*5801 was previously reported to be associated with NPC (27, 30). Given the tight linkage disequilibrium between HLA-C*w**0302 and HLA-B*5801, we cannot evaluate the individual contributions to risk of NPC of these two HLA alleles. In contrast to HLA-C*w**0302, HLA-C*w**0401 is not in strong linkage disequilibrium with any HLA allele previously observed to be associated with NPC. A modest correlation (σ = 0.12) was observed here between C*w**0401 and HLA-A*1101 (an allele previously observed to reduce risk of NPC; ref. 27). However, individuals with HLA-C*w**0401 in the absence of A*1101 had a

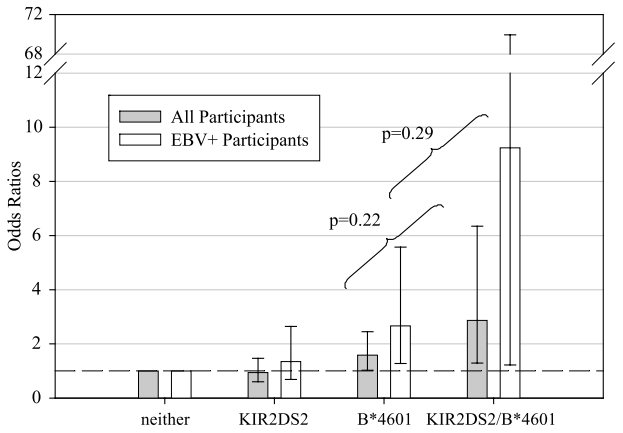

Figure 2. Joint effect of KIR2DS2 and HLA-B*4601. There are 532 subjects with both KIR2DS2 and HLA-B*4601 data including 134 cases and 133 controls with neither KIR2DS2 nor B*4601, 52 cases and 56 controls with KIR2DS2 alone, 75 cases and 47 controls with B*4601 alone, and 26 cases and 9 controls with both. For the 360 EBV+ individuals, 134 cases and 48 controls had neither KIR2DS2 nor B*4601, 52 cases and 14 controls had KIR2DS2 alone, 75 cases and 10 controls had B*4601 alone, and 26 cases and 1 control had both. Individuals are considered EBV+ if they are positive for viral capsid antigen IgA, Epstein-Barr nuclear antigen 1 IgA or anti-DNase. *Bars,* 95% CI. Pearson's χ^2 test was used to examine the significance of associations between carriers of B*4601 alone and carriers of both KIR2DS2 and B*4601 for all participants and EBV+ participants, respectively. Two-sided *P* values are reported.

nearly 3-fold reduction in NPC risk relative to individuals missing both alleles (OR, 0.37; 95% CI, 0.11-1.3), suggesting that the effect of HLA-C*w**0401 is independent of the HLA-A*1101 effect.

Discussion

Genetic susceptibility factors are known to be important in NPC pathogenesis. Given the strong link between EBV and NPC, and the role of *HLA* gene products in the presentation of viral antigens to the adaptive immune response, *HLA* genes have been extensively studied as risk factors for NPC. In the present report, we have extended previous findings by demonstrating an association between two HLA-C*w* alleles and NPC: HLA-C*w**0302 and HLA-C*w**0401. Whereas the association with HLA-C*w**0302 might mirror the previously reported association between HLA-B*5801 and NPC (due to tight linkage disequilibrium between the two alleles), the association between HLA-C*w**0401 and NPC seems to be independent of previously reported associations.

In contrast to the extensive work done to date to evaluate the role of adaptive immune response in the etiology of NPC, surprisingly little is known about the role of innate immunity in the control of EBV infection and its contribution to NPC risk. To address this issue, we investigated *KIR* genes and their HLA class I ligands. Two distinct hypotheses could explain the possible role of KIRs in the etiology of NPC and other viral-associated cancers (31). First, activating KIRs (and/or reduction in inhibitory KIRs) may be protective against NPC as a consequence of increased cytolysis of EBV-infected cells by activated NK cells. Alternatively, activating KIRs (and/or reduction in inhibitory KIRs) might increase risk of NPC through nonspecific inflammatory responses, such as oxidative DNA damage, triggered by activated NK cells. Recent findings examining KIRs in the context of human papillomavirus–related cervical intraepithelial neoplasia support the latter hypothesis (13). In that study, the presence of the activating KIR3DS1, particularly when found in the absence of certain inhibitory KIR-HLA ligand pairs was associated with increased risk of high-grade cervical intraepithelial neoplasia and cervical cancer (31).

Despite the fact that large differences were not observed between subjects included and excluded from our analyses, our inability to genotype all participants is a limitation as the resulting study size has diminished statistical power. Furthermore, we are restricted by our incomplete understanding of KIR biology and knowledge of their ligands. As *KIR* can vary at both the allele and gene level, we are presently only able to determine the absence or presence of each *KIR* gene and we are not able to determine whether a given gene is present on one or both chromosomes. As a consequence, we are not able to infer haplotypes without family data. Still, the finding that NPC risk is augmented with increasing number of activating KIRs particularly in EBV+ subjects provide support to the hypothesis that activation of innate immune effector cells

Table 1. HLA-C*w* allele frequencies among NPC cases and community controls

Allele frequencies (%)				
HLA-C allele	NPC cases (2 *n* = 424)*	Controls (2 *n* = 396)	All participants, OR (95% CI) †	EBV+ participants, OR (95% CI) ‡
102	23.8	17.9	1.4 (0.92-2.0)	1.4 (0.73-2.6)
103	0.24	0.25	0.94 (0.06-15.1)	
302	13.7	8.3	1.9 (1.19-3.2)	2.0 (0.89-4.5)
303	2.8	5.1	0.54 (0.26-1.1)	0.39 (0.15-1.1)
304	10.1	12.9	0.75 (0.46-1.2)	0.67 (0.33-1.4)
401	3.1	6.3	0.46 (0.23-0.92)	0.37 (0.14-0.93)
403	1.7	0.25	6.8 (0.82-55.3)	
501	0.0	0.51		
602	1.4	1.8	0.80 (0.26-2.4)	1.5 (0.18-12.8)
701	0.47	0.51	0.94 (0.13-6.7)	
702	24.3	20.5	1.3 (0.87-1.9)	1.3 (0.68-2.3)
704	0.47	0.51	0.94 (0.13-6.7)	0.49 (0.04-5.5)
801	8.0	11.1	0.71 (0.43-1.2)	0.70 (0.33-1.5)
1202	3.8	4.0	0.93 (0.45-1.9)	1.0 (0.32-3.1)
1203	0.0	0.76		
1402	1.9	3.8	0.45 (0.18-1.1)	0.42 (0.12-1.5)
1403	0.0	0.25		
1502	4.2	5.3	0.79 (0.41-1.5)	0.89 (0.31-2.5)

*The effective sample size was 2 *n* with each individual contributing two separate alleles.
†ORs and 95% CI's were calculated with the referent group being those individuals that failed to carry that specific HLA-C allele.
‡Individuals are considered EBV+ if they are positive for viral capsid antigen IgA, Epstein-Barr nuclear antigen 1 IgA, and anti-DNase.

increases the risk of virally associated cancers such as NPC. This is consistent with previous assertions that restriction of the control group in case-control studies to individuals who are at risk of disease (in this case by virtue of having an EBV seroprofile shown to be linked to NPC susceptibility; ref. 26) helps to clarify disease associations (32).

In conclusion, the observed tendency of activating KIRs to be associated with increased risk of NPC is consistent with the hypothesis that increases in the level of innate immune response stimulation may contribute to increased risk of some virus-associated cancers, potentially through an amplified inflammatory response triggered by NK cells (or other effector cells) expressing activating KIRs. The present study represents a first attempt to link KIRs, HLA-C*w*, and therefore innate immunity, to NPC pathogenesis and clearly requires confirmation in independent studies.

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