รายงานวิจัยฉบับสมบูรณ์

โครงการ

ความหลากหลายของจำนวนคิลเลอร์เซลล์อิมมูโนโกลบูลินไลค์รีเซพเตอร์ยีนในประชากรภาคตะวันออกเฉียงเหนือของไทย และความสัมพันธ์ของจำนวนคิลเลอร์เซลล์อิมมูโนโกลบูลินไลค์รีเซพเตอร์ยีนกับความรุนแรงในการติดเชื้อเดงกี่

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ตุลาคม 2560

สัญญาเลขที่ **TRG5880208**

รายงานวิจัยฉบับสมบูรณ์

โครงการ
ความหลากหลายของจำนวนคิลเลอร์เซลล์อิมมูโนโกลบูลินไลค์รีเซพเตอร์ยีนในประชากรภาคตะวันออกเฉียงเหนือของไทย และความสัมพันธ์ของจำนวนคิลเลอร์เซลล์อิมมูโนโกลบูลินไลค์รีเซพเตอร์ยีนกับความรุนแรงในการติดเชื้อเดงกี่

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มหาวิทยาลัยธรรมศาสตร์ ศูนย์รังสิต

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยธรรมศาสตร์
(ความเห็นในรายงานนี้เป็นของผู้วิจัย
สกว.และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

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Abstract

Killer cell immunoglobulin-like receptors (KIRs) are cell surface receptors on natural killer (NK) cells and subsets of T cells. Genetic variations of *KIRs* have been reported in different populations worldwide. The impact of *KIR* polymorphisms on immune response has been concerned in several diseases, particularly viral infections. This study investigated genetic variations of *KIR* copy number in individuals who were dengue infections and healthy controls (northeastern Thais, NETs). The quantitative Polymerase Chain Reaction (qPCR) method was used to indentify KIR copy number. Firstly, the novel expanded and contracted *KIR* copy number profiles were identified at cumulatively high frequencies. These all comprise haplotypes with duplication (6.9%) or deletion (2.7%) of *KIR3DL1/S1* along with adjacent genes. Five expanded *KIR* profiles comprised haplotypes with duplications of *KIR2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1/S1* and *2DS1/4*, whereas two contracted profiles contained only a single copy of *KIR3DP1*, *3DL1/S1* and *2DL4*. Using a KIR haplotype prediction program (KIR Haplotype Identifier), 14% of NET haplotypes carried atypical haplotypes based on the gene copy number data. Later, one hundred and thirty nine dengue infections were identified *KIR* copy number to compare with healthy controls. The results showed only four *KIR* loci associated with dengue infection. Individuals who had two copies of *KIR2DL2* and *2DS2* were significantly protective to dengue infection with p=0.001. In contrast, two copies of *KIR2DL1* and *2DL3* were susceptible to dengue infection with p=0.02 and p=0.001. The association of *KIR* copy number and severity of dengue infections (DF *vs* DHF) were not found in this study. However, statistical analysis needs to perform for more data precision and correction.

Keywords : *KIR* copy number; dengue infection; *KIR* haplotype

บทคัดย่อภาษาไทย

KIRs เป็นรีเซพเตอร์ที่พบบนผิวของเซลล์ NK และบางกลุ่มประชากรของเซลล์ T ได้มีการรายงานความหลากหลายทางพันธุกรรมของ *KIR* ในกลุ่มประชากรต่างๆทั่วโลกและอิทธิพลของความหลากหลายของยีน *KIR* ในแต่ละบุคคลมีความเกี่ยวข้องกับการตอบสนองทางภูมิคุ้มกันของโรคต่างๆ โดยเฉพาะการติดเชื้อไวรัส การศึกษาครั้งนี้ได้ทำการตรวจหาความหลากหลายด้านจำนวน copy ของยีน KIR เพื่อหาความสัมพันธ์กับการติดเชื้อไวรัสเด็งกี่และมีกลุ่มควบคุมคือประชากรที่อาศัยอยู่ที่ภาคตะวันออกเฉียงเหนือของไทย โดยใช้วิธี quantitative Polymerase Chain Reaction (qPCR) ผลการศึกษาความหลากหลายจำนวน copy ของยีน KIR ในกลุ่มประชากรภาคตะวันออกเฉียงเหนือของไทยพบ KIR genotypes ใหม่ที่ยังเคยไม่มีการรายงานมาก่อนและมีความถี่สูง โดยประกอบด้วย *KIR* profiles 2 แบบ ได้แก่ แบบ expansion จำนวน 6.9% (n=5) และ แบบ contraction จำนวน 2.7% (n=2) ทั้งนี้ *KIR* profiles ที่เป็นแบบ expanded profiles พบว่ามี duplication ของยีน *KIR2DP1, 2DL1, 3DP1, 2DL4, 3DL1/S1* และ *2DS1/4* ส่วน profiles ที่เป็นแบบ contracted profiles จะพบยีน *KIR3DP1, 3DL1/S1* และ *2DL4* มีเพียง 1 copy (ปกติคือ 2 copies) จากนั้นเมื่อทำการวิเคราะห์ KIR haplotype โดยใช้ KIR haplotype prediction program (KIR Haplotype Identifier) พบว่า KIR haplotypes ของประชากรภาคตะวันออกเฉียงเหนือ ประกอบด้วย atypical haplotypes จำนวน 14% จากนั้นทำการเปรียบเทียบกับกลุ่มคนไข้ที่ติดเชื้อไวรัสเด็งกี่จำนวน 139 ราย พบว่ามี KIR เพียง 4 loci ที่สัมพันธ์กับการติดเชื้อไวรัสเด็งกี่ โดยพบว่า ผู้ที่มีจำนวนยีน *KIR2DL2* และ *2DS2* จำนวน 2 copies สัมพันธ์กับการป้องกันอาการที่เกิดจากเชื้อไวรัสเด็ง (p=0.001) และผู้ที่มีจำนวน 2 copies ของยีน *KIR2DL1* และ *2DL3* จะสัมพันธ์ต่อการเกิดอาการที่เกิดจากเชื้อไวรัสเด็งที่ (p=0.02 และ p=0.001 ตามลำดับ) การศึกษาในครั้งนี้ไม่พบว่าความสัมพันธ์ระหว่าง *KIR* copy number กับความรุนแรงของการติดเชื้อไวรัสเด็งกี่ (DF *vs* DHF) อย่างไรก็ตาม การวิเคราะห์ข้อมูลดังกล่าวยังต้องวิเคราะห์ร่วมข้อมูลอื่นๆเพื่อความถูกต้องและแม่นยำก่อนการรายงานต่อไป

คำสำคัญ: *KIR* copy number; การเชื้อไวรัสเด็ง; KIR haplotype

The copy number variation of killer immunoglobulin like receptors *(KIRs)* in Northeastern Thais and the association of *KIR* copy number to severity of dengue infections

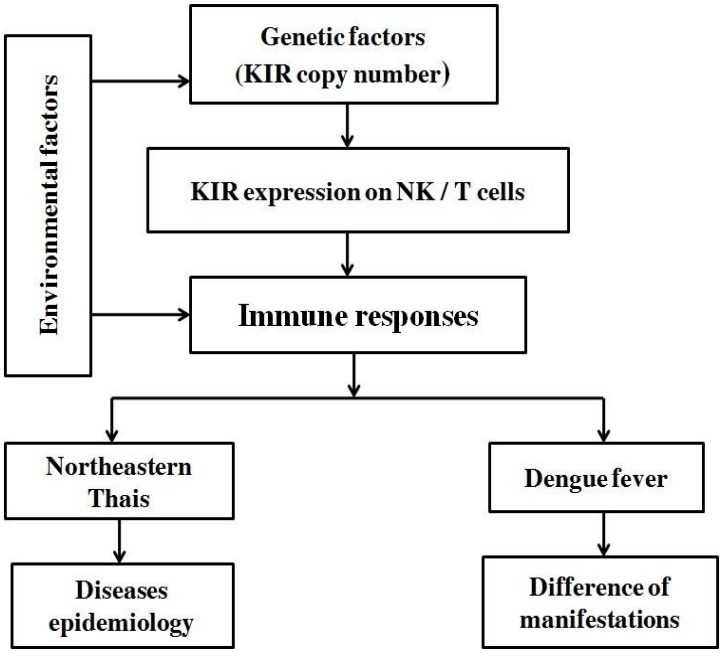
1. Introduction to the research problem and its significance

Killer immunoglobulin like receptors (KIRs) are members of surface receptors on several immune cells especially NK cells and subpopulation of T lymphocytes. KIRs can be inhibitory or activating receptors based on their structures, except KIR2DL4 which can mediate both activating and inhibitory signals to NK cells.[1] In addition to other receptors on NK cells, the interactions between KIRs and HLA can regulate NK cell function, based on inhibitory or activating signal transduction. Diversity of *KIR* are achieved through allelic polymorphism, a combination of genes content, and gene copy number.[2, 3] The population diversity and rapid evolution of the *KIR* genes strongly suggest that they are under pathogen-mediated selections.[1, 4] An extreme degree of heterogeneity among individual *KIR* contributes to diversity of immune responses. As previous studies, activating *KIR* genes have been associated with autoimmune diseases,[5-8] whereas the influence of inhibitory *KIR* genes associates with the outcome of infectious diseases especially in viral infections.[9-11] Dengue infection is the most common of mosquito borne viral diseases in tropical and subtropical regions around the world especially in Thailand. Several studies indicated the role of NK cells in dengue virus infection especially early activity in primary infection.[12] The activities of NK cells were also significantly associated with mild dengue clinical manifestations.[13] Evidently, NK cells are active in defences against dengue virus infections and might play some roles in pathogenesis of DHF/DSS through the mechanism of NK cell-mediated cytotoxicity and cytokine production.[14-16]

Copy number variation (CNV) of *KIR* are derived from non-reciprocal recombination, the mechanism initiates form unequal crossing between non-allelic of *KIR* genes (high

similarity sequences) generating a novel hybrid genes, CNV, and expansion/contraction of *KIR* loci.[17] KIR expression on NK cells is correlated with the copy number of that gene, which may affect function of NK cells.[18] Sequentially, copy number variations of *KIR* were identified in the United Kingdom and United States. The results indicated that the *KIR* B haplotypes were commonly found to have gene copy number variation.[19] In addition, the genetic association and functional study indicating that copy number of *KIR3DS1* was associated with lower HIV-1 viral load as well as inhibited HIV-1 replication more robustly.[20] Consistent with previous report, activating *KIR* copy number of *KIR3DH* was associated with simian immunodeficiency virus (SIV) replication in Mamu-A*01^{-} rhesus monkeys, controlling SIV spread.[21, 22] Interestingly, copy number variation of *KIR3DH* was also associated with releasing of granzyme B by NK cells during primary SIV infection[22]. Thus, the conventional KIR typing is inadequate to study disease associations and the gene copy number should be taken into account.

Genetic variations of *KIR* and *HLA ligands* in norteastern Thais (NET) have been reported in my previous study.[23] The genetic variations of *KIR* have been completely characterized except for gene copy number variation. Accordingly, the study of CNVs of *KIR*s in NET and dengue infections would essentially clarify the genetics of KIRs in NET and the role of KIRs in dengue infections. In addition, *KIR* copy number variations are good candidates as susceptibility factors contributing to the severity of dengue viral infections as well as other viral infection models in further study as shown in a diagram below. This study will first clarify the up-to-date genetic variations of *KIR* copy number in Thais and then explore the role of *KIR* copy number in dengue virus infections.



The diagram presents the significance of research project

2. Literature review

Killer cell Immunoglobulin like receptors (KIRs) are cell surface receptor on several immune cells including NK cells and subset of T cells. KIRs are encoded on chromosome 19q13.4,[24, 25] within a 100-200 Kb region of the Leukocyte Receptor Complex (LRC). The *KIR* gene family is composed of 17 genes (*KIR2DL1-4, 2DL5A, 2DL5B, 2DS1-5, 3DL1-3, 3DS1, 2DP1,* and *3DP1*) with *KIR2DL* and *3DL* encoding the inhibitory KIRs, *KIR2DS* and *KIR3DS* encoding activating KIRs and two pseudogenes (*KIR2DP1*and *KIR3DP1*) encoding no cell surface receptors. *KIR2DL4* is the only receptor which can be both inhibitory and activating functions. The HLA class I molecules on target cells are ligands of KIRs,[26] HLA-C group 1 is a ligand for KIR2DL2 and KIR2DL3, whereas HLA-C group 2 provides ligand for KIR2DL1. KIR3DL1 has specificity for the HLA-Bw4 epitope that exists on many of the HLA-B alleles and some HLA-A molecules. KIR3DL2 binds to HLA-A3 and HLA-A11 allele families. HLA-A11 is also a ligand of KIR2DS4,[27] however the ligands of the remainder of KIRs are still unknown.

Genetic variation of *KIR* is achieved through allelic polymorphism, a combination of genes content and gene copy number.[2, 3] *KIR* haplotypes can be classified into two groups, namely A and B.[28] Four framework genes are conserved in both group A and B (*KIR3DL3-3DP1-2DL4-3DL2*). Group A is fixed with nine genes (*KIR3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2*) and mostly contains inhibitory *KIR* except *KIR2DS4*. Allelic polymorphism is commonly found in this group. In contrast, group B has variable gene content and contains dominantly activating *KIR* genes.

The diversity of *KIRs* is studied and recorded in "The Allele Frequency Net Database"[29] to provide a central source database of *KIR*s including gene frequencies, allele frequencies, *KIR* genotypes, linkage disequilibrium, and disease associations. Presently, the data of one hundred and forty six populations with 17,852 individuals are in *KIR* database including my previous study. The distributions of *KIR* among populations have revealed the variations of A and B haplotypes.[3, 30-33] A haplotype is overrepresented in Northeast Asians (Japanese, Korean and Chinese),[32, 34, 35] whereas the B haplotype has high frequencies in the native habitants of India, Australia, and America.[3, 33, 36, 37] However, they are approximately equal in Caucasians and Africans.

The great variations of *KIR*s are derived from reciprocal recombination and non-reciprocal recombination. Reciprocal recombination at the central region of *KIR* haplotypes (between *KIR3DP1* and *KIR2DL4,* hotspot) results in centromeric and telomeric gene combinations and form new variant *KIR* haplotypes.[38] On the other hand, non-reciprocal recombination mechanism initiates from unequal crossing between non-allelic *KIR* genes (sequences with high similarity) generating gene copy number variations (CNVs) resulting in a novel hybrid gene, and expansion/contraction of *KIR* haplotypes. Currently, the CNVs and novel hybrid genes in *KIR* complex have been investigated and proposed to be an evolutionary strategy of the immune system.[39] CNVs of KIRs are important factors relating to the KIR expression on NK and T cells, which lead to different immune responses.

Consistent with previous reports, the *KIR* copy number relates with immune responses in HIV-1 and simian immunodeficiency virus (SIV).[20, 21] Consequently, the CNVs and hybrid genes need to be considered when study population genetics as well as associations of *KIR* and diseases.

Genetic studies of *KIR* and diseases have been mainly concerned in viral infections, autoimmune diseases, and transplantation. The role of KIRs in the immune responses depends on extensive genomic diversity and their ligands (HLA class I allotypes), which are heterogeneity among individuals. It is conceivable that *KIR* gene variation affects resistance and susceptibility to infections and pathogenesis. *KIR* genes and infectious diseases become an attractive target for association studies. The influence of *KIR* polymorphisms in human diseases has recently covered in several reviews.[40-42]

Dengue viruses cause a variety of manifestations in human ranging from asymptomatic infection to a self-limiting febrile illness and severe dengue.[43] Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more severe forms, life-threatening manifestations of dengue infections. It is generally believed that primary infections elicit lifelong protective immunity. However, the risk of dengue severity is in secondary infections with distinct serotypes.[44, 45]

Several studies have shown the role of NK cells in dengue virus infections especially their early activities in primary infections.[12] The high activities of NK cells are significantly associated with mild dengue clinical manifestations.[13] Moreover, Kurane and colleagues'[14] study demonstrating that dengue virus infected cells were responded by human NK cells via direct cytolysis and antibody-dependent cell-mediated cytotoxicity. Recently, the evidence showing that NK cell degranulation can be activated by interaction of NKp44 with the flavivirus envelope protein.[15] In addition, DCs and NK cells establish a private conversation by being in close physical contact promoting NK cell proliferation, NK-mediated shattering of iDCs and NK-dependent DC maturation in responses against pathogens.[16] These data

imply that NK cells are active in defense against dengue virus infections and might play some roles in pathogenesis of DHF/DSS through the mechanism of NK cell-mediated cytotoxicity and cytokine production.

3. Objectives

3.1 To determine the copy number of *KIR* and predict *KIR* haplotypes (haplotye ID, centromeric motif, telomeric motif, unusual haplotypes) in northeastern Thai population and compare with previous studies of other populations

3.2 To study the association of *KIR* copy number and severity of dengue infections

4. Methodology

4.1 Materials

4.1.1 Study Population

4.1.1.1 Copy number variation of *KIRs* in Northeastern Thais

Seventy-three samples were recruited from unrelated healthy individuals who are Thais and living in northeastern of Thailand for at least two generations with ethical approval. Genomic DNA samples have been provided by Associate Professor Dr. Amornrat Romphruk, the Blood Transfusion Center, Faculty of Medicine, Khon Kaen University, Thailand.

4.1.1.2 The association of *KIR* copy number and severity of dengue infections

One hundred and thirty nine samples for association study of *KIR*s with severity of dengue infections were provided from Network of Biomedical Research in Dengue Hemorrhagic fever and Dengue viruses in collaborations with Dr. Prida Malasit (M.D.), Faculty of Medicine, Siriraj Hospital.

4.2 Methods

4.2.1 Prediction of A and B haplotypes

Classification of group A and B haplotypes was predicted on the basis of a previous study (Rajalingam *et al.*, 2008).[31] Group A haplotype is individuals who carry only nine genes include *KIR3DL3, 2DL3, 2DL1, 3DP1, 2DP1, 2DL4, 3DL1, 2DS4,* and *3DL2*, which was considered to carry two copies of group A haplotype (AA genotype). Whereas individuals who lack the four variable genes (*KIR2DL3, 2DL1, 3DL1,* and *2DS4*) were regarded as carry two copies of group B haplotype (BB genotype) and the remainder of individuals was considered as heterozygote which carry both group A and B haplotypes (AB genotype). The frequency of group A and B haplotypes was calculated as the following formula: group A = (2NAA+NAB)/2n and group B = (2NBB+NAB)/2n, when NAA, NAB, and NBB are the number of AA, AB, and BB genotype and n is the total number of individuals.

4.2.2 Copy number determination

Quantitative PCR was used to identify copy number for all 20 *KIR* genes in northeastern Thais and dengue samples by Roche LightCycler 480 at *Cambridge* Institute for Medical Research (CIMR), University of Cambridge, UK in collaborations with Dr James Traherne. The data were analyzed by myself.

4.2.2.1 DNA and master mixed preparation

Ten nanograms of gDNA (2.5 ml of 4 ng/ul) were prepared in 384 well plates using the Matrix Hydra II robot (Thermo Scientific). Primers and probes sequences for analysis all *KIR* loci were developed from previous study (Jiang *et al.*, 2012).[19]

4.2.2.2 Multiplex Quantitative PCR assay

The multiplex quantitative PCR reactions were carried out in a triplex format which included three probes targeting, two *KIR* genes and one reference gene. Fluorescent signals were collected at the end of each cycle. FAM, Cy5 and Dragonfly Orange probes were used in multiplex assays.

4.2.2.3 Copy number calculation

Cq values were calculated using the Second Derivartive Maximum Method and the Fit Points Method using the built-in software. The copy number was measured by relative quantification analysis of the target and reference gene (STAT6) using comparative Cq method. The ΔΔCq was used to calculate *KIR* copy number. The first ΔCq was calculated by the cycle threshold difference between the target and reference assay of the same sample. The second ΔCq was calculated by the difference of previous ΔCq values from a test sample and calibrator sample which the copy number of target is known.

The copy caller software from Applied Biosystems was used to correct *KIR* copy number.

4.2.2.4 Corrected *KIR* copy number

Based on LD of *KIR* haplotypes, *KIR* copy number was checked for normal and novel haplotypes (extended and deleted haplotypes) using following formula; *KIR3DL3*=2, *2DS2=2DL2*, *2DL2+2DL3*=2, *2DP1=2DL1*, *3DP1=2DL4*, *3DP1+2DL4* = 4, *3DL1E4* = *3DL1E9*, *3DL1E4+3DL1E9* = 2, *3DL1E9+3DS1*=2, *2DS3+2DS5=2DL5*, *2DS4TOTAL+2DS1*=2, *2DS4WT+2DS4DEL* = *2DS4TOTAL*, *3DL2E4+3DL2E9*= 4, *3DL2E4=3D*

4.2.2.5 *KIR* haplotype prediction

The *KIR* haplotypes were calculated by KIR haplotype identifier program, CIMR bioinformatics, University of Cambridge. The program can predict KIR haplotypes, including group A, group B, centromeric motif, telomeric motif, and unusual haplotypes. Segregation analysis of *KIR* was investigated to identify aberrant *KIR* haplotypes to confirm CNVs.

4.2.3 Statistical Analysis

The differences of KIR haplotypes amongst populations were tested by the Chi-Square, using data derived from direct measurements of haplotypes in the populations.

The Chi-Square was used for nonrandom association between pairs of genes copy number, $p < 0.05$ is considered to statistically significant using the SPSS 19.0 software.

Odd ratios, 95% CI were used to study the association study in dengue infections using the SPSS 19.0 software and Microsoft excel 2013.

Bonferroni correction will be used to correct *p* value to avoid false positive.

5. Scope of research

This project will identify *KIR* copy number, percentage of genes duplication and absence, haplotype prediction (group A, group B, centromeric motif, telomeric motif, and unusual haplotypes) in northeastern Thais and dengue infected patients. The association of *KIR* copy number in dengue patients will be investigated.

Results

1. KIR copy number variations in Northeastern Thais

1.1 *KIR* genotypes in NETs

KIR genotypes were identified based on quantitative PCR data. Twenty genotypes were characterized in the NET samples (n=73) that had copy number typing for all *KIR* genes as shown in Figure 1. Genotypes 1, 2, 3, 4, 5, 7 and 8 were commonly found (>5% frequency) as seen in previous studies of the Thai population. To compare *KIR* genotype with other populations, the genotype frequencies of Asians (Chinese Han, Mongolian and N. Indian), Europeans (Italian and English) and Amerindian (N.Brazilian) were included, showing that genotype IDs 1 and 2 were the two most common genotypes (>10% frequency) in Amerindian, Europeans and most of Asians (NETs, Thais, Chinese Han and Mongolian), whereas other genotypes varied among populations. Individually, the frequencies of all *KIR* genotypes in N. Indians were less than 10%, with the most common genotype found at 9.3%. Eighty per cent of the genotypes, based on qPCR, were in accordance with those obtained using the PCR-SSP typing method. However, inconsistent data between PCR-SSP typing and quantitative PCR were found in *KIR2DS1*. Originally, we thought that the discordances probably could be explained by allelic polymorphisms of *KIR2DS1* that affected PCR-SSP primer binding sites. Subsequently, we have performed DNA sequencing and DNA sequence analysis of the samples (n=4). The discordances of the samples were typing errors of the PCR-SSP method because of high homology sequences between *KIR2DL1* and *KIR2DS1* as well as low quality of the DNA templates. Accordingly, there is no variant of *KIR2DS1* in this population.

Genotype ID	Framework / Pseudogenes					A haplotype member				B haplotype-specific							Present study			Asians					Europeans		Amerindian
																	n=73			NETs	Thais	Chinese Han	Mongolian	N.Indian	Italian	English	N.Brazilian
																				n=235	n=500	n=503	n=90	n=867	n=217	n=584	n=377
	3DL2	3DL3	2DL4	2DP1	3DP1	2DL1	2DL3	3DL1	2DS4	2DL2	2DL5	2DS1	2DS2	2DS3	2DS5	3DS1	haplo.	N	%	%	%	%	%	%	%	%	%
1	■	■	■	■	■	■	■	■	■	□	□	□	□	□	□	□	*AA*	*13*	*17.81*	*35.7*	*40.8*	*54.7*	*37.8*	*9.3*	*28.5*	*27.4*	*31*
4	■	■	■	■	■	■	■	■	■	■	□	□	■	□	□	□	*Bx*	*9*	*12.33*	*9.8*	*6.8*	*5.2*	*5.5*	*2.9*	*8.3*	*13.5*	*7.4*
2	■	■	■	■	■	■	■	■	■	□	■	■	□	□	■	■	*Bx*	*8*	*10.96*	*10.2*	*11.2*	*12.3*	*13.3*	*2.9*	*12.9*	*14*	*15.4*
3	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	*Bx*	*7*	*9.59*	*2.6*	*4.4*	*3.2*	*4.4*	*1.7*	*9.2*	*6.7*	*6*
5	■	■	■	■	■	■	■	■	■	■	■	□	■	■	□	□	*Bx*	*6*	*8.22*	*1.3*	*6*	*3.2*	*NA*	*5.2*	*NA*	*7.4*	*5.6*
8	■	■	■	■	■	■	■	■	■	□	■	■	□	■	□	■	*Bx*	*5*	*6.85*	*8.1*	*6*	*7*	*3.3*	*0.9*	*NA*	*1.7*	*1.6*
7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	*Bx*	*5*	*6.85*	*6*	*3.6*	*1.4*	*1.1*	*0.6*	*3.6*	*4.1*	*2.6*
69	■	■	■	■	■	■	■	□	□	□	■	■	□	□	■	■	*Bx*	*3*	*4.11*	*1.7*	*NA*	*0.6*	*NA*	*NA*	*NA*	*1.2*	*1.1*
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*Bx*	*3*	*4.11*	*3.4*	*4*	*1.6*	*NA*	*2.8*	*4.6*	*4.5*	*2.4*
76	■	■	■	□	■	□	□	■	■	■	■	■	■	□	■	■	*Bx*	*2*	*2.74*	*1.7*	*NA*	*0.4*	*NA*	*NA*	*NA*	*NA*	*0.8*
71	■	■	■	■	■	■	□	■	■	■	■	□	■	■	□	□	*Bx*	*2*	*2.74*	*0*	*NA*	*0.4*	*NA*	*0.8*	*NA*	*1.5*	*2.4*
70	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	*Bx*	*2*	*2.74*	*0.9*	*2.6*	*0.6*	*NA*	*2*	*NA*	*1.7*	*0.8*
91	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	□	*Bx*	*1*	*1.37*	*0.4*	*NA*	*NA*	*NA*	*NA*	*NA*	*0.2*	*1.1*
75	■	■	■	■	■	■	■	□	□	□	■	■	□	■	■	■	*Bx*	*1*	*1.37*	*0.9*	*NA*	*1.2*	*NA*	*0.5*	*NA*	*0.3*	*0.3*
68	■	■	■	■	■	■	■	□	□	■	■	■	■	□	■	■	*Bx*	*1*	*1.37*	*0.4*	*NA*	*0.6*	*1.1*	*3.6*	*NA*	*NA*	*1.8*
94	■	■	■	■	■	■	□	■	■	■	■	□	■	■	□	■	*Bx*	*1*	*1.37*	*0*	*NA*	*NA*	*NA*	*0.8*	*NA*	*NA*	*0.8*
204	■	■	■	□	■	□	□	■	■	■	■	■	■	■	□	■	*Bx*	*1*	*1.37*	*0*	*NA*	*0.2*	*NA*	*NA*	*NA*	*NA*	*NA*
235	■	■	■	□	■	■	□	■	■	■	■	□	■	□	■	□	*Bx*	*1*	*1.37*	*0*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
72	■	■	■	□	■	□	□	■	■	■	□	□	■	□	□	□	*Bx*	*1*	*1.37*	*1.3*	*NA*	*NA*	*NA*	*1.4*	*NA*	*NA*	*0.3*
28	■	■	■	■	■	■	■	■	■	□	■	■	□	■	■	■	*Bx*	*1*	*1.37*	*1.3*	*NA*	*NA*	*1.1*	*0.1*	*NA*	*NA*	*NA*
CF	*1*	*1*	*1*	*0.7*	*1*	*0.8*	*0.6*	*0.7*	*0.7*	*0.3*	*0.4*	*0.3*	*0.3*	*0.2*	*0.2*	*0.3*											

Figure 1: *KIR* genotypes in 73 NETs and other populations. Twenty *KIR* genotypes were identified with genotype ID assigned based on The Allele Frequency Net Database (AFND). Filled and opened boxes represent presence and absence of *KIR* genes, respectively. Hap. refers to KIR haplotype group. Carrier frequency (CF) and genotype frequencies (%) are given.

1.2 KIR copy number frequency in NETs

Copy numbers of seventeen *KIR* genes, including *KIR3DL3, 2DS2, 2DL2, 2DL3, 2DP1,2DL1, 3DP1,2DL4, 3DL1, 3DS1, 2DS3, 2DS5,2DL5, 2DS1, 2DS4F, 2DS4D,* and *3DL2*, were determined in 73 NETs as shown in Table 1. Copy number data for most samples matched standard centromeric/telomeric haplotype configurations but copy number characteristics of expanded and contracted haplotypes, i.e. unusual structural variation, was also observed. This data is consistent with an earlier study that reported expanded haplotypes in Thais. For example, the central framework genes, *KIR3DP1* and *2DL4*, which typically present with 2 copies, were found at 1, 3 or 4 copies in 1.4-5.5% of NETs. Duplication of other genes was also identified with three copies of *2DP1, 2DL1, 3DL1or 3DS1* observed in approximately 1% of NETs (Figure 2).

Table 1. *KIR* copy number frequencies in 73 NETs

***KIR* copy**	**Framework and Pseudogenes**					**A haplotype member**					**B haplotype-specific**						
number	***3DL3***	***2DP1***	***3DP1***	***2DL4***	***3DL2***	***2DL1***	***2DL3***	***3DL1***	***2DS4WT***	***2DS4DEL***	***2DS2***	***2DL2***	***3DS1***	***2DL5***	***2DS3***	***2DS5***	***2DS1***
0	0.0	6.8	0.0	0.0	0.0	5.5	12.3	9.6	45.2	45.2	42.5	42.5	45.2	31.5	61.6	58.9	45.2
1	0.0	35.6	2.7	2.7	0.0	37.0	46.6	46.6	45.2	38.4	45.2	45.2	41.1	50.7	37.0	37.0	43.8
2	100.0	56.2	90.4	90.4	100.0	56.2	41.1	42.5	9.6	16.4	12.3	12.3	12.3	17.8	1.4	4.1	11.0
3	0.0	1.4	5.5	5.5	0.0	1.4	0.0	1.4	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0
4	0.0	0.0	1.4	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

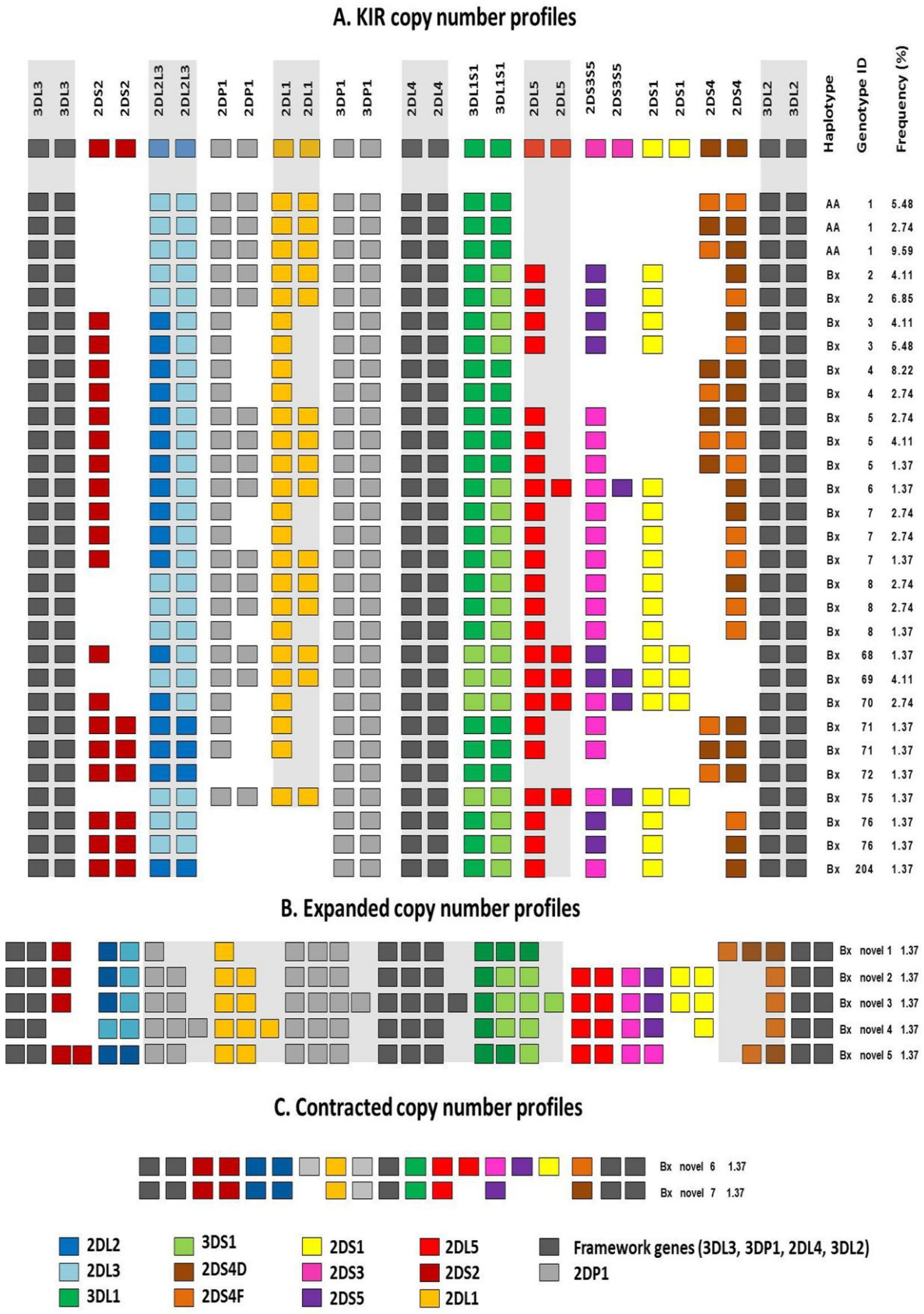


Figure 2: *KIR* copy number profiles in NETs. Seventy-three individuals were characterized for *KIR* copy number profiles. Thirty-six copy number profiles were found; A. Usual KIR copy number, B. Expanded copy number profiles, C. Contracted copy number profiles

1.3 *KIR* copy number profiles in NETs

To provide additional resolution beyond conventional methods, *KIR* copy number profiles were constructed from the copy number data. Thirty-six copy number profiles were identified (Figure 2). A variation at *2DS4* (*2DS4F/2DS4D*) subdivided into nine genotypes (Genotypes 1, 2, 3, 4, 5, 7, 8, 71, and 76) and was largely responsible for the expansion of genotypes from the twenty, identified by PCR-SSP, to thirty-six *KIR* copy number profiles. Genotypes 7 and 8 were further subdivided by copy number of *2DL1* and *2DP1*, varying between one or two copies for each gene. Unusual *KIR* genotypes displaying expanded and contracted copy number profiles were identified, and are listed as novel genotypes 1-7 (Figure 2). Novel genotypes 1-5 included expanded copy number profiles comprising 3-4 copies of *KIR3DP1*, *2DL4* and *3DL1/S1*. Individuals carrying four copies of these genes likely carry two expanded haplotypes. Copy number variation of novel genotypes 1- 3 included three copies of *2DS1/4*. Novel genotype 4 included three copies of *2DP1* and *2DL1*. Novel genotypes 6 and 7 comprised contracted copy number which were defined by only one copy of *KIR3DP1* and *2DL4* along with one copy of *2DS1/S4* for novel genotype 7, which also misses a copy of *2DP1* (Figure 2). Copy number data was concordant between exons 4 and 9 of *3DL1* and *3DL2*. The contracted haplotype carrying *3DL1/2v* was, therefore, not seen in NETs. Together expanded or contracted copy number profiles, representing non-standard structural variations, included for 9.6% of all *KIR* genotypes (6.9% expanded and 2.7% contracted). Linkage Disequilibrium between *KIR* genes was constructed using Cramer's V statistics (Wn*) to investigate the non-random association between KIR genes, as shown in Figure3.

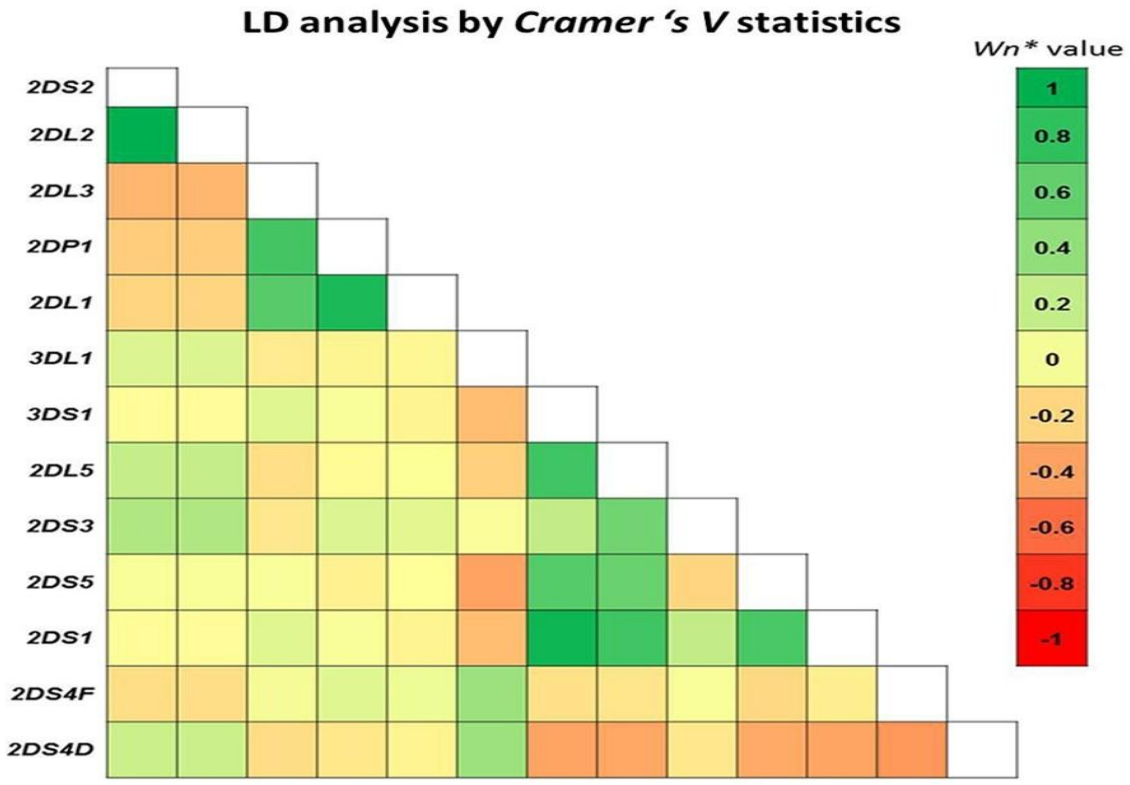


Figure 3: Linkage Disequilibrium (LD) distribution in NETs.

The strongest positive LD **(***Wn**=1**)** is shown in dark green and the strongest negative LD, **(***Wn**= -1**)** is shown in dark red with different shades in between as displayed in the key**.** LD of 13 *KIR* genes was constructed using Cramer's V statistics (*Wn**) to investigate the non-random association between *KIR* genes. As in previous studies, strong positive LD was found within both centromeric (e.g. between genes *2DS2* and *2DL2*) and telomeric regions, for example, between *3DS1* and *2DS1*. *KIR2DS3/5* can be present either in centromeric or telomeric regions. In African populations, *2DS5* is found on the centromeric side, whereas outside Africa *2DS3* is more frequently associated with the centromeric region and *2DS5* with the telomeric end. In the NET population, *2DS5* showed strong LD with *2DS1* and *3DS1* and negligible LD with centromeric genes, consistent with telomeric location, whereas *2DS3* showed weak LD signals with both centromeric (*2DS2* and *2DL2*) and telomeric genes (*2DS1* and *3DS1*), suggesting *2DS3* is present in both regions. This arrangement of *2DS3/5* within NET haplotypes is typical of populations outside of Africa.

1.4 *KIR* haplotype prediction in NETs

Haplotypes in the NET samples were imputed using the KIR Haplotype Identifier program (Methods). Eighty six percent of NET haplotypes were predicted to be common haplotypes that comprised pairing of standard centromeric (CA01, CB01, CB02) and telomeric (TA01, TB01) motifs. The remaining 14% of haplotypes were classified as having non-standard structural variations that do not fit the standard motif structures (Figure 4), accounting for the expanded and contracted profiles described above.

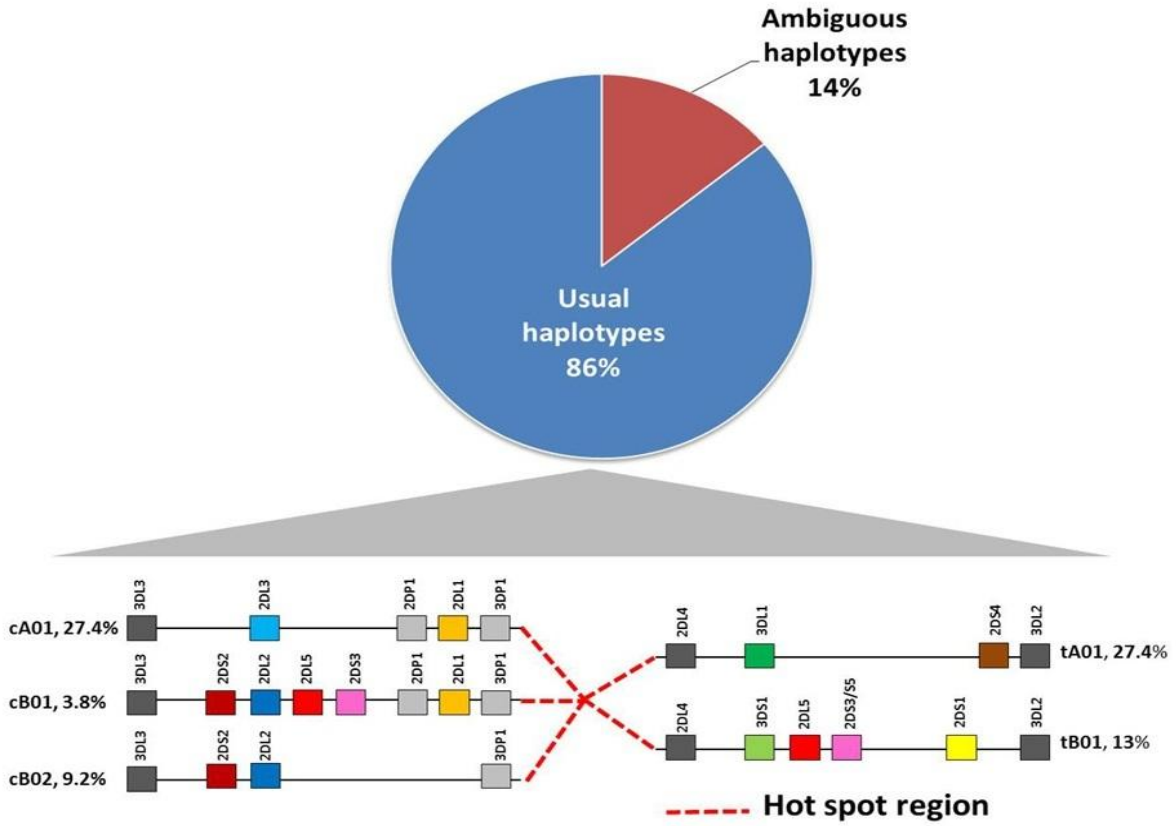


Figure 4: Prediction of KIR haplotype in NETs. KIR haplotypes were predicted by the KIR Haplotype Identifier program. The majority of haplotypes *(*86*%)* were generated by pairings between three centromeric motifs *(*cA01, cB01, cB02*)* and two telomeric motifts *(*tA01, tB01*).* The remaining *(*14*%)* were classified as carrying at least one haplotype with non-typical structure*.*

2. Association of KIR copy number variations with dengue infections

One hundred and thirty nine dengue infections were identified *KIR* copy number to compare with control group as showed in table 2. The negative association was significantly found in case – control study for 2 copies of *KIR2DL2* and *2DS2*. Two copies of *KIR2DL2* associated with DEN, DF, and DHF with (95% CI) and p-value of 0.18 (0.0553 - 0.5571) and 0.001, 0.1 (0.0122 - 0.7505) and 0.007, and 0.25 (0.0675 - 0.9018) and 0.024, respectively. Two copies of *KIR2DS2* associated with both in DEN and DHF with (95% CI) and p-value of 0.11 (0.0231 - 0.5061) and 0.001, and 0.2 (0.0429 - 0.9574) and 0.028, respectively. In contrast, *KIR2DL1* and *2DL3* were significantly positive associated in case - control study. Two copies of *KIR2DL3* associated with DEN, DF, and DHF with (95% CI) and p-value of 2.49 (1.4418 - 4.3084) and 0.001, 1.93 (1 - 3.7093) and 0.049, and 3.07 (1.6105 - 5.8501) and 0.001, respectively. Two copies of *KIR2DL1* associated with DEN and DHF with (95% CI) and p-value of 1.93 (1.1004 - 3.3824) and 0.021 and 2.2 (1.1249 - 4.3027) and 0.021, respectively. However, the association of *KIR* copy number and severity of dengue infections were not found in this study.

Table 2 The copy number variation of *KIR* in dengue infections (Cont.)

Dengue infection	Copy number	Frequencies of *KIR* copy number									
		3DS1	***2DL5***	***2DS3***	***2DS5***	***2DS1***	***2DS4***	***2DS4F***	***2DS4D***	***3DL2E4***	***3DL2E9***
DEN (N =139)	**0 copy**	58.99	52.17	77.70	66.91	58.39	4.32	36.96	33.09	0.00	0.00
	1 copy	34.53	35.51	15.83	32.37	37.96	38.13	52.17	54.41	1.46	0.72
	2 copies	5.04	10.14	5.76	0.72	3.65	56.83	10.87	12.50	98.54	99.28
	3 copies	1.44	2.17	0.72	0.00	0.00	0.72	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DF (N= 63)	**0 copy**	60.32	49.21	74.60	68.25	58.06	4.76	38.10	32.79	0.00	0.00
	1 copy	33.33	38.10	17.46	31.75	37.10	38.10	52.38	54.10	1.61	0.00
	2 copies	6.35	11.11	7.94	0.00	4.84	57.14	9.52	13.11	98.39	100.00
	3 copies	0.00	1.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DHF (N=75)	**0 copy**	57.33	54.05	80.00	65.33	58.11	4.00	36.49	33.78	0.00	0.00
	1 copy	36.00	33.78	14.67	33.33	39.19	38.67	51.35	54.05	1.35	1.33
	2 copies	4.00	9.46	4.00	1.33	2.70	56.00	12.16	12.16	98.65	98.67
	3 copies	2.67	2.70	1.33	0.00	0.00	1.33	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2 The copy number variation of *KIR* in dengue infections (Cont.)

Dengue infection	Copy number	Frequencies of *KIR* copy number									
		3DS1	***2DL5***	***2DS3***	***2DS5***	***2DS1***	***2DS4***	***2DS4F***	***2DS4D***	***3DL2E4***	***3DL2E9***
DEN (N =139)	**0 copy**	58.99	52.17	77.70	66.91	58.39	4.32	36.96	33.09	0.00	0.00
	1 copy	34.53	35.51	15.83	32.37	37.96	38.13	52.17	54.41	1.46	0.72
	2 copies	5.04	10.14	5.76	0.72	3.65	56.83	10.87	12.50	98.54	99.28
	3 copies	1.44	2.17	0.72	0.00	0.00	0.72	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DF (N= 63)	**0 copy**	60.32	49.21	74.60	68.25	58.06	4.76	38.10	32.79	0.00	0.00
	1 copy	33.33	38.10	17.46	31.75	37.10	38.10	52.38	54.10	1.61	0.00
	2 copies	6.35	11.11	7.94	0.00	4.84	57.14	9.52	13.11	98.39	100.00
	3 copies	0.00	1.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DHF (N=75)	**0 copy**	57.33	54.05	80.00	65.33	58.11	4.00	36.49	33.78	0.00	0.00
	1 copy	36.00	33.78	14.67	33.33	39.19	38.67	51.35	54.05	1.35	1.33
	2 copies	4.00	9.46	4.00	1.33	2.70	56.00	12.16	12.16	98.65	98.67
	3 copies	2.67	2.70	1.33	0.00	0.00	1.33	0.00	0.00	0.00	0.00
	4 copies	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

3. Conclusion and Discussion

The impact of *KIR* variations on human immune responses is being interested worldwide. Presently, genetic variations of *KIR* have been reported in many populations as well as diseases association. This study, *KIR* copy number was investigated using a quantitative PCR in northeastern Thais (NETs) and dengue patients in order to understand complexity of *KIR* and influence of *KIR* copy number on dengue infections. For *KIR* copy number in NETs, the result showed that framework genes usually presented 2 copies. *KIR* belonging A haplotype presented 1-2 copies, whereas *KIR* member of B haplotype presented 0-1 copy. *KIR* copy number profiles were constructed in NETs which extended and contracted profiles were defined (9.6%) as well as unusual KIR haplotypes (14%). In addition, the association of *KIR* copy number and dengue infection were investigated, showing that only four *KIR* loci (*KIR2DL2*, *2DS2*, *2DL1* and *2DL3*) associated with dengue infections. Two copies of *KIR2DL2* and *2DS2* were significantly protective to dengue infection, whereas two copies of *KIR2DL1* and *2DL3* were susceptible to dengue infection. Later, to study *KIR* copy number variation and severity of dengue infection, the dengue fever (DF) were compared with dengue hemorrhagic fever (DHF). Unfortunately, the relationship between *KIR* copy number and severity of dengue infections (DF *vs* DHF) were not found. However, further investigation needs to be performed to overcome limitations of this study. Moreover, data analysis with sequential infection, infection serotype as well as increasing sample size could help for further study.

A quantitative PCR can help to understand genetic organization of *KIR* in NETs which is involved in immune responses and epidemiology. Moreover, this study could provide useful information for identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation (HSCT) to improve clinical outcome, in KIR-disease association studies and imputation analyses of NETs.

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Novel *KIR* genotypes and gene copy number variations in northeastern Thais

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Summary

KIR (Killer Immunoglobulin-like Receptor) variants influence immune responses and are genetic factors in disease susceptibility. Using sequence-specific priming PCR, we have previously described the diversity of *KIR* genes in term of presence/absence in northeastern Thais (NETs). To provide additional resolution beyond conventional methods, quantitative PCR was applied to determine *KIR* copy number profiles. Novel expanded and contracted *KIR* copy number profiles were identified at cumulatively high frequencies. These all comprise haplotypes with duplication (6·9%) or deletion (2·7%) of *KIR3DL1/S1* along with adjacent genes. Five expanded *KIR* profiles comprised haplotypes with duplications of *KIR2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1/S1* and *2DS1/4*, whereas two contracted profiles contained only a single copy of *KIR3DP1*, *3DL1/S1* and *2DL4*. Using a KIR haplotype prediction program (KIR Haplotype Identifier), 14% of NET haplotypes carried atypical haplotypes based on the gene copy number data.

Keywords: haplotypes; *KIR* copy number; quantitative PCR.

Introduction

Killer Immunoglobulin-like Receptors (KIRs) are expressed on natural killer (NK) cells and subsets of T cells. KIRs play an important role in mediating self-tolerance and facilitating the cytotoxic activity of NK cells against pathogenic cells. Interactions of KIRs and HLA class I molecules on target cells can modulate the function of NK cells. The *KIR* gene family is located within the leucocyte receptor cluster on chromosome 19q13.4.[1,2] The *KIR* nomenclature reflects the number of extracellular immunoglobulin-like domains: two domains (*KIR2D*), three domains (*KIR3D*), followed by short (S) or long (L) cytoplasmic tail. Seventeen *KIR*s have been characterized, of which 15 are functional as activating or inhibitory *KIRs* (*KIR2DL1–4*, *2DL5A*, *2DL5B*, *3DL1–3*, *2DS1–5* and *3DS1*) and two pseudogenes (*KIR2DP1*, *3DP1*). Extensive polymorphism of *KIR* has been reported worldwide,[3] including allelic polymorphisms, gene content (presence/absence polymorphism) and copy number variations. *KIR* haplotypes can be classified into groups A and B. The number of genes in group A haplotypes is fixed by the presence of four framework genes (*KIR3DL3*, *3DP1*, *2DL4* and *3DL2*), pseudogene (*KIR2DP1*) and inhibitory (*KIR2DL3*, *2DL1*, *3DL1*), as well as one activating gene, *KIR2DS4*. Conversely, group B haplotypes consist of variable numbers of *KIR* genes and one or more activating *KIR* genes. However, A and B haplotypes with distinctive

gene content may be generated by shuffling of centromeric and telomeric motifs. The centromeric motif is defined by all *KIR* located between *3DL3* and *3DP1*, and the telomeric motif by *KIR* located between *2DL4* and *3DL2*. A recombination hotspot sited between *KIR3DP1* and *KIR2DL4* delineates centromeric and telomeric haplotype motifs of the *KIR* gene cluster.[4] Most haplotypes comprise pairings between three common centromeric (cA01, cB01, cB02) and two telomeric (tA01, tB01) motifs.[5] In addition, the expression of *KIRs* may be subject to epigenetic regulation through the promoter region[6,7] as well as pre- and post-transcriptional regulation,[8] resulting in variegated expression of KIRs on NK cells.[9] Genetic variations of *KIR* are clearly involved in disease susceptibility and resistance, including infectious diseases,[10,11] autoimmune diseases,[12] pregnancy disorders[13,14] and cancers,[15,16] and they influence haematopoietic stem cell transplantations.[17,18]

To understand the influence of *KIR* in immune responses, the presence and absence of individual *KIR* genes has been investigated in normal populations[19] as well as disease cohorts.[20] Different *KIR* distributions in different geographical regions may have been selected through NK cell control of local diseases.[21] *KIR* genes are highly homologous and are closely arranged head-to-tail, facilitating unequal crossing over during meiosis and generation of novel *KIR* haplotypes, including gene duplications and hybrid genes.[22,23] Several studies have linked genetic associations of diseases to *KIR* copy number.[24–27]

Recently, genetic variation of *KIR* and their *HLA* ligands in northeastern Thais (NETs) was reported,[28–31] by conventional methods [sequence-specific priming–PCR (PCR-SSP) typing]. However, gene copy number variations of *KIR* have not previously been reported in Thais. Hence, to better understand the *KIR* diversity in NETs, *KIR* copy number was ascertained by quantitative PCR (qPCR).

Materials and methods

Study population

Seventy-three healthy blood donors were obtained from the Blood Transfusion Centre, Faculty of Medicine, Khon Kaen University, Thailand. Interviews were conducted for volunteers; they were confirmed to be of Thai ancestry living in the northeastern part of Thailand for at least two generations.[32,33] Ethical permission was obtained for the investigation from the Human Research Ethics Committee of Thammasart University No. 1 (COA 057/59).

KIR *genotyping and copy number identification*

The salting-out method[34] was used to extract DNA from peripheral blood. To prepare genomic DNA for *KIR* copy number identification, 10-ng samples of genomic DNA (2·5 μl of 4 ng/μl) were aliquoted into 384-well plates using the Hydra 96 microdispenser (Art Robbins, San Jose, CA). *KIR* copy number typing was performed using a quantitative multiplex PCR assay described by Jiang *et al.*[35]

The typing results of 17 *KIR* genes obtained using PCR-SSP were previously reported[28] and compared with those obtained with the qPCR method.[35] Copy numbers for all *KIR* genes (*KIR2DL1–5*, *2DS1–5*, *2DP1*, *3DP1*, *3DL1*, *3DL2*, *3DL3* and *3DS1)* were determined in the Thai samples using a Roche LightCycler 480 at Cambridge Institute for Medical Research (CIMR), University of Cambridge, UK. Using multiplex assays, the copy number was measured by relative quantification analysis of the target gene (*KIR*) and reference gene (signal transducer and activator of transcription 6; *STAT6*) using the comparative Cq method.[36] Cq value is the qPCR cycle at which fluorescence from amplification exceeds the background fluorescence (also referred to as threshold cycle, Ct). The ΔΔCq was used to calculate KIR copy number. The first ΔCq was calculated by the cycle threshold difference between the target and reference assay of the same sample. The second ΔCq was calculated by the difference of ΔCq values from a test sample and a calibrator sample with known copy number of the target. Two assays for both *3DL1* and *3DL2* genes that target different exons were included. These assays were designed to help detect a known fusion gene of *3DL1*, *3DL1/2v*,[37] which is carried on a truncated haplotype (with *2DS4* completely deleted) seen in individuals of African descent. There is a drop in copy number for exon 9 of *3DL1* and exon 4 of *3DL2* (i.e. discordance between the exon 4 and exon 9 copy numbers in the same gene) when the fusion gene is present. Assays for *2DS4* variants, *2DS4D* (a 22-bp deletion in exon 5 that causes a frameshift mutation) and *2DS4F* (full-length gene), were also included.

CopyCaller software from Applied Biosystems (Foster City, CA) was used to score *KIR* copy number. When the Cq of the reference gene was greater than 32 or where a data point was more than 4 SD from the mean ΔCq of four replicates, the reaction was not analysed. KIR copy number frequencies were calculated from the 73 NET samples.

KIR *genotypes and predicted haplotypes*

The *KIR* genotypes (IDs) were ascertained according to the Allele Frequency Net Database (http://www.allelefrequencies.net)[3]. The *KIR* haplotypes (centromeric–telomeric motif pairings) in NETs were predicted by the KIR Haplotype Identifier program (www.bioinformatics.cimr.cam.ac.uk/haplotypes/) using the determined copy number for each *KIR* gene.[35] *KIR* haplotypes were

predicted in samples (n = 73) that had copy number typing for all *KIR* genes.

Results

KIR genotypes in NETs

KIR genotypes were identified based on quantitative PCR data. Twenty genotypes were characterized in the NET samples (n = 73) that had copy number typing for all *KIR* genes as shown in Fig. 1. Genotypes 1, 2, 3, 4, 5, 7 and 8 were commonly found (> 5% frequency) as seen in previous studies[28,38] of the Thai population. To compare *KIR* genotype with other populations, the genotype frequencies of Asians (Chinese Han, Mongolian and north Indian), Europeans (Italian and English) and Amerindian (north Brazilian) were included,[28,38–44] showing that genotype IDs 1 and 2 were the most common genotypes (> 10% frequency) in Amerindian, Europeans and most Asians (NETs, Thais, Chinese Han and Mongolian), whereas other genotypes varied among populations. Individually, the frequencies of all *KIR* genotypes in north Indians were < 10%, with the most common genotype found at 9·3%. Eighty per cent of the genotypes, based on qPCR, were in accordance with those obtained using the PCR-SSP typing method. However, inconsistent data between PCR-SSP typing and quantitative PCR were found in *KIR2DS1*. Originally, we thought that the discordances could probably be explained by allelic polymorphisms of *KIR2DS1* that affected PCR-SSP primer binding sites. Subsequently, we have performed DNA sequencing and DNA sequence analysis of the samples (n = 4). The discordances of the samples were typing errors of the PCR-SSP method because of high homology sequences between *KIR2DL1* and *KIR2DS1* as well as low quality of the DNA templates. Accordingly, there is no variant of *KIR2DS1* in this population.

Genotype ID	Framework/Pseudogenes					A haplotype member				B haplotype-specific							Present study			Asians					Europeans		Amerindian
																		n = 73		NETs n = 235	Thais n = 500	Chinese Han n = 503	Mongolian n = 90	N· Indian n = 867	Italian n = 217	English n = 584	N· Brazilian n = 377
	3DL2	3DL3	2DL4	2DP1	3DP1	2DL1	2DL3	3DL1	2DS4	2DL2	2DL5	2DS1	2DS2	2DS3	2DS5	3DS1	haplo·	*N*	%	%	%	%	%	%	%	%	%
1	■	■	■	■	■	■	■	■	■	□	□	□	□	□	□	□	AA	13	17·81	35·7	40·8	54·7	37·8	9·3	28·5	27·4	31
4	■	■	■	■	■	■	■	■	■	■	□	□	■	□	□	□	Bx	9	12·33	9·8	6·8	5·2	5·5	2·9	8·3	13·5	7·4
2	■	■	■	■	■	■	■	■	■	□	■	■	□	□	■	■	Bx	8	10·96	10·2	11·2	12·3	13·3	2·9	12·9	14	15·4
3	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	Bx	7	9·59	2·6	4·4	3·2	4·4	1·7	9·2	6·7	6
5	■	■	■	■	■	■	■	■	■	■	□	■	■	■	□	□	Bx	6	8·22	1·3	6	3·2	NA	5·2	NA	7·4	5·6
8	■	■	■	■	■	■	■	■	■	□	■	■	□	■	■	■	Bx	5	6·85	8·1	6	7	3·3	0·9	NA	1·7	1·6
7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Bx	5	6·85	6	3·6	1·4	1·1	0·6	3·6	4·1	2·6
69	■	■	■	■	■	■	■	□	□	□	■	■	□	□	■	■	Bx	3	4·11	1·7	NA	0·6	NA	NA	NA	1·2	1·1
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Bx	3	4·11	3·4	4	1·6	NA	2·8	4·6	4·5	2·4
76	■	■	□	■	■	□	□	■	■	■	■	■	■	□	■	■	Bx	2	2·74	1·7	NA	0·4	NA	NA	NA	NA	0·8
71	■	■	■	■	■	■	□	■	■	■	■	□	■	■	□	□	Bx	2	2·74	0	NA	0·4	NA	0·8	NA	1·5	2·4
70	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	Bx	2	2·74	0·9	2·6	0·6	NA	2	NA	1·7	0·8
91	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	□	Bx	1	1·37	0·4	NA	NA	NA	NA	NA	0·2	1·1
75	■	■	■	■	■	■	■	□	□	□	■	■	□	■	■	■	Bx	1	1·37	0·9	NA	1·2	NA	0·5	NA	0·3	0·3
68	■	■	■	■	■	■	■	□	□	■	■	■	■	□	■	■	Bx	1	1·37	0·4	NA	0·6	1·1	3·6	NA	NA	1·8
94	■	■	■	■	■	■	□	■	■	■	■	□	■	■	□	■	Bx	1	1·37	0	NA	NA	NA	0·8	NA	NA	0·8
204	■	■	■	□	■	□	□	■	■	■	■	■	■	■	□	■	Bx	1	1·37	0	NA	0·2	NA	NA	NA	NA	NA
235	■	■	■	□	■	■	□	■	■	■	■	□	■	□	■	■	Bx	1	1·37	0	NA	NA	NA	NA	NA	NA	NA
72	■	■	■	□	■	□	□	■	■	■	□	□	■	□	□	□	Bx	1	1·37	1·3	NA	NA	NA	1·4	NA	NA	0·3
28	■	■	■	■	■	■	■	■	■	□	■	■	□	■	■	■	Bx	1	1·37	1·3	NA	NA	1·1	0·1	NA	NA	NA
CF	1	1	1	0·7	1	0·8	0·6	0·7	0·7	0·3	0·4	0·3	0·3	0·2	0·2	0·3											

Figure 1. Killer immunoglobulin-like receptor gene (*KIR*) genotypes in 73 northeastern Thais (NETs) and other populations. Twenty *KIR* genotypes were identified with genotype ID assigned based on The Allele Frequency Net Database. Filled and opened boxes represent presence and absence of *KIR* genes, respectively. Hap. refers to KIR haplotype group.[3] Carrier frequency (CF) and genotype frequencies (%) are given.

Table 1. Killer immunoglobulin-like receptor gene (*KIR*) copy number frequencies in 73 northeastern Thais

KIR copy number	Framework and pseudogenes					A haplotype member					B haplotype-specific						
	3DL3	*2DP1*	*3DP1*	*2DL4*	*3DL2*	*2DL1*	*2DL3*	*3DL1*	*2DS4WT*	*2DS4DEL*	*2DS2*	*2DL2*	*3DS1*	*2DL5*	*2DS3*	*2DS5*	*2DS1*
0	0·0	6·8	0·0	0·0	0·0	5·5	12·3	9·6	45·2	45·2	42·5	42·5	45·2	31·5	61·6	58·9	45·2
1	0·0	35·6	2·7	2·7	0·0	37·0	46·6	46·6	45·2	38·4	45·2	45·2	41·1	50·7	37·0	37·0	43·8
2	100·0	56·2	90·4	90·4	100·0	56·2	41·1	42·5	9·6	16·4	12·3	12·3	12·3	17·8	1·4	4·1	11·0
3	0·0	1·4	5·5	5·5	0·0	1·4	0·0	1·4	0·0	0·0	0·0	0·0	1·4	0·0	0·0	0·0	0·0
4	0·0	0·0	1·4	1·4	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0

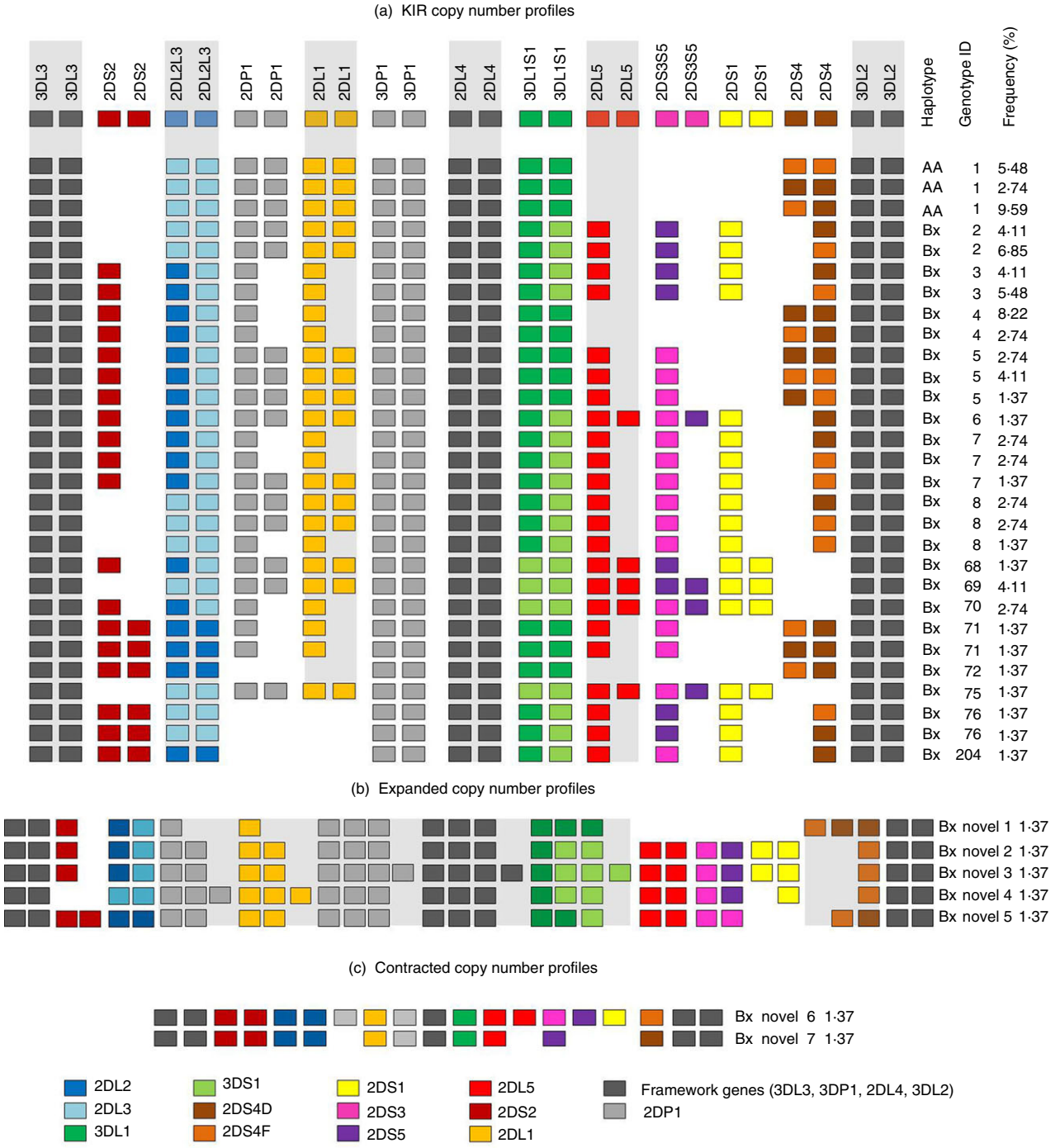

Figure 2. Killer immunoglobulin-like receptor gene (*KIR*) copy number profiles in northeastern Thais (NETs). Seventy-three individuals were characterized for *KIR* copy number profiles. Thirty-six copy number profiles were found; (a) usual KIR copy number, (b) expanded copy number profiles,[26,40] (c) contracted copy number profiles.[26,40] [Colour figure can be viewed at wileyonlinelibrary.com]

KIR copy number frequency in NETs

Copy numbers of 17 *KIR* genes, including *KIR3DL3*, *2DS2*, *2DL2*, *2DL3*, *2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1*, *3DS1*, *2DS3*, *2DS5*, *2DL5*, *2DS1*, *2DS4F*, *2DS4D* and *3DL2*, were determined in 73 NETs as shown in Table 1. Copy number data for most samples matched standard centromeric/telomeric haplotype configurations but copy number characteristics of expanded and contracted haplotypes, i.e. unusual structural variation, was also observed. These data are consistent with an earlier study that reported expanded haplotypes in Thais.[37] For example, the central framework genes,

KIR3DP1 and *2DL4*, that typically present with two copies, were found at one, three or four copies in 1·4–5·5% of NETs. Duplication of other genes was also identified with three copies of *2DP1*, *2DL1*, *3DL1* or *3DS1* observed in approximately 1% of NETs (Fig. 2).

KIR copy number profiles in NETs

To provide additional resolution beyond conventional methods, *KIR* copy number profiles were constructed from the copy number data. Thirty-six copy number profiles were identified (Fig. 2). A variation at *2DS4* (*2DS4F/2DS4D*) subdivided into nine genotypes (Genotypes 1, 2, 3, 4, 5, 7, 8, 71 and 76) and was largely responsible for the expansion of genotypes from the 20, identified by PCR-SSP, to 36 *KIR* copy number profiles. Genotypes 7 and 8 were further subdivided by copy number of *2DL1* and *2DP1*, varying between one or two copies for each gene. Unusual *KIR* genotypes displaying expanded and contracted copy number profiles were identified, and are listed as novel genotypes 1–7 (Fig. 2). Novel genotypes 1–5 included expanded copy number profiles comprising three or four copies of *KIR3DP1*, *2DL4* and *3DL1/S1*. Individuals carrying four copies of these genes probably carry two expanded haplotypes. Copy number variation of novel genotypes 1–3 included three copies of *2DS1/4*. Novel genotype 4 included three copies of *2DP1* and *2DL1*. Novel genotypes 6 and 7 comprised contracted copy numbers, which were defined by only one copy of *KIR3DP1* and *2DL4* along with one copy of *2DS1/S4* for novel genotype 7, which also misses a copy of *2DP1* (Fig. 2). Copy number data were concordant between exons 4 and 9 of *3DL1* and *3DL2*. The contracted haplotype carrying *3DL1/2v*[37] was, therefore, not seen in NETs. Together, expanded or contracted copy number profiles, representing non-standard structural variations, included 9·6% of all *KIR* genotypes (6·9% expanded and 2·7% contracted). Linkage disequilibrium between *KIR* genes

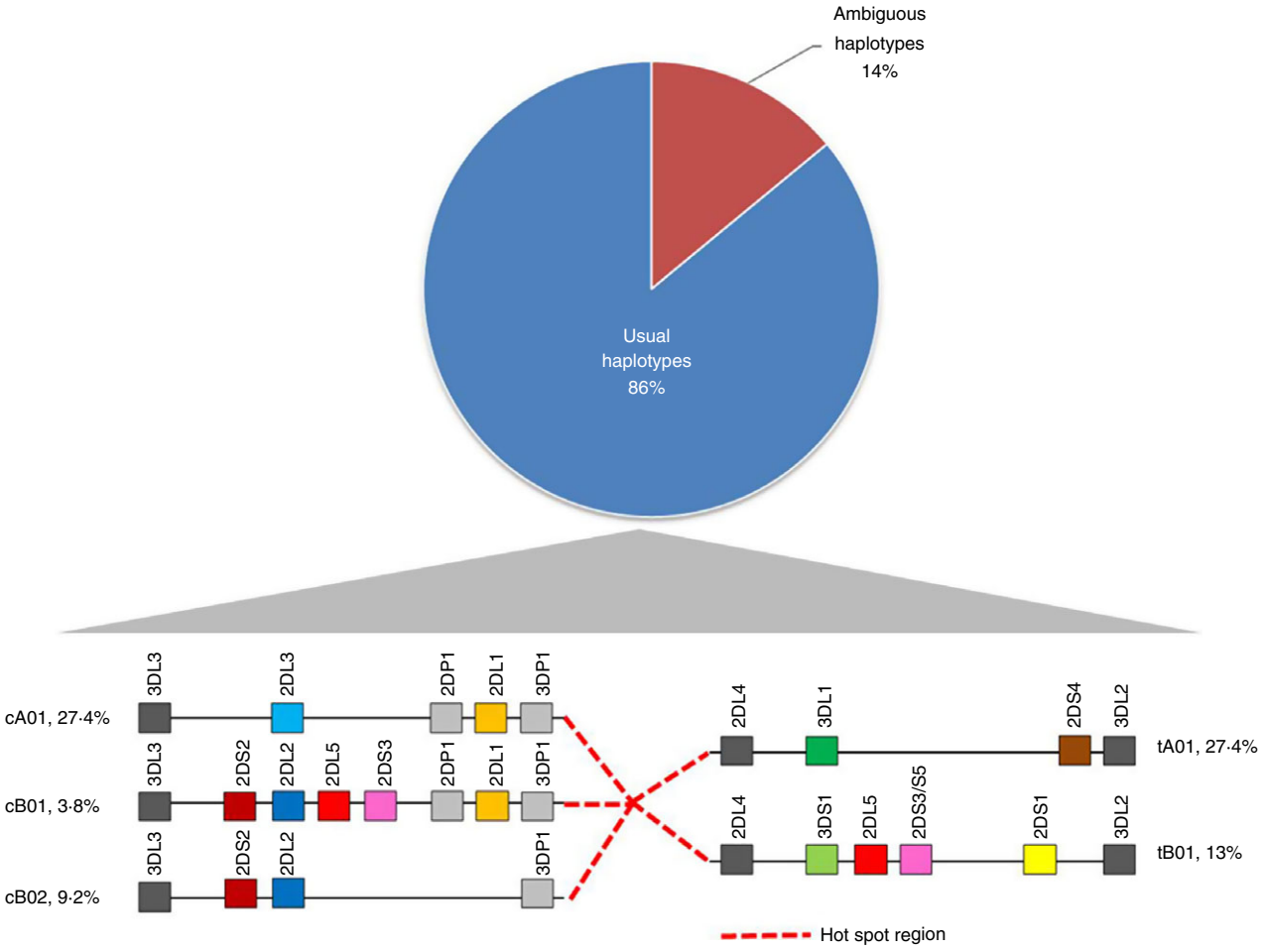

Figure 3. Prediction of killer immunoglobulin-like receptor (KIR) haplotype in northeastern Thais (NETs). KIR haplotypes were predicted by the KIR Haplotype Identifier program. The most haplotypes (86%) were generated by pairings between three centromeric motifs (cA01, cB01, cB02) and two telomeric motifts (tA01, tB01). The remaining (14%) were classified as carrying at least one haplotype with non-typical structure. [Colour figure can be viewed at wileyonlinelibrary.com]

was constructed using Cramer's V statistics (Wn*) to investigate the non-random association between KIR genes, as shown in the Supplementary material (Fig S1).

KIR haplotype prediction in NETs

Haplotypes in the NET samples were imputed using the KIR Haplotype Identifier program (see Materials and methods). Eighty-six per cent of NET haplotypes were predicted to be common haplotypes that comprised pairing of standard centromeric (CA01, CB01, CB02) and telomeric (TA01, TB01) motifs. The remaining 14% of haplotypes were classified as having non-standard structural variations that do not fit the standard motif structures (Fig. 3), accounting for the expanded and contracted profiles described above.

Discussion

Recently, genetic variations of *KIR* and their ligands in NETs were reported by presence and absence of 17 *KIRs*.[28] To better understand the complexity of *KIR*, qPCR was used to investigate copy number of *KIR* genes in NETs. To compare with conventional methods, the *KIR* genotype, copy number and haplotype were analysed. Variations of *KIR* copy number ranged from zero to four copies. Individuals who were previously classified as having the same genotype by PCR-SSP typing, were subdivided by *KIR* copy number variations (Fig. 2). Twenty *KIR* genotypes (Fig. 1) and 36 copy number profiles (Fig. 2) were identified. Most haplotypes could be considered to have arisen from three common centromeric (cA01, cB01, cB02) and two common telomeric (tA01, tB01) motifs. However, seven expanded and contracted *KIR* profiles were identified in the NET population (Fig. 2). Remarkably, five expanded *KIR* profiles (novel genotypes 1–5) comprised haplotypes with duplications of centromeric and telomeric genes including *2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1/S1* and *2DS1/S4*. Two contracted profiles (novel genotypes 6 and 7) contained one copy of *KIR3DP1*, *2DL4* and *3DL1/S1*. All the unusual copy number profiles comprised haplotypes with deletion/duplication of *3DL1/S1* and *2DL4* located in the central region of the *KIR* cluster.[37,45] Similar haplotypes have been described in Caucasians. Our data indicate that these haplotypes could be more frequent in the NET population at 9·6%, but this requires further verification in a larger set of samples. The frequencies of these haplotypes could relate to population history or a region-specific selective advantage for particular expanded/deleted haplotypes, for example. In this regard, *KIR2DS1/S4* contributes to successful pregnancy in humans[46] and *KIR3DL1/S1* copy number is linked to retroviral control.[24] Furthermore, this study could provide useful information for identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation (HSCT) to improve clinical outcome, in *KIR*–disease association studies and imputation analyses of NETs.[47]

A study of *KIR* copy number in families from the UK and USA, indicated that B haplotypes were subject to gene duplication or deletion and the data here are consistent with this observation.[48]

Acknowledgements

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Disclosures

The authors declare that they have no conflict of interest.

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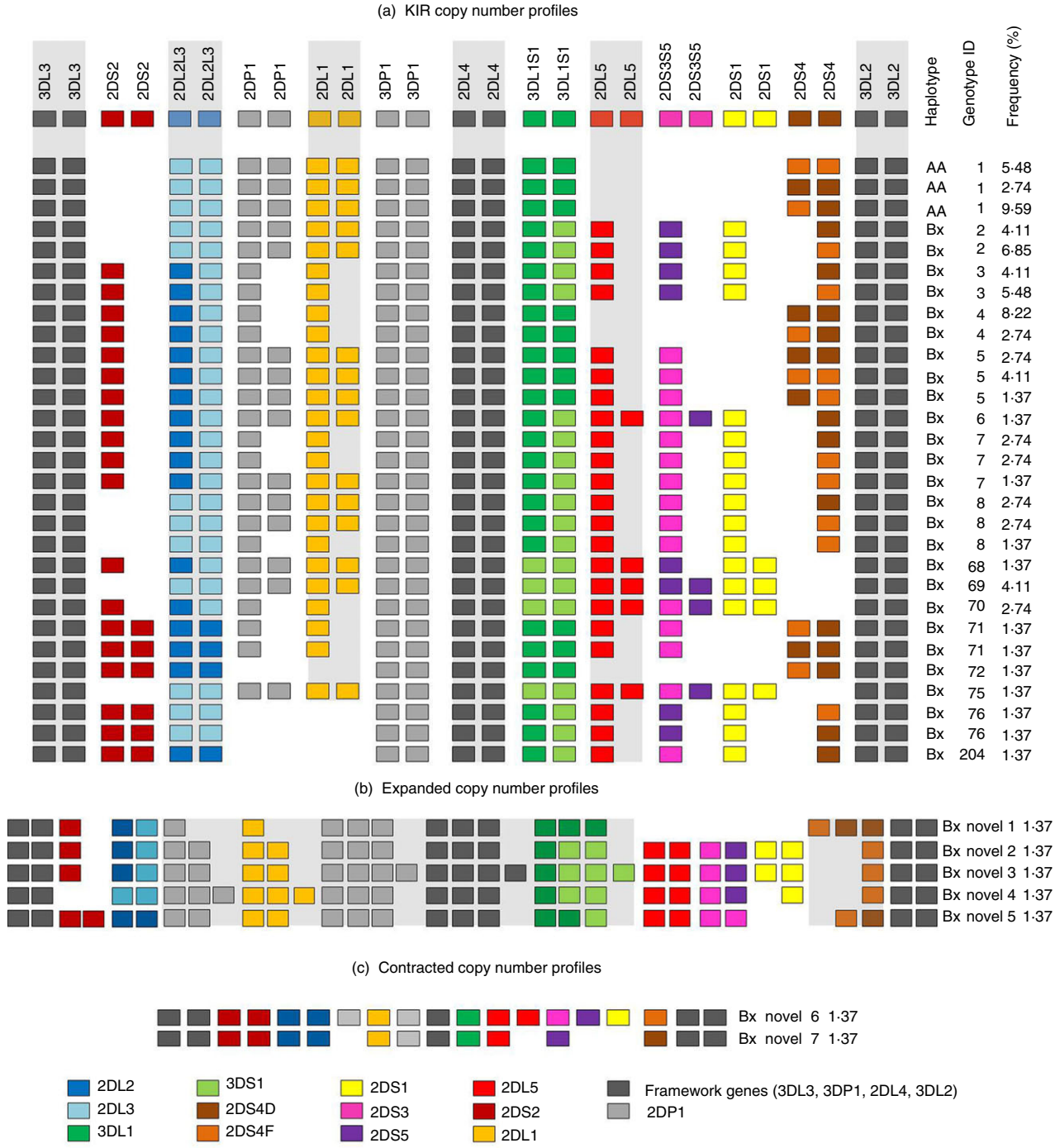
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linkage disequilibrium distribution in northeastern Thais.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.



Gene copy number variations of killer immunoglobulin-like receptors (KIRs) identified novel KIR copy number profiles in northeastern Thais. These included expanded and contracted KIR profiles.

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Short population report

Genetic study of *KIR* and *HLA* ligands in 235 individuals from Northeastern Thailand

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ABSTRACT

The diversity of *17 KIR* and *HLA* ligands (*HLA-C1*, *C2*, *Bw4*, *A11*) were investigated in two hundred and thirty-five unrelated healthy donors in Northeastern Thais (NETs) by the polymerase chain reaction with sequence-specific primer (PCR-SSP) method. The Hardy-Weinberg Equilibrium (HWE) was used to verify genotyping method for dimorphic *KIR* and *HLA*. They were in HWE ($p > 0.05$). *KIR* and *HLA* ligands frequencies, genotypes, haplotypes and linkage disequilibrium (LD) were presented. The genetic data are available in allele Frequencies Net Database.

The northeast of Thailand, known as "Isan", is the largest region of Thailand bordered by Loa to the east and north, and Cambodia to the south east. Northeastern Thailand consists of 20 provinces and contains a population of approximately 22 million, forming a third of the whole Thai population. The majority of Northeastern Thais (NETs) are Loatian ethnic that migrated to Isan between mid-fourteenth and the late-eighteenth centuries, whereas other ethnics, including Khmer, Suay, Phu Tai, and Vietnamese are the minorities [1]. NETs usually speak "Thai-Isan" as a dialect that is similar to the Loatian language, whereas Thai language is still spoken and used for education. Like the neighboring countries, most cultures of NETs are similar to Loa, and some of them have been adopted to be a part of Thai culture.

To study the genetic variations of *KIR* and *HLA* ligands in NETs, two hundred and thirty-five unrelated healthy blood donors from 20 provinces of the northeast of Thailand were collected from the Blood Transfusion Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. The blood donors were from Thai people who live in both rural and urban of the north eastern region of Thailand for at least two generations. For ethical approval, the subjects were informed and written consent forms were obtained for genetic study.

The diversity of 17 *KIR* and *HLA* ligands (*HLA-C1*, *C2*, *Bw4*, *A11*) was investigated by the polymerase chain reaction with the sequence-specific primer (PCR-SSP) method [2], with two steps amplification modified from Tajik et al. (2010) [3]. Most *KIR* and *HLA* primers were designed to amplify specific exon sequences of *KIR* and *HLA* [3–5]. Identification of *HLA* polymorphisms was performed using the HLA typing method as previous described [6,7]. To confirm ambiguous *KIR* typing, *KIR2DS4* and *KIR3DP1* subtyping were also performed by using different primer binding sites of the exon as previously described [5,8]. The *KIR* and *HLA* primers were tested on the standard genomic DNA samples to investigate specificity. These included the DNA samples from IHW 9021, IHW 9016, IHW 9024, IHW 9031, and NK92.

In this study, all individuals were typed for *KIR*, *HLA-C1*, *-C2*, *-A11* and *-Bw4*, and only 108 individuals were typed for *HLA-Bw4* subgroups (Supplementary Tables S1, S2). To verify *KIR* and *HLA* genotyping, the Hardy-Weinberg equilibrium proportion was tested in dimorphic *KIR* (*KIR3DP variant1/ KIR3DP1full*, $p = 0.1$) and *HLA* (*HLA-C1/HLA-C2*, $p = 0.93$). The *HLA-Bw4* group which is a KIR3DL1 ligand was identified as *HLA-A(Bw4)*, *HLA-Bw4(I80)*, and *HLA-Bw4(T80)*, with the frequencies of 39%, 28% and 28%, respectively. The *KIR* and *HLA* frequencies in the population were determined by direct counting and the formula: $GF = 1 - \sqrt{1-f}$, which f is the observed frequency (OF). AA and Bx genotypes in NETs have also been classified (Supplementary Table S3) [9] and the genotypes are available in allelefrequencies.net under the population name Thailand North East KIR and population number

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3373 [9]. Furthermore, to study the associations between *KIR* loci, linkage disequilibrium (LD) of the 16 *KIR* genes were also constructed using Cramer's *V* [10] and chi-square statistics (Supplementary Table S4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2017.04.002.

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