**Research paper**

**KIR copy number variations in dengue-infected patients from northeastern Thailand**

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**ABSTRACT**

Killer immunoglobulin-like receptors (KIRs) are a family of receptors expressed on Natural killer (NK) cells. The extensive polymorphism of KIR is involved in the immune responses of NK cells and influences dengue infections. We investigated the diversity of KIR copy numbers in dengue-infected patients from northeastern Thailand. Copy numbers of KIRs were determined by quantitative polymerase chain reaction in 137 dengue-infected patients, comprising 63 dengue fever (DF) and 74 dengue hemorrhagic fever (DHF). The distribution of KIRs was observed to be between 0-4 copies. The KIR AA genotype with heterozygous KIR2DS4D/WT was the most common in dengue patients, 25.4% DF and 23 % DHF. Forty KIR profiles were determined in dengue patients, including 31 usual, 6 expanded, and 3 contracted profiles. Investigation of KIR copy number and dengue severity indicated that two copies of *KIR2DL3* combined with HLA-C1C1 associated with an increased risk of DHF (OR 2.32, 95% CI 1.159 - 4.624, P=0.016), whereas one copy of *KIR2DL2* and *KIR2DL3* together with *HLA-C1C1* associated with a reduced risk of DHF (OR 0.17, 95% CI 0.058 - 0.482, P<0.001). The outcomes of this study will contribute to the understanding of KIR complexity and innate immune responses in dengue infections.

**Keywords**: *KIR* copy number; dengue infection; dengue fever; dengue hemorrhagic fever; dengue severity

1. **Introduction**

Dengue is a mosquito-borne infectious disease that is endemic in tropical and subtropical countries. It has been estimated that 400 million people worldwide were annually infected by Dengue virus (DENV) [1]. In addition, the number of dengue cases reported to the World Health Organization (WHO) from 2000 to 2019 has increased over eightfold [2]. Dengue infections can result in a spectrum of clinically distinct outcomes, ranging from asymptomatic infection to a severe life-threatening illness [3]. Symptomatic dengue illness is clinically classified to a self-limiting febrile illness or dengue fever (DF), and a severe illness, known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The pathogenesis of DHF is not thoroughly characterized but the host innate immune response has been implicated [4]. Natural killer (NK) cells are a group of innate immune cells that play a critical role against dengue infections and clinical outcomes [5-7]. The functional heterogeneity of NK cells is influenced by an expression of surface receptors on NK cells. Among NK receptors, killer immunoglobulin-like receptors (KIRs) are an essential regulator of NK cell activity which can either deliver activating or inhibitory signal to NK cells. The *KIR* gene family includes six genes encoding activating KIRs with the short cytoplasmic tails (*KIR2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, and *3DS1*), and eight inhibitory genes encoding receptors with long cytoplasmic tails (*KIR2DL1*, *2DL2*, *2DL3*, *2DL5A*, *2DL5B*, *3DL1*, *3DL2*, and *3DL3*), whereas *KIR2DL4* can either deliver an activating or inhibitory signal to NK cells [8, 9]. *KIR2DP1* and *3DP1* are pseudogenes. The diversity of *KIRs* is generated through a combination of gene contents, allelic polymorphisms, copy number variations, and gene expressions [10]. Based on gene content variations, *KIRs* are classified into A and B haplotypes [11]. KIR-A haplotype is characterized by the presence of nine genes, including *KIR2DL1, 2DL3, 3DL1, 2DS4* and framework/pseudogenes (*3DL3, 2DP1, 3DP1, 2DL4*, and *3DL2*). The *KIR2DS4* is only a single activating gene on haplotype A which encodes either a wild-type form (*KIR2DS4WT*) serving as a membrane-bound receptor or a deleted form (*KIR2DS4D*) yielding a truncated soluble protein [12]. Conversely, KIR-B haplotype has a variable gene content and usually contains multiple activating *KIR* genes. The human leukocyte antigen (HLA) class I molecules on target cells are recognized by KIRs on NK cells [13]. KIR2DL1 recognizes HLA-C group 2, while KIR2DL2 and KIR2DL3 bind to HLA-C group 1. KIR3DL1 recognizes HLA-A and HLA-B allotypes with the Bw4 motif (HLA-Bw4), defined by amino acid residues 77–83 in the α1 domain [14]. Based on a dimorphism at position 80, HLA-Bw4 can be either divided into isoleucine (Bw4 80I) or threonine (Bw4 80T). KIR3DL2 has specificity for HLA-A3 and HLA-A11. The effect of KIR and their ligands has been implicated in viral infections and disease pathogenesis [15-17]. Evidently, studies have described the associations of *KIR* with dengue infection and clinical outcomes [18-20]. Recently, our previous studies demonstrated the effect of *KIR* gene-content variations on dengue severity in northeastern Thais [21]. Therefore, the present study continuously investigates *KIR* copy number in dengue-infected patients with a quantitative polymerase chain reaction method called qKAT [22, 23]. Regarding the complexity of *KIRs*, this study will help elucidate the further dimensions of *KIR* genetics in dengue-infected patients from northeastern Thailand.

1. **Materials and methods**

**2.1 Study subjects**

The dengue-infected patients consisted of 137 unrelated individuals from the northeastern provinces of Thailand. Dengue patients were classified as DF and DHF based on the World Health Organization criteria [24]. Genomic DNA of all dengue patients were provided by the Dengue Hemorrhagic Fever Research Unit, Office for Research and Development, Siriraj Hospital, Faculty of Medicine, Mahidol University, Bangkok, Thailand. This investigation was approved for ethical permission by the Human Research Ethics Committee of Thammasat University No.1 (COA 057/59).

**2.2 HLA typing and KIR copy number identification**

The HLA typing method of the dengue samples was adapted from Tajik et al., 2010 [25], which used polymerase chain reactions with sequence-specific primers (PCR-SSP) to identify KIR ligands as previously described [26]. HLA ligands were grouped into HLA-C1, HLA-C2, HLA-Bw4, HLA-A (Bw4), HLA-Bw4 (I80), and HLA-Bw4 (T80). The KIR+HLA ligand pairs were analyzed based on individual *KIR* copy numbers as follows: KIR2DL1+C2, KIR2DL2+C1, KIR2DL3+C1, KIR2DS1+C2, KIR2DS2+C1, KIR3DL1+Bw4, and KIR3DS1+Bw4. For KIR copy number identification, all *KIR* genes (*KIR2DL1-5*, *2DS1-5*, *2DP1, 3DP1, 3DL1, 3DL2, 3DL3*, and *3DS1*) were determined in 137 dengue patients (DF = 63, DHF = 74) using a Roche LightCycler 480 at the Cambridge Institute for Medical Research (CIMR), University of Cambridge, UK as previously described [22].

**2.3 KIR genotypes and haplotypes**

The KIR genotypes (IDs) were ascertained according to the Allele Frequency Net Database (AFND) (<http://www.allelefrequencies.net>)[27]. The KIR haplotypes (centromeric–telomeric motif pairings) in dengue patients were predicted by the KIR Haplotype Identifier program (www.bioinformatics.cimr.cam.ac.uk/haplotypes/) using the determined copy number for each *KIR* gene [22] . KIR haplotypes were predicted in 137 samples that had copy number typing for all *KIR* genes.

**2.4 Statistical analysis**

The influence of multiple *KIR* copy numbers on dengue severity was investigated by comparing one copy vs. 2-4 copies of each *KIR* between DHF and DF. The difference in *KIR* copy number distribution between the dengue group and Thais was investigated by directly counting the number of individuals containing *KIRs* with 1, 2, 3, and 4 copies, and compared the copy number of each *KIR* (1-4 copies) using the chi-square test. The associations were calculated using the chi-square test with Yates correction or Fisher exact test as applicable. P-value and the odds ratio (OR) with a confidence interval (CI) of 95% were calculated using the IBM SPSS19.0 software (IBM Corporation, New York, USA) and Microsoft Excel 2019. A P value <0.05 was considered statistically significant. The P-value was corrected for multiple comparisons by the Bonferroni inequality method and expressed as Pc value.

1. **Results**

**3. 1 Frequencies of *KIR* copy number in dengue patients**

One hundred and thirty seven dengue patients (63 DF and 74 DHF) were typed for copy number of *KIR* genes, including *KIR3DL3, 2DS2, 2DL2, 2DL3, 2DP1,2DL1, 3DP1, 2DL4, 3DL1, 2DL5, 2DS3, 2DS5, 2DS1, 2DS4D, 2DS4WT* and *3DL2* as shown in **Table 1**. Consistent with our previous study, the results demonstrated that two copies of the framework/pseudogenes (*KIR3DL3, 2DP1, 3DP1, 2DL4,* and *3DL2*) and KIR A haplotype member (*KIR2DL1, 2DL3, 3DL1*, and *2DS4)* were frequently observed, whereas genes of the KIR B haplotype (*2DL2, 3DS1, 2DL5, 2DS3, 2DS5,* and *2DS1*) were typically identified with one copy [28]. The distributions of all copies for each *KIR* in dengue patients are mostly similar to individuals from northeastern Thais [28]. Of those, only one copy of *2DS3* was significantly low in dengue patients (15.3%) compared to northeastern Thais (37%) and this survived the Bonferroni correction (Pc=0.016). Additionally, three and four copies of *KIR* geneswere identified in dengue-infected patients with frequencies between 1.4% - 5.4%. Of those, three copies of KIR B haplotype-specific genes *KIR2DS2* and *3DS1*, and four copies of *KIR3DP1, 2DL4,* and *2DL1* were only identified in DHF (all 1.4% frequency, n=1). According to the strong linkage disequilibrium of closely linked *KIR loci*, discordant frequencies of *KIR* copy numbers were observed in three pairs of *KIR*s, *KIR2DL1-2DP1*, *2DL2-2DL3*, and *2DL2-2DS2*. Given the inconsistent data, the raw data of these *KIR* genes were firstly checked on the copy number analysis software by comparing the cycle threshold (Ct) of the target gene to the reference gene (*STAT6* and standard genomic DNA with known *KIR* copy number) of the quantitative PCR, and secondly by repeated typing by PCR-SSP method using different primer sets in samples containing zero copy versus one or more copies of respective genes. These results were consistent between the two methods, however, gene sequencing analysis to rule out potential allelic polymorphisms affecting discordance of *KIR* copy number results in individuals would give further verification.

* 1. **KIR profiles and haplotypes in dengue patients**

KIR copy number profiles were characterized according to the Allele Frequency Net Database (AFND) as shown in **fig.1**. Fortyprofiles were observed in dengue-infected patients, of which 29 and 28 profiles were identified in DF and DHF, respectively. Based on copy number of framework genes, KIR profiles were further classified into 31 usual (**fig.1A**), 6 expanded (**fig.1B**), and 3 contracted profiles (**fig.1C**). Of those, twelve profiles were only found in DF, and eleven were only identified in DHF. On account of expanded *KIR* profiles, the rare haplotype was identified in Thai population with 1.4% frequency in DHF as shown in genotype no. 6 of fig.1B. This rare haplotype was first found in Europeans, and the haplotype was generated by a duplication of *KIR2DS3, 2DP1, 2DL1, 3DP1* *2DL4*, *3DS1*, and *2DL5* [29]. Additionally, the AA haplotype (genotype no.1) with heterozygous *KIR2DS4D/WT* was the most common in DF and DHF with frequencies of 25.4% and 22.97%, respectively. For haplotype analysis, the centromeric–telomeric motif pairings of *KIR* haplotypes in dengue patients were predicted based on *KIR* copy number data as shown in **Table 2**. The results showed that the pairing of CA01 motif (*KIR3DL3, 2DL3, 2DP1, 2DL1*, and *3DP1*) and TA01 motif (*KIR2DL4, 3DL1, 2DS4*, and *3DL2*) was the most common pairing in dengue patients with a frequency of 59.5% in DF and 57.4% in DHF. The frequency of unusual haplotypes was observed at similar frequency in DF and DHF (13.5%). The influence of KIR-AA haplotype and HLA ligand on dengue outcomes was investigated, and an association was not found (**Table S1**).

* 1. **Influence of multiple KIR copy numbers on dengue severity**

1. The effect of *KIR* copy numbers on clinical outcomes was investigated in dengue patients by comparing one copy vs multiple copies (2-4 copies) of each *KIR* between DF and DHF as shown in **Table 3**. An association was not observed in this investigation. Additionally, the influence of *KIR* carriage versus no copies on dengue severity was examined by comparing zero copy vs one or more copies grouped, and an association was not found (Table S2). The combinations of *KIR* copy numbers with known *HLA* ligands and clinical outcomes were investigated as shown in **Table 4**. Of note, the results indicated that an individual possessing one copy of *KIR2DL2* and *2DL3* with homozygous *HLA-C1* (*HLA-C1C1*) was significantly associated with a decreased risk of developing DHF (OR 0.17, 95% CI 0.058 - 0.482, P<0.001). Conversely, two copies of *KIR2DL3* and *HLA-C1C1* were significantly associated with an increased risk of developing DHF with OR 2.32, 95% CI 1.159 - 4.624, P=0.016. Notably, the Bonferroni correction (Pc) was applied and showed that only the *KIR2DL2L3+C1C1* survived with Pc=0.005. **Discussion**

The role of *KIR* in viral infections is of medical research interest since the diversity of *KIR* generates heterogeneity of NK cell responses. The hypothesis is that genetic diversity of individual *KIRs* is related to disease susceptibility and clinical manifestations. As in the previous study, the association of *KIR* gene content polymorphisms and dengue infections was carried out [21]. This study was a continued investigation in which we applied a high- resolution method to determine copy number of *KIRs* in 137 dengue patients from northeastern Thailand. The results showed a distinction in *KIR* copy numbers in dengue patients, specifically 3 copies of certain KIR B haplotype-specific genes (*KIR2DS2*, *2DS3*, and *3DS1*) were observed in DHF but not in DF. This suggests that the activating KIRs would be involved in the immune responses of NK cells and/or T cells relevant to the pathogenesis of the disease [30].

The pairings of centromeric and telomeric motifs were predicted indicating the combination of CA01 and TA01 motifs (CA01TA01) was the most common in dengue patients (59.5% DF, 57.4% DHF) and northeastern Thais (54.8%)[28].  Interestingly, this study identified a duplication of *KIR2DS3, 2DP1, 2DL1, 3DP1,* *2DL4*, *3DS1* and *2DL5* in an individual with DHF (1.4%) consistent with an expanded profile. The duplications of these genes form a novel haplotype in Europeans as previously described by Amorim et al. [29], and this is the first time such a haplotype has been seen in the Thai population. Moreover, this investigation demonstrated that two copies of *KIR2DL3* with *HLA-C1C1* were associated with an increased risk of developing DHF with P= 0.016. In contrast, one copy of *KIR2DL2* and *2DL3* with *HLA-C1C1* was associated with a decreased risk of developing DHF with P<0.001 and Pc=0.005.

In comparison with our previous study, this investigation presents a further dimension of KIR variations in association with the clinical outcomes of dengue infections. As previously published, the association of *KIR3DS1+Bw4* was determined and suggested to be a protective genotype to developing DHF [21]. In the present study, the effect of *KIR3DS1*+*Bw4* on dengue severity was not found, while the combination of *KIR2DL2/3* and *HLA-C1* was significantly associated with clinical outcomes of dengue infections. On account of the associative study, a small sample size would impact our analysis and the power of the study. For instance, the association was investigated by one or a few individuals between DF and DHF, particularly individuals who contain a combination of *KIR3DS1* and *3DS1* with *HLA+Bw4* observed in Table 4. Of those, although the present study did not observe the effect of *KIR3DS1*+*Bw4* on dengue severity due to the limitation of sample size, this finding suggests that KIR2DL2/3 would play an essential role in providing an immune response to dengue infections relevant to the clinical outcomes.

To our knowledge, this study is the first to highlight the influence of *KIR2DL2* and *2DL3* copy numbers on dengue severity. Based on the interaction of KIR2DL2/3 and HLA-C1, the affinity of KIR2DL3+C1 is weaker than KIR2DL2+C1 resulting in less inhibition and higher NK cell responsiveness of KIR2DL3+C1 compared to KIR2DL2+C1 [31, 32]. With this reasoning, two copies of *KIR2DL3* with *HLA*-*C1C1* would promote the immune response of NK cells in dengue infections by releasing IFN-gamma causing disease severity, while one copy of *KIR2DL2* and *2DL3* with *HLA-C1C1* is associated with a decreased risk of developing DHF. Similarly, our previous study demonstrated an increased frequency of *KIR2DL3* in dengue patients compared to heathy individuals [21]. Remarkably, the combination of *KIR2DL2*+*C1* was highly found in Thai populations compared to dengue patients as well as this was highly observed in DF compared to DHF [21]. These results would indicate the role of KIR2DL3 and 2DL2 in dengue infections and pathogenesis of the disease. However, given the limitation of this study, the larger sample size and allelic genotyping could increase the impact of our results, and further investigation of *KIR* expression and functional study would assist in explaining NK cell response in dengue infection.

Presently, the role of *KIR2DL3+C1* combination on diseases has been well described in hepatitis C virus (HCV) infection [16, 33, 34]. On account of KIR and HLA interaction, although both KIR2DL2 and 2DL3 can recognize HLA-C1 on the target cell [31], the binding affinity is dependent on the allotypic differences in *KIR2DL2/2DL3*, their HLA-C ligands, and their associated peptide that is related to the quality of inhibitory signals to NK cells [35]. Taken together, NK cell responses in KIR2DL2- and 2DL3- positive individuals are more sensitive to the peptide presented by HLA-C1, suggesting changes in peptide repertoire during viral infection potentially modulate NK cell reactivity [36]. Evidently, Ziegler et al. demonstrated that HIV-1 infection could induce the changes of peptide presented by HLA-C\*03:04 to reduce the engagement with KIR2DL3, leading to the viral escape of NK cell-mediated immune responses [37]. Like HIV, it could be that dengue viruses can alter peptide repertoire to interfere with the engagement of peptide-sensitive inhibitory KIR2DL2/3 and modulate the immune response of NK cells involving pathogenesis of disease and clinical outcomes. Interestingly, HLA-B\*46:01 is defined as HLA-C1 allele binding to KIR2DL3, which the high frequency of HLA-B\*46:01 is observed in Southeast Asians [38]. With this regard, this HLA-B allele combined with KIR2DL2/3 would be interesting to investigate how they are involved in the pathogenesis of diseases, particularly dengue infections, for a future large cohort.

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In summary, this study is the first to determine the copy number of *KIRs* in dengue patients. We dissected KIR copy numbers, profiles, and haplotypes in 137 dengue patients from northeastern Thailand. Further, we identified that *KIR2DL2/2DL3* combined with HLA-C1 homozygosity was associated with a decreased risk of developing DHF. Resolving the molecular basis of KIR-mediated NK cell responses against dengue-infected cells will lead to better understanding of the innate immunopatholgy of the disease.

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1. **Conflict of Interest**

The authors declare that they have no conflict of interest.

1. **References**

[1] Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CLet al. : The global distribution and burden of dengue. Nature 2013;496:504.

[2] Organization WH: Dengue and severe dengue. 2020.

[3] Rodenhuis-Zybert IA, Wilschut J, Smit JM: Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci 2010;67:2773.

[4] Uno N, Ross TM: Dengue virus and the host innate immune response. Emerg Microbes Infect 2018;7:167.

[5] Zimmer CL, Cornillet M, Sola-Riera C, Cheung KW, Ivarsson MA, Lim MQet al. : NK cells are activated and primed for skin-homing during acute dengue virus infection in humans. Nat Commun 2019;10:3897.

[6] Azeredo EL, De Oliveira-Pinto LM, Zagne SM, Cerqueira DI, Nogueira RM, Kubelka CF: NK cells, displaying early activation, cytotoxicity and adhesion molecules, are associated with mild dengue disease. Clin Exp Immunol 2006;143:345.

[7] St John AL, Rathore AP, Yap H, Ng ML, Metcalfe DD, Vasudevan SGet al. : Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. Proc Natl Acad Sci U S A 2011;108:9190.

[8] Ponte M, Cantoni C, Biassoni R, Tradori-Cappai A, Bentivoglio G, Vitale Cet al. : Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. Proc Natl Acad Sci U S A 1999;96:5674.

[9] Rajagopalan S, Fu J, Long EO: Cutting edge: induction of IFN-gamma production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. J Immunol 2001;167:1877.

[10] Shilling HG, Young N, Guethlein LA, Cheng NW, Gardiner CM, Tyan Det al. : Genetic control of human NK cell repertoire. J Immunol 2002;169:239.

[11] Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss Bet al. : Human diversity in killer cell inhibitory receptor genes. Immunity 1997;7:753.

[12] Middleton D, Gonzalez A, Gilmore PM: Studies on the expression of the deleted KIR2DS4\*003 gene product and distribution of KIR2DS4 deleted and nondeleted versions in different populations. Hum Immunol 2007;68:128.

[13] Lanier LL: NK cell recognition. Annu Rev Immunol 2005;23:225.

[14] Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P: The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. J Exp Med 1995;181:1133.

[15] Boelen L, Debebe B, Silveira M, Salam A, Makinde J, Roberts CHet al. : Inhibitory killer cell immunoglobulin-like receptors strengthen CD8(+) T cell-mediated control of HIV-1, HCV, and HTLV-1. Sci Immunol 2018;3.

[16] Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell Aet al. : Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology 2010;51:1168.

[17] Rizzo R, Gentili V, Rotola A, Bortolotti D, Cassai E, Di Luca D: Implication of HLA-C and KIR alleles in human papillomavirus infection and associated cervical lesions. Viral Immunol 2014;27:468.

[18] Beltrame LM, Sell AM, Moliterno RA, Clementino SL, Cardozo DM, Dalalio MMet al. : Influence of KIR genes and their HLA ligands in susceptibility to dengue in a population from southern Brazil. Tissue Antigens 2013;82:397.

[19] Alagarasu K, Bachal RV, Shah PS, Cecilia D: Profile of killer cell immunoglobulin-like receptor and its human leucocyte antigen ligands in dengue-infected patients from Western India. Int J Immunogenet 2015;42:432.

[20] Petitdemange C, Wauquier N, Jacquet JM, Theodorou I, Leroy E, Vieillard V: Association of HLA class-I and inhibitory KIR genotypes in Gabonese patients infected by Chikungunya or Dengue type-2 viruses. PLoS One 2014;9:e108798.

[21] Chaisri S, Jumnainsong A, Romphruk A, Leelayuwat C: The effect of KIR and HLA polymorphisms on dengue infection and disease severity in northeastern Thais. Med Microbiol Immunol 2020;209:613.

[22] Jiang W, Johnson C, Simecek N, Lopez-Alvarez MR, Di D, Trowsdale Jet al. : qKAT: a high-throughput qPCR method for KIR gene copy number and haplotype determination. Genome Med 2016;8:99.

[23] Jayaraman J, Kirgizova V, Di D, Johnson C, Jiang W, Traherne JA: qKAT: Quantitative Semi-automated Typing of Killer-cell Immunoglobulin-like Receptor Genes. J Vis Exp 2019.

[24] (WHO) WHO: Dengue haemorrhagic fever: diagnostic, treatment, prevention and control Geneva, 1997.

[25] Tajik N, Shahsavar F, Nasiri M, Radjabzadeh MF: Compound KIR-HLA genotype analyses in the Iranian population by a novel PCR-SSP assay. Int J Immunogenet 2010;37:159.

[26] Chaisri S, Kitcharoen K, Romphruk AV, Romphruk A, Witt CS, Leelayuwat C: Polymorphisms of killer immunoglobulin-like receptors (KIRs) and HLA ligands in northeastern Thais. Immunogenetics 2013;65:645.

[27] Gonzalez-Galarza FF, McCabe A, Santos E, Jones J, Takeshita L, Ortega-Rivera NDet al. : Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. Nucleic Acids Res 2020;48:D783.

[28] Chaisri S, Traherne JA, Jayaraman J, Romphruk A, Trowsdale J, Leelayuwat C: Novel KIR genotypes and gene copy number variations in northeastern Thais. Immunology 2018;153:380.

[29] Amorim LM, Augusto DG, Nemat-Gorgani N, Montero-Martin G, Marin WM, Shams Het al. : High-Resolution Characterization of KIR Genes in a Large North American Cohort Reveals Novel Details of Structural and Sequence Diversity. Front Immunol 2021;12:674778.

[30] Prakash S, Ranjan P, Ghoshal U, Agrawal S: KIR-like activating natural killer cell receptors and their association with complicated malaria in north India. Acta Trop 2018;178:55.

[31] Winter CC, Gumperz JE, Parham P, Long EO, Wagtmann N: Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. J Immunol 1998;161:571.

[32] Carrington M, Wang S, Martin MP, Gao X, Schiffman M, Cheng Jet al. : Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. J Exp Med 2005;201:1069.

[33] Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski Jet al. : HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872.

[34] Thoens C, Berger C, Trippler M, Siemann H, Lutterbeck M, Broering Ret al. : KIR2DL3(+)NKG2A(-) natural killer cells are associated with protection from productive hepatitis C virus infection in people who inject drugs. J Hepatol 2014;61:475.

[35] Moradi S, Stankovic S, O'Connor GM, Pymm P, MacLachlan BJ, Faoro Cet al. : Structural plasticity of KIR2DL2 and KIR2DL3 enables altered docking geometries atop HLA-C. Nat Commun 2021;12:2173.

[36] Cassidy S, Mukherjee S, Myint TM, Mbiribindi B, North H, Traherne Jet al. : Peptide selectivity discriminates NK cells from KIR2DL2- and KIR2DL3-positive individuals. Eur J Immunol 2015;45:492.

[37] Ziegler MC, Nelde A, Weber JK, Schreitmuller CM, Martrus G, Huynh Tet al. : HIV-1 induced changes in HLA-C\*03 : 04-presented peptide repertoires lead to reduced engagement of inhibitory natural killer cell receptors. AIDS 2020;34:1713.

[38] Hilton HG, McMurtrey CP, Han AS, Djaoud Z, Guethlein LA, Blokhuis JHet al. : The Intergenic Recombinant HLA-B \*46:01 Has a Distinctive Peptidome that Includes KIR2DL3 Ligands. Cell Rep 2017;19:1394.

**Table 1.** *KIR* copy number frequencies in 137 dengue-infected patients

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ***KIR* copy** | ***KIR* gene frequencies (%)** | | | | | | | | | | | | | | | | | | | | |
| **number** | **Framework and pseudogenes** | | | | |  | **A haplotype member** | | | | | |  | **B haplotype-specific** | | | | | | | |
|  | ***3DL3***  n  (%) | ***2DP1***n  (%) | ***3DP1***  n  (%) | ***2DL4***  n  (%) | ***3DL2***  n  (%) |  | ***2DL1***  n  (%) | ***2DL3*** n  (%) | ***3DL1***n  (%) | ***2DS4W***  n  (%) | ***2DS4D*** n  (%) | ***2DS4Total***  n  (%) |  | ***2DS2*** n  (%) | ***2DL2*** n  (%) | ***3DS1*** n  (%) | ***2DL5*** n  (%) | ***2DS3*** n  (%) | ***2DS5*** n  (%) | ***2DS1*** n  (%) |
| **DF (N=63)** | |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 0 copy | 0  (0.0) | 0  (0.0) | 0  (0.0) | 0  (0.0) | 0  (0.0) |  | 0  (0.0) | 0  (0.0) | 3  (4.8) | 24  (38.1) | 20  (31.8) | 3  (4.8) |  | 38  (60.3) | 38  (60.3) | 38  (60.3) | 31  (49.2) | 47  (74.6) | 43  (68.3) | 36  (57.1) |
| 1 copy | 0  (0.0) | 17  (27.0) | 3  (4.8) | 3  (4.8) | 0  (0.0) |  | 17  (27.0) | 26  (41.3) | 25  (39.7) | 33  (52.4) | 35  (55.6) | 24  (38.1) |  | 25  (39.7) | 24  (38.1) | 21  (33.3) | 24  (38.1) | 11  (17.5) | 20  (31.8) | 24  (38.1) |
| 2 copies | 63  (100) | 43  (68.3) | 58  (92.1) | 58  (92.1) | 63  (100) |  | 43  (68.3) | 36  (57.1) | 34 (54.0) | 6  (9.5) | 8  (12.7) | 36  (57.1) |  | 0  (0.0) | 1  (1.6) | 4  (6.4) | 7  (11.1) | 5  (7.9) | 0  0.0 | 3  (4.8) |
| 3 copies | 0  (0.0) | 3  (4.8) | 2  (3.2) | 2  (3.2) | 0  (0.0) |  | 3  (4.8) | 1  (1.6) | 1  (1.6) | 0  (0.0) | 0  (0.0) | 0  (0.0) |  | 0  (0.0) | 0  (0.0) | 0  (0.0) | 1  (1.6) | 0  (0.0) | 0  (0.0) | 0  (0.0) |
| 4 copies | 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  (0.0) | 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  (0.0) |
| **DHF (N=74)** | |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 0 copy | 0  (0.0) | 2  (2.7) | 0  (0.0) | 0  (0.0) | 0  (0.0) |  | 2  (2.7) | 3  (4.1) | 3  (4.1) | 27  (36.5) | 24  (32.4) | 3  (4.1) |  | 51  (68.9) | 51  (68.9) | 43  (58.1) | 40  (54.1) | 60  (81.1) | 48  (64.9) | 43  (58.1) |
| 1 copy | 0  (0.0) | 14  (18.9) | 1  (1.4) | 1  (1.4) | 0  (0.0) |  | 14  (18.9) | 21  (28.4) | 29  (39.2) | 38  (51.4) | 41  (55.4) | 28  (37.8) |  | 20  (27.0) | 20  (27.0) | 26  (35.1) | 25  (33.8) | 10  (13.5) | 25  (33.8) | 29  (39.2) |
| 2 copies | 74  (100) | 56  (75.7) | 68  (91.9) | 68  (91.9) | 74  (100) |  | 55  (74.3) | 50  (67.6) | 41  (55.4) | 9  (12.2) | 9  (12.2) | 42  (56.8) |  | 2  (2.7) | 3  (4.1) | 3  (4.1) | 7  (9.5) | 3  (4.1) | 1  (1.4) | 2  (2.7) |
| 3 copies | 0  (0.0) | 2  (2.7) | 4  (5.4) | 4  (5.4) | 0  (0.0) |  | 2  (2.7) | 0  (0.0) | 1  (1.4) | 0  (0.0) | 0  (0.0) | 1  (1.4) |  | 1  (1.4) | 0  (0.0) | 2  (2.7) | 2  (2.7) | 1  (1.4) | 0  (0.0) | 0  (0.0) |
| 4 copies | 0  (0.0) | 0  (0.0) | 1  (1.4) | 1  (1.4) | 0  (0.0) |  | 1  (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  (0.0) | 0  (0.0) | 0  (0.0) | 0  (0.0) |

DF: dengue fever cases; DHF: dengue hemorrhagic fever cases; *2DS4WT*: a wide-type variant; *2DS4D*: a deleted variant; *2DS4* total: *2DS4 (WT+D)*; N: total number; n: number of individuals

**Table 2.** KIR haplotype prediction in dengue-infected patients

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Haplotypes** | **DF** | |  | **DHF** | |
| **n** | **%** |  | **n** | **%** |
| CA01TA01 | 75 | 59.5 |  | 85 | 57.4 |
| CA01TB01 | 14 | 11.1 |  | 21 | 14.2 |
| CB01TA01 | 5 | 4.0 |  | 3 | 2.0 |
| CB01TB01 | 2 | 1.6 |  | 3 | 2.0 |
| CB02TA01 | 7 | 5.6 |  | 13 | 8.8 |
| CB02TB01 | 6 | 4.8 |  | 3 | 2.0 |
| Unusual haplotypes | 17 | 13.5 |  | 20 | 13.5 |

DF: dengue fever cases; DHF: dengue hemorrhagic fever cases; n: number of individuals

**Table 3.** The influence of multiple KIR copy numbers (2-4 copies vs 1 copy) on dengue severity

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***KIR*** | **Dengue-infected patients** | |  | **Tests of association** | | |
| **DF**  **n (%)** | **DHF**  **n (%)** |  | **OR** | **95%CI** | **P** |
| *2DL1* | 46 (73) | 58 (80.6) |  | 1.53 | (0.684 - 3.429) | 0.299 |
| *2DL2* | 1 (4) | 3 (13) |  | 3.60 | (0.347 - 37.362) | 0.257 |
| *2DL3* | 37 (58.7) | 50 (70.4) |  | 1.67 | (0.818 - 3.420) | 0.157 |
| *2DL5* | 8 (25) | 9 (26.5) |  | 1.08 | (0.358 - 3.260) | 0.891 |
| *3DL1* | 35 (58.3) | 42 (59.2) |  | 1.03 | (0.515 - 2.079) | 0.924 |
| *2DS1* | 3 (11.1) | 2 (6.5) |  | 0.55 | (0.085 - 3.577) | 0.528 |
| *2DS3* | 5 (31.3) | 4 (28.6) |  | 0.88 | (0.183 - 4.226) | 0.873 |
| *2DS4WT* | 6 (15.4) | 9 (19.1) |  | 1.30 | (0.419 - 4.046) | 0.647 |
| *2DS4DEL* | 8 (18.6) | 9 (18) |  | 0.96 | (0.335 - 2.755) | 0.940 |
| *3DS1* | 4 (16) | 5 (16.1) |  | 1.01 | (0.240 - 4.240) | 0.990 |

DF: dengue fever cases; DHF: dengue hemorrhagic fever cases; N: total number; n: number of individuals; OR: odds ratio; CI: confidence interval; P: P-value for test of association; *2DS2* and *2DS5*, that contained only 0 and 1 copy, were not applicable.

**Table 4**. The influence of *KIR* copy numbers and known *HLA* ligands on dengue severity

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **KIR-HLA** | **Dengue-infected patients** | |  | **Tests of association** | | |
| **DF** | **DHF** |  | **OR** | **95%CI** | **P** |
| **n (%)** | **n (%)** |  |
| *2DL1* and *2DL1+C2C2* | 1 (1.6) | 2 (2.7) |  | 1.72 | (0.152 - 19.453) | 0.657 |
| *2DL1* and *2DL1+C1C2* | 12 (19.1) | 12 (16.2) |  | 0.82 | (0.341 - 1.987) | 0.664 |
| *2DL2* and *2DL2+C1C1* | 1 (1.6) | 1 (1.4) |  | 0.85 | (0.052 - 13.862) | 0.909 |
| *2DL2* and *2DL3+C1C1* | 19 (30.2) | 5 (6.8) |  | **0.17** | **(0.058 - 0.482)\*** | **<0.001** |
| *2DL2* and *2DL3+C1C2* | 12 (19.1) | 7 (9.5) |  | 0.44 | (0.163 - 1.208) | 0.106 |
| *2DL3* and *2DL3+C1C1* | 22 (34.9) | 41 (55.4) |  | **2.32** | **(****1.159 - 4.624)\*\*** | **0.016** |
| *2DL3* and *2DL3+C1C2* | 10 (15.9) | 7 (9.5) |  | 0.55 | (0.198 - 1.552) | 0.256 |
| *2DS1* and *2DS1+C1C2* | 1 (1.6) | 1 (1.4) |  | 0.85 | (0.052 - 13.862) | 0.909 |
| *3DL1* and *3DL1+Bw4* | 26 (42.6) | 32 (45.1) |  | 1.11 | (0.554 - 2.202) | 0.778 |
| *3DL1* and *3DL1+ A (Bw4)* | 16 (26.2) | 15 (21.1) |  | 0.75 | (0.336 - 1.687) | 0.49 |
| *3DL1* and *3DL1+Bw4 (I80)* | 12 (19.7) | 13 (18.3) |  | 0.92 | (0.383 - 2.189) | 0.842 |
| *3DL1* and *3DL1+Bw4 (T80)* | 9 (14.8) | 15 (21.1) |  | 1.55 | (0.624 - 3.839) | 0.344 |
| *3DL1* and *3DS1+Bw4* | 14 (23) | 22 (31) |  | 1.51 | (0.691 - 3.29) | 0.301 |
| *3DL1* and *3DS1+ A (Bw4)* | 11 (18) | 14 (19.7) |  | 1.12 | (0.465 - 2.682) | 0.805 |
| *3DL1* and *3DS1+Bw4 (I80)* | 3 (4.9) | 9 (12.7) |  | 2.81 | (0.724 - 10.878) | 0.122 |
| *3DL1* and *3DS1+Bw4 (T80)* | 5 (8.2) | 6 (8.5) |  | 1.03 | (0.299 - 3.571) | 0.958 |
| *3DS1* and *3DS1+Bw4* | 3 (4.9) | 2 (2.8) |  | 0.56 | (0.091 - 3.469) | 0.528 |

DF: dengue fever cases; DHF: dengue hemorrhagic fever cases; N: total number; n: number of individuals; OR: odds ratio; CI: confidence interval; P: P-value for test of association; Values in bold indicate significant association. *2DL2* and *2DL2+C1C2, 2DS1* and *2DS1+C2C2, 2DS2* and *2DS2+C1C1, 2DS2* and *2DS2+C1C2,3DS1* and *3DS1+ A (Bw4), 3DS1*and *3DS1+Bw4 (I80)* and *3DS1*and *3DS1+Bw4 (T80)* were not applicable due to absence of data on KIR-HLA combinations. *3DL1S1+Bw4* combinations were analyzed in 61 DF and 71 DHF.

Pc: Bonferroni corrected; \*Pc=0.005, \*\*Pc=0.272.

**Figure caption**

**Figure 1. The *KIR* copy number profiles identified in dengue-infected patients**

KIR profiles were characterized based on the Allele Frequency Net Database (AFND). Copy number of *KIR* were represented in different shades of grey, ranging from 0 copies (white) to 4 copies (black). The usual profile is an individual who had two copies of each framework gene (**fig.1A**), whereas an individual who had 3-4 copies of any framework gene was identified as having an expanded profile (**fig.1B**), and an individual who had one copy of any framework genes was identified as having a contracted profile (**fig.1C**). Haplo: haplotype; Gen: genotype; DF: dengue fever cases; DHF: dengue hemorrhagic fever cases; CNV: copy number variation.