Possible association of killer cell immunoglobulin-like receptor genotypes and haplotypes with dry eye disease in a Han Chinese population

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Purpose: The objective of this study was to explore whether killer immunoglobulin-like receptor (KIR) genotypes and haplotypes are associated with dry eye disease (DED) in a Han Chinese population.

Methods: Polymerase chain reaction with sequence-specific primers (PCR-SSP) method was used to genotype KIR genes in 106 patients with DED and 220 healthy controls.

Results: Twenty-three KIR genotypes were observed in the DED patient and healthy control groups, ten of which had not been described previously. The genotype G and haplotype 4 were associated with increased risk of DED, and the odds ratio (OR) and 95% confidence interval (95% CI) were 2.35 and 1.09–5.10, respectively. Genotype B/B was also associated with increased risk of DED, and the OR and 95% CI were 2.38 and 1.03–5.49. However, all frequencies of these KIR genotypes and haplotypes were no longer statistically significant between the two groups after the Bonferroni correction was applied for multiple testing.

Conclusions: There was a possible association between certain KIR genotypes and haplotypes with DED in a Han Chinese population. However, additional confirmation is required.

Dry eye disease (DED) is one of the most common ocular disorders, affecting 10–30% of the population [1]. Patients with DED experience visual disturbance, eye dryness, irritation, foreign body sensation, light sensitivity, itching, and even blindness [2,3].

Recent studies have shown that DED is an inflammatory disease that shares similar characteristics with autoimmune diseases [4-6], although the exact pathogenesis of DED remains unclear. The immunological responses of the host have been supposed to play a critical role in the clearance of inflammation, including cytokine production, antigen participation, and receptor recognition, which depends on the host's genetic background.

Killer immunoglobulin-like receptor (KIR) molecules are encoded by the KIR gene family, which exhibit genetic variation in the number and type of the genes presented on a haplotype [7]. The KIR haplotypes can be resolved into two broad haplotypes termed A and B, which consist of four conserved framework genes, KIR2DL4 (ID 3805; OMIM 604945), 3DL2 (ID 3812; OMIM 604947), 3DL3 (ID 115653; OMIM 610095), and 3DP1 (ID 548594; OMIM 610604). Haplotype B contains a variety of genes, including KIR2DL2 (ID 3803; OMIM 604937), 2DL5 (ID 100125575; OMIM 605305), 2DS1 (ID 3806; OMIM 604952), 2DS2 (ID 100132285; OMIM 604953), 2DS3 (ID 3808; OMIM 604954), 2DS5 (ID 3810; OMIM 604956), or 3DS1 (ID 3813; OMIM 604946), while haplotype A is characterized by the absence of all these genes. KIR genes are found in two adjacent clusters where the framework genes flank each cluster: KIR3DL3 and 3DP1 flank the centromeric (Cen) cluster while KIR2DL4 and 3DL2 flank the telomeric (Tel) cluster [7]. KIR gene motifs are defined based on the combination of KIR genes present in the Cen or Tel segment on the haplotype, and KIR haplotypes A and B, with different distributions among ethnic groups, have distinctive Cen and Tel gene-content motifs [8-10].

Natural killer (NK) cells are a vital component of the innate and adaptive immune systems, expressing several cell surface receptors (KIRs, C-type lectin superfamily receptors, and natural cytotoxicity receptors) [11]. NK cell activity is regulated by an extensive repertoire of regulatory receptors including the most polymorphic KIR family [12]. Several studies in KIR gene diversity were associated with infectious and noninfectious diseases [12,13]. KIR molecules
play an important role in anti-inflammation immunity by providing activating or inhibitory signals to regulate the activation of NK cells and T cell subsets [13,14]. The KIR genotype with distinctive gene content from maternal and paternal haplotypes produces diversity among individuals, which may influence the individuals’ immunity to different diseases. Interestingly, the associations between diseases and KIR genotypes have been observed in several clinical studies [14-16]. For example, individuals with KIR genotype A/A were reported to be relatively protected against psoriatic vulgaris and idiopathic bronchiectasis [14,15], and individuals with genotype A/B were significantly more likely to remain seronegative among long-term HIV-exposed subjects than those with genotype A/A [16]. Previously, we showed that KIR2DS2 was associated with the susceptibility to severe DED [17]. However, until now, the role of the overall KIR genotype in patients with DED has remained unclear. The aim of this study was to investigate the distribution of KIR genotypes and haplotypes in a Han Chinese population of 106 patients with DED and 220 healthy controls with polymerase chain reaction with sequence-specific primers (PCR-SSP).

METHODS

 Subjects: A total of 106 patients with DED, consisting of 47 men and 59 women, were recruited from the Ophthalmology Department and 220 unrelated healthy subjects from the health examination center of the 4th People’s Hospital of Jinan as a control group described by Ren et al. [17]. Of the 106 patients, 27 were classified with moderate DED and 79 (31 male, 48 female) with severe DED. These DED patients had no other diseases. Briefly, the patients with DED were diagnosed by Schirmer’s I test, which was advised by the National Eye Institute workshop. A 35.5-mm filter paper strip was used to measure the amount of tears that were produced over a period of 5 min. More than 10-mm wetting of the filter paper was normal. Patients with moderate dry eyes had wetting values between 10 and 5 mm. Patients with severe dry eyes had wetting values of less than 5 mm. Meanwhile, healthy subjects had wetting values of more than 10 mm in Schirmer’s I test. Valid informed consent was obtained from each participant, and this study was approved by the human ethics committee of the 4th People’s Hospital of Jinan. And the study adhered to the tenets of the Declaration of Helsinki and the ARVO statement on human subjects.

KIR genotyping: KIR genotyping was performed using PCR-SSP in all samples collected from the recruited subjects for the following 16 KIR genes: 2DL1–5, 3DL1–3, 2DS1–5, 3DS1, 2DP1, and 3DP1. The PCR sequence-specific polymorphism primers used for the detection of KIR genes, and the PCR amplification methods were previously described [17]. Briefly, 20–50 ng DNA was amplified in 10 ml volume containing 0.2 mM dNTP, 0.5U Taq DNA polymerase (Promega Corporation), 0.4 mM primers (except for KIR2DS1, 0.8 mM), and 1× PCR buffer. PCR amplification was carried out in a 9700 thermal cycler (PerkinElmer) under the following conditions: initial denaturing at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 90 s, plus a final extension at 72 °C for 10 min. Partial annealing temperatures were changed as follows: KIR2DS2 (63 °C), KIR2DS3 (63 °C), KIR2DS4 (61 °C), and KIR2DS5 (63 °C).

Genotype and haplotype analysis: Each genotype was given the putative haplotype combination according to the model described by Hsu et al. [18]. In assigning genes to a specific haplotype, the following assumptions were made: 1) All haplotypes contained KIR3DL3, 2DL4, and 3DL2; 2) the haplotypes contained either 2DL2 or 2DL3, but not both; and 3) the haplotypes contained either 3DP1 or 3DP1 variant (3DP1v), but not both [18]. In the assessment of the KIR haplotypes, haplotype A was defined by the presence of one or more of the following genes: KIR2DL5, 2DS1, 2DS2, 2DS3, 2DS5, and 3DS1. Conversely, haplotype A was defined by the absence of all these genes [19]. Four conserved framework genes divide the KIR locus into similarly sized Cen and Tel segments that differ in gene content [8]. Genotypes for the Cen and Tel parts of the KIR locus were defined according to Cooley et al.’s description [8]. Briefly, Cen-A/A contains 2DL3 only, Cen-A/B contains 2DL3 with 2DS2 and/or 2DL2, and Cen-B/B contains 2DS2 and/or 2DL2 but no 2DL3, whereas Tel-A/A has 3DL1 and 2DS4 only, Tel-A/B has 3DL1 and 2DS4 with 3DS1 and/or 2DS1 and Tel-B/B lacks 3DL1 and/or 2DS4.

Statistical analysis: Genotype frequencies were determined by directly counting the number of individuals who were positive in some particular KIR phenotype specificity. Each genotype was given the putative haplotype combination according to the model described by Hsu et al. [18]. A chi-square was used to test for the statistical significance of the genotypes or haplotypes between the DED patient group and the healthy control group. P values less than 0.05 were regarded as statistically significant. The strength of the association was estimated by calculating the odds ratio (OR) and the 95% confidence interval (95% CI). Statistical analysis was performed using the SPSS 15.0 (SPSS Inc., Chicago, IL) software package. Bonferroni correction for multiple testing was applied in the comparison of the genotype and haplotype frequencies between the two groups. The corrected p values (Pe) are shown in the tables.
RESULTS

KIR genotype frequencies in control subjects and patients:
In our study, all tested KIR genes were detected in the healthy control and DED patient groups. Framework genes (KIR2DL4, 3DL2, 3DL3, and 3DP1) were observed in all individuals. All KIR genotypes and putative haplotypes were determined according to Hsu et al.’s description [18].

In this study, no significant differences in the frequencies of the KIR genotypes and haplotypes were found between the patients with moderate DED and the healthy controls with the chi-square analysis (data not shown). However, certain KIR genotypes and haplotypes were associated with increased or reduced risk of severe DED (Table 1 and Table 2). Twenty-three KIR genotypes were observed in both groups, including ten new genotypes of NF1-NF10, which has not been observed in Caucasians thus far [18]. Among these genotypes, 20 were determined in healthy controls and 21 in patients with DED (Table 1). In the healthy controls, the three KIR genotypes with higher frequencies in rank order were AJ (37.73%), AH (11.36%), and AF (10.45%). In the patients with DED, genotypes AJ (27.85%), G (13.92%), and P (10.12%) were the three higher genotypes. Genotype G was associated with increased risk of DED, and the OR and 95% CI were 2.58 and 1.10–6.02, respectively. However, the frequency of genotype G was not statistically significant between the two groups after the Bonferroni correction was applied for multiple testing. The other KIR genotypes did not show any associations between the two groups.

KIR haplotype frequencies in control subjects and patients:
In this study, all 23 KIR genotypes were resolved into corresponding pairs of haplotypes (Table 2). There were 16 different haplotypes of which haplotype 2 was the most common (56.14%), followed by haplotype 1 (15.91%) and 5

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### Table 1. The frequencies of KIR genotype in healthy controls and DED patients.

| Genotype | Haplotype | Healthy controls | DED patients | OR(95%CI) | p value | Pc (n=23) |
|----------|-----------|-----------------|--------------|-----------|---------|-----------|
| E        | 1,3       | 2.091           | 1.127        | 1.40(0.13–15.63) | 0.785   |
| F        | 1,4       | 3.136           | 2.53         | 1.88(0.31–11.46) | 0.487   |
| G        | 4,5       | 5.911           | 13.92        | 2.58(1.10–6.02) | 0.025*  |
| H        | 2,4       | 2.273           | 3.8          | 1.70(0.40–7.27) | 0.471   |
| I        | 5,8       | 1.045           | 1.27         | 2.81(0.17–45.43) | 0.448   |
| M        | 2,8       | 1.042           | 2.53         | 2.83(0.39–20.45) | 0.282   |
| P        | 2,17      | 1.040           | 0.91         | 1.01(0.43–2.38) | 0.974   |
| AE       | 1,6       | 3.641           | 2.53         | 0.69(0.14–3.31) | 0.64    |
| AF       | 1,2       | 10.45           | 7.59         | 0.70(0.28–1.80) | 0.461   |
| AG       | 1,9       | 4.090           | 3.8          | 0.93(0.24–3.51) | 0.909   |
| AH       | 2,5       | 11.36           | 7.59         | 0.64(0.25–1.63) | 0.346   |
| AI       | 1,5       | 7.273           | 5.06         | 0.68(0.22–2.10) | 0.5     |
| AJ       | 2,2       | 37.73           | 27.85        | 0.64(0.36–1.12) | 0.115   |
| NF1      | 2,9       | 0.451           | 1.27         | 2.81(0.17–45.43) | 0.448   |
| NF2      | 4,13      | 0.451           | 1.27         | 2.81(0.17–45.43) | 0.448   |
| NF3      | 2,6       | 0.451           | 0           | -         | 0.548   |
| NF4      | 4,8       | 0.451           | 1.27         | 2.81(0.17–45.43) | 0.448   |
| NF5      | 2,11      | 0.911           | 0           | -         | 0.395   |
| NF6      | 2,23      | 0.011           | 1.27         | -         | 0.095   |
| NF7      | 6,6       | 0.011           | 1.27         | -         | 0.095   |
| NF8      | 1,4       | 0.011           | 1.27         | -         | 0.095   |
| NF9      | ??,       | 0.451           | 1.27         | 2.81(0.17–45.43) | 0.448   |
| NF10     | ??,       | 0.451           | 1.27         | 2.81(0.17–45.43) | 0.448   |

+ positive numbers; gf, genotype frequency; OR, odds ratio; 95% CI, 95% confidence interval; *, indicates statistical significance (p<0.05) according Chi Square analysis; Pc, corrected p value after Bonferroni correction; ?, unclassed haplotype.
(13.92%) in the healthy controls and the patients with DED. Haplotype 4 was associated with increased risk of DED, and the OR and 95% CI were 2.48 and 1.31–4.69, respectively; while haplotype 2 appeared to have an inverse association with the disease (OR, 0.64; 95% CI, 0.44–0.92). However, the frequencies of haplotypes 4 and 2 were not statistically significant between the two groups after the Bonferroni correction was applied for multiple testing. The other KIR haplotypes did not show any associations between the two groups.

The distributions of KIR haplotype A and B are shown in Table 3. The frequency of haplotype A was higher than haplotype B in the healthy controls and the patients with DED. Genotype B/B was associated with increased risk of DED, and the OR and 95% CI were 2.352 and 1.09–5.10, respectively. Interestingly, when compared the distributions of the Cen and Tel segments of the KIR genotype in the two groups (Table 4), Cen-B/B was also associated with increased risk of DED (OR, 2.380; 95% CI, 1.03–5.49). However, the frequencies of genotype B/B and Cen-B/B were not statistically significant between the two groups after the Bonferroni correction was applied for multiple testing. The other Cen and Tel motifs of the KIR genotypes did not show any associations between the two groups.

### DISCUSSION

The KIR gene system spans a region of about 150 kb on chromosome 19 [11]. The number and type of KIR genes arranged on the haplotypes vary greatly, which produces substantial differences in KIR gene content among individuals [7]. Genetic diversity within the KIR locus can moderate the NK cells and T cell subset responses to a diverse array of diseases and thus suggests that KIR diversity may influence susceptibility or resistance to different diseases [20]. Recent studies showed that KIR genotypes are associated with autoimmune diseases such as idiopathic bronchiectasis [15] and psoriasis vulgaris [14] and microbial pathogen diseases caused by hepatitis B virus [21], Ebola virus [22], and HIV [16]. However, little is known about the association of KIR genotypes with DED, which is characterized by sustained inflammation on the ocular surface. Interestingly, this is the first study to analyze KIR genotypes associated with DED. In our study, patients with DED and healthy controls were identified as having KIR genotype A/A, A/B, or B/B based on the multiple KIR genes the individuals possessed. Similar distributions of KIR genotypes were observed between the two groups (Table 3). Interestingly, individuals with genotype B/B had an increased risk of severe DED, which was consistent with, to some extent, our previous report that KIR2DS2 was associated with susceptibility to DED [17] since genotype B/B contains KIR2DS2. It was similar to the results that genotypes with haplotype B were found to

### Table 2. The frequencies of KIR haplotypes in healthy controls and DED patients.

| Haplotype | Healthy controls | DED patients | OR(95%CI) | p value | \(P_c\) (n=13) |
|-----------|-----------------|--------------|-----------|---------|---------------|
|           | + h(f)%         | + h(f)%      |           |         |               |
| 1         | 70 (56.14)      | 22 (44.94)   | 0.86 (0.51–1.44) | 0.553   |               |
| 2         | 247 (56.14)     | 71 (44.94)   | 0.64 (0.44–0.92) | 0.016*  | >0.05         |
| 3         | 2 (0.45)        | 1 (0.63)     | 1.40 (0.13–15.49) | 0.786   |               |
| 4         | 23 (5.23)       | 19 (12.03)   | 2.48 (1.31–4.69) | 0.004*  | >0.05         |
| 5         | 55 (12.5)       | 22 (13.92)   | 1.13 (0.67–1.93) | 0.647   |               |
| 6         | 9 (2.05)        | 4 (2.53)     | 1.24 (0.38–4.10) | 0.719   |               |
| 8         | 4 (0.91)        | 4 (2.53)     | 2.83 (0.70–11.46) | 0.128   |               |
| 9         | 1 (0.23)        | 1 (0.63)     | 2.80 (0.17–44.97) | 0.449   |               |
| 11        | 2 (0.45)        | 0 (0)        | -          |         |               |
| 13        | 1 (0.23)        | 1 (0.63)     | 2.80 (0.17–44.97) | 0.449   |               |
| 17        | 22 (5)          | 8 (5.06)     | 1.01 (0.44–2.32) | 0.975   |               |
| 23        | 0 (0)           | 1 (0.63)     | -          |         |               |
| ?         | 4 (0.91)        | 4 (2.53)     | 2.83 (0.70–11.46) | 0.128   |               |

+, positive numbers; h(f), haplotype frequency; OR, odds ratio; 95% CI, 95% confidence interval; *, indicates statistical significance (p<0.05) according Chi Square analysis; \(P_c\), corrected p value after Bonferroni correction; ?, unclassed haplotype.
be increased in Japanese psoriasis vulgaris cases [14]. The structurally distinctive characteristic of haplotype B is the multiplicity of activating KIRs that might increase chances of accidental activation of immunocytes over the threshold. Moreover, this inaccurate activation would occur especially in patients who do not possess the proper combinations of inhibitory KIRs [14]. Recent data supported the hypothesis that DED is a localized autoimmune disease resulting from the imbalance between protective immunoregulatory and proinflammatory mechanisms [6]. Combined with our data, these findings supported previously proposed models of KIR-mediated autoimmunity [23].

Dividing these KIR haplotypes into the Cen and Tel motifs showed that homozygosity for Cen-B/B was also associated with increased risk of DED (Table 4). Interestingly, it was recently shown that Cen-B/B was associated with a lower risk of relapse after unrelated transplantation for acute myelogenous leukemia [8]. These data suggested that individuals with the Cen-B/B motif might generate different immune responses to different diseases.

We further refined KIR genotypes [18] to analyze the association between patients with DED and the healthy controls. In our study, ten new genotypes NF1–10 that have not been observed in Caucasians thus far were identified [18]. The distinctive distributions of the KIR genotypes between

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**Table 3. The frequencies of KIR genotypes A/A, A/B and B/B and haplotypes A and B, observed in healthy controls and DED patients.**

| Genotyping          | Healthy controls | DED patients | OR (95% CI) | p value | \(P_c\) |
|---------------------|------------------|--------------|-------------|---------|--------|
| KIR genotypes       | +                | +            |             |         |        |
| A/A                 | 116              | 35           | 0.71 (0.43–1.20) | 0.199   |        |
| A/B                 | 87               | 31           | 0.99 (0.58–1.67) | 0.962   |        |
| B/B                 | 17               | 13           | 2.35 (1.09–5.10) | 0.027*  | >0.05  |
| KIR haplotypes      | +                | +            |             |         |        |
| A                   | 319              | 101          | 0.67 (0.46–0.98) | 0.043*  | >0.05  |
| B                   | 121              | 57           | 1.49 (1.01–2.19) | 0.043*  | >0.05  |

+, positive numbers; gf, genotype frequency; hf, haplotype frequency; OR, odds ratio; 95% CI, 95% confidence interval; *, indicates statistical significance (p<0.05) according Chi Square analysis; \(P_c\), corrected p value after Bonferroni correction; ?, unclassed haplotype.

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**Table 4. The frequencies of KIR centromeric (Cen) and telomeric (Tel) gene content motifs in healthy controls and EDE patients.**

| Motifs | Healthy controls | DED patients | OR (95% CI) | p value | \(P_c\) (n=6) |
|--------|------------------|--------------|-------------|---------|---------------|
| Cen-A/A| 163              | 56           | 0.85 (0.48–1.51) | 0.581   |               |
| Cen-A/B| 43               | 12           | 0.74 (0.37–1.48) | 0.391   |               |
| Cen-B/B| 14               | 11           | 2.38 (1.03–5.49) | 0.037*  | >0.05         |

Tel-A/A 149 67.73 51 64.56 0.87 (0.51–1.49) 0.608
Tel-A/B 38 17.27 17 21.52 1.31 (0.69–2.49) 0.403
Tel-B/B 33 15 11 13.92 0.92 (0.44–1.91) 0.817

#: KIR centromeric (Cen) and telomeric (Tel) gene content motifs were defined according to the description of Cooley et al. [8]. Briefly, Cen-A/A contains 2DL3 with 2DS2 and/or 2DL2, and Cen-B/B contains 2DS2 and/or 2DL2 but no 2DL3, whereas Tel-A/A has 3DL1 and 2DS4 only, Tel-A/B has 3DL1 and 2DS4 with 2DS1 and/or 2DS4 and Tel-B/B lacks 3DL1 and/or 2DS4. +, positive numbers; gf, genotype frequency; OR, odds ratio; 95% CI, 95% confidence interval; *, indicates statistical significance (p<0.05) according Chi Square analysis; \(P_c\), corrected p value after Bonferroni correction; ?, unclassed haplotype.

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952
Han Chinese and Caucasian populations suggested the role of the natural selection of certain genes over the others. Interestingly, our data in Table 1 and Table 2 show that genotype G and haplotype 4 were associated with increased risk of DED, whereas haplotype 2 appeared to have an inverse association with the disease. These data suggested that different KIR genotypes/haplotypes might use the combinations of synergistic receptors to mediate different natural cytotoxicity here. Lu et al. [24] reported that individuals with genotype M or FZ1 or haplotype 4 had an increased risk of hepatitis B virus infection, whereas individuals with genotype AH or haplotype 5 facilitated the clearance of the hepatitis B virus. These data suggested that different KIR genotypes/haplotypes might contribute to the different immune responses to autoimmune and virus infectious diseases and that certain KIR gene combinations could provide an immune microenvironment to get rid of or be susceptible to different diseases.

All frequencies of these KIR genotypes and haplotypes were no longer significant between the healthy controls and the patients with DED after the Bonferroni correction analysis. The Bonferroni correction is a critical statistical method for mitigating the risk of making false-positive conclusions, and appropriate use can ensure the integrity of studies in which a large number of significance tests are used, although it tends to be a bit too conservative and increases the risk of generating false negatives [25].

Our findings might help develop biomarkers to monitor the ocular surface inflammatory status and provide a new interesting therapeutic strategy for considering the molecular basis of DED, which will not only improve our knowledge to fully understand the mechanisms that lead to DED but also provide directions for developing effective and safe anti-inflammatory treatments that will be beneficial for patients with DED.

In conclusion, our study showed that KIR genotype G, haplotype 4, and Cen-B/B were possibly associated with increased risk of DED, whereas haplotype 2 was possibly associated with a reduced risk of DED in a Han Chinese population. These studies should be subjected to multivariate analysis and repeated in patients of other ethnic origins from the same and different regions.

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