Molecular genetic analyses of human
NKG2C (KLRC2) gene deletion

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Abstract

Human NKG2A, NKG2C and NKG2E genes are located on 12p13 in the NK gene complex. We recently identified deletion of NKG2C in a Japanese population. This study was performed to identify the breakpoint, and to examine the association of NKG2C deletion with susceptibility to rheumatoid arthritis and systemic lupus erythematosus. The location of the breakpoint was determined to be 1.5–1.8 kb telomeric from the 3′ end of NKG2A by comparing sequences of the intergenic segments upstream and downstream of the NKG2C gene in the common haplotype with the intergenic sequence between NKG2A and NKG2E in the deletion haplotype. Based on this information, a genotyping system was developed. The frequency of NKG2C deletion haplotype was 20.2% in Japanese and 20.0% in Dutch populations. The frequency of homozygous deletion was 4.1% in Japanese and 3.8% in Dutch. Evidence for an association with rheumatic diseases was not detected. These results indicated that NKG2C deletion is commonly present in Japanese and Dutch, suggesting that NKG2C is not essential for survival and reproduction, and is not associated with rheumatic diseases.

Introduction

Human NK cells are regulated by opposing signals from HLA class I receptors of two distinct families, the Ig superfamily and the C-type lectin superfamily, that activate or inhibit effector functions (1–5). Imbalance of such signals may possibly be involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

Human NKG2 molecules belong to the C-type lectin family. To date, seven members including splicing isoforms have been identified (6–9). Among the NKG2 family, NKG2C and NKG2E are expressed as heterodimers with CD94. Upon contacting HLA-E (10), they deliver activating signals via adaptor molecules such as DAP12 (11). NKG2 genes are located on chromosome 12p13 in the NK gene complex (NKC) (12,13), one of the candidate susceptibility regions of RA and SLE identified by linkage analyses (14–16). Although only suggestive evidence for linkage with RA and SLE with 12p13 has been reported, the detection power of the linkage analyses is highly limited in the case of complex diseases, where each susceptibility gene is assumed to confer modest to moderate relative risk (18,19). In fact, disease-associated polymorphisms have often been identified by association studies in chromosomal regions where only modest or no linkage was demonstrated (19). For example, association of mannose-binding lectin polymorphisms with SLE has been repeatedly reported, in spite of absence of significant linkage [reviewed in (20)]. Since NK cells have been functionally implicated in rheumatic diseases (21), genetic association with disease susceptibility needs to be examined at least for the polymorphisms with potential functional relevance.

In a previous study, in the process of variation screening of NKG2 genes, we found that a proportion of the Japanese population lacks the NKG2C gene (also designated as KLRC2), due to homozygous deletion of an ~16-kb genomic region encompassing NKG2C (17).
Genetics of human NKG2C deletion

To establish a genotyping system and to determine the frequency of the NKG2C deletion haplotype, it was essential to map the precise breakpoint. In the present study, we attempted to identify the breakpoint, to determine the frequency of the NKG2C deletion haplotype, and to examine its association with susceptibility to RA and SLE, in Japanese and Dutch populations.

Methods

Subjects

Genotyping was performed using the genomic DNA from 245 healthy Japanese (mean age 32.9 ± 9.4 years) and 105 healthy Dutch individuals. For the case-control association studies, 174 Japanese patients with RA (58.3 ± 11.1 years), and 155 Japanese (41.3 ± 14.1 years) and 89 Dutch patients with SLE (44.8 ± 14.4 years) were also genotyped. Diagnoses of RA, SLE and the classification for the presence of lupus nephritis were based on the American College of Rheumatology criteria (22,23). The subjects were recruited in Tokyo and Groningen respectively. This study was approved by the Institutional Review Boards of the participating institutions.

Determination of the breakpoint

The location of the breakpoint was determined by comparing sequences of the intergenic segments upstream and downstream of the NKG2C gene in the common haplotype with the intergenic sequence between NKG2A (KLRC1) and NKG2E (KLRC3) in the deletion haplotype. High-mol.-wt genomic DNA was isolated from the peripheral blood from individuals previously determined to be homozygous for the common or the deletion type (17), using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

To amplify the intergenic segment upstream of the NKG2C in the common haplotype, primers were placed within the 3'-untranslated region (3'UTR) of NKG2A and the promoter region of NKG2C. The forward and reverse primers were NKG2C1 (5'-GCATTGCGATGTCATGACGATAAAATTG-3') and NKG2C2 (5'-CGGTCTCTTCTCTAAATCATGACCTTATTGTCA-3') respectively. The primers to amplify the intergenic segment downstream of the NKG2C in the common haplotype were placed within the 3'UTR of NKG2C and the promoter region of NKG2E. The forward and reverse primers were NKG2C3 (5'-TTGCGTTTACAGTCATGACGATAAAATTG-3') and NKG2C4 (5'-GTGTTCTTCTCTATAATCATGACCTTATTGTCA-3') respectively. In the sample homozygous for the deletion haplotype, primers NKG2C1 and NKG2C4 were used to amplify the intergenic segment between NKG2A and NKG2E of the deletion haplotype. Each reaction was performed using the Takara LA Taq kit (Takara, Otsu, Japan). The PCR condition consisted of initial denaturation at 96°C for 10 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 66.5°C for 30 s and extension at 72°C for 10 min. The sequences were then determined by direct sequencing using commercial reagents (ABI Prism dRhodamine Terminator Cycle Sequencing-Ready Reaction kit; Applied Biosystems, Foster City, CA).

Genotyping for the deletion haplotype

The principle of the genotyping method is described later in the Results. The primers for PCR#1 were BREAK-F (5'-ACTCCTGTTTCTATTTGATGC-3') and BREAK-R (5'-ACACAGTGTATAAAGAAAG-3'). The PCR condition consisted of initial denaturation at 96°C for 10 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 40 s. The primers for PCR#2 were NKG2CT/F (5'-ATCAATTTAGAATAGGATGC-3') and NKG2CT/R (5'-CGCAAGTTACACCACATCATC-3'). PCR was performed similarly for 27 cycles with the annealing temperature at 57°C. The primers for NKG2A (internal control) were NKG2A3F (5'-TGATCCTACCTCTCTTTCG-3') and NKG2A4R (5'-TATGTAACAGCCTAAGATCAG-3') (17). The diploype frequencies were compared between patients and healthy individuals using χ²-test at the significance level of 5%.

Linkage disequilibrium (LD) analysis

Eight polymorphisms previously described in the NKG2A gene and one in the CD94 gene (17) were analyzed for the LD with NKG2C deletion in the 177 Japanese healthy individuals. The polymorphisms analyzed were: c.±169G → A, c.±11A → G, c.238T → A, c.284→67→62delAAACTT, c.338-90A → G, c.1015C → T, c.1077C → T and c.1146→1150delGATT of NKG2A, and c.±134A → T of CD94. The extent of LD was assessed by Lewontin’s D' (24), using the software package SNPalyze version 2.2 (Dynacom, Tokyo, Japan).

Results

Determination of the breakpoint of the NKG2C deletion

The intergenic segments upstream and downstream of the NKG2C gene were highly homologous (Figs 1 and 2a); therefore, scattered nucleotide substitutions that characterize each sequence were used to determine the location of the breakpoint. The centromeric portion of the intergenic sequence of the deletion haplotype was identical with that of the corresponding region of the upstream segment of the NKG2C in the common haplotype, while the telomeric portion of the intergenic sequence of the deletion haplotype was identical with the downstream segment of the NKG2C in the common haplotype. The breakpoint was found to be within the 292-bp region 1.5–1.8 kb telomeric from the 3'UTR of NKG2A. Around the breakpoint, several nucleotides unique to the deletion haplotype were observed.

Genotyping for the NKG2C deletion haplotype

Based on these findings, we developed a typing system for the NKG2C deletion. The diploype for the presence or absence of NKG2C was determined by the combination of two sets of PCR (Fig. 2a). In PCR#1, the primers were placed at both sides of the breakpoint. Amplification of a 411-bp fragment is observed only in the presence of the NKG2C deletion. In PCR#2, the primers were placed within exon 6 of NKG2C to detect the presence of the common haplotype. Amplification of a 363-bp fragment is observed only in the presence of NKG2C. When amplification was observed both in PCR#1 and #2, the subject was typed as heterozygous for the deletion (Fig. 2b).
Using this system, the diplotype frequencies in the Japanese and Dutch populations were determined (Table 1). Approximately 4% of the general population lacks NKG2C.

The frequency of the NKG2C deletion haplotype was remarkably similar in both populations: 20.2% in Japanese and 20.0% in Dutch. Significant association with the susceptibility to SLE and RA (Table 1) or with lupus nephritis (data not shown) was not observed.

**LD analysis**

To elucidate the extent of the haplotype block that contains the NKG2C deletion, LD with eight NKG2A polymorphisms described in the previous paper (17) were examined. Only the two polymorphisms in the 3'UTR (c.1077C → T and c.1146_1150delGATT) showed strong LD with the NKG2C deletion (D' > 0.9), while the remainder showed only modest to moderate LD (D' < 0.6), indicating that the centromeric boundary of the haplotype containing the NKG2C deletion is within the NKG2A locus. Similarly, only weak LD was observed between the NKG2C deletion and CD94 c.-134A → T (D' = 0.19).

**Discussion**

In this study, we identified the breakpoint of the human NKG2C gene deletion, which we recently found to be commonly present in the general population (17). By comparing the genomic sequences between NKG2A and NKG2E, the breakpoint was determined to be 1.5–1.8 kb telomeric from the 3' end of NKG2A. NKG2 genes are postulated to have developed through gene duplication events and several Alu repeats are present in this region (25,26). The NKG2C deletion is most likely a result of an unequal crossover event; however, although Alu repeats are often involved in gene recombination events (27), the breakpoint of the NKG2C deletion was not located in the Alu sequence.

Around the breakpoint, several nucleotides unique to the deletion haplotype were present, suggesting that these nucleotide substitutions have occurred after the divergence of this haplotype. Interestingly, direct sequencing revealed that the nucleotides unique to the deletion haplotype were shared among Japanese and Dutch individuals, suggesting that the NKG2C deletion haplotype in these populations is a result of a common ancestral event. LD analysis demonstrated...
that the centromeric boundary of the haplotype containing the NKG2C deletion lies within the NKG2A gene and the telomeric boundary does not extend to the CD94 gene, located 110 kb apart from NKG2C. Whether the NKG2C deletion haplotype is in LD with NKG2E or NKG2D polymorphisms needs to be examined in future studies.

We established a genotyping method for the NKG2C deletion, and determined the frequencies of the NKG2C deletion haplotype in Japanese and Dutch populations, which turned out to be remarkably similar in both populations. Although the functional consequences of the NKG2C deficiency remain unclear, the fact that ~4% of the general

Table 1. Diplotype frequency of the NKG2C deletion in the Japanese and Dutch patients and controls

<table>
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<th>RA (n = 176)</th>
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Percentages are shown in parentheses. Statistically significant difference was not observed. Diplotype frequencies of healthy individuals were compatible with the Hardy–Weinberg equilibrium in both populations.

Fig. 2. (A) The position of the breakpoint and the strategy for the NKG2C deletion genotyping. The breakpoint was mapped to the 1.5–1.8 kb telomeric region from the 3′UTR of NKG2A. The diplotype for the presence or absence of NKG2C was determined by the combination of two sets of PCR. In PCR#1, the primers were placed at both sides of the breakpoint. Amplification of a 411-bp fragment is observed only in the presence of the NKG2C deletion. In PCR#2, the primers were placed within exon 6 of NKG2C to detect the presence of the common haplotype. Amplification of a 363-bp fragment is observed only in the presence of NKG2C. As an internal control, primers that amplify the 780-bp fragment of NKG2A exon 3 and exon 4 were used in both reactions. (B) Genotyping for NKG2C deletion haplotype. The diplotype of each individual was determined by using PCR#1 and #2 in combination. Lane 1, common type homozygote (+/+); lane 2, heterozygote (+/del); lane 3, deletion homozygote (del/del). Molecular weight marker used was a 100-bp ladder.
population lacks NKG2C indicates that NKG2C is not essential for survival and reproduction. It is conceivable that lack of NKG2C is compensated by other activating receptors, possibly NKG2E (or its alternatively spliced form NKG2H). Such a possibility is supported by the fact that human NKG2C and NKG2E genes are highly homologous across the carbohydrate recognition domain (92% nucleotide and 90% amino acid by BLAST search), where ligand interactions would occur. In addition, a previous study that reported the normal function and number of NK cells in patients with DAP12 deficiency suggests 'functional redundancy or developmental compensation in target recognition by NK cells' (28).

In the chimpanzee genome, two NKG2C genes (Pt-NKG2C1 and Pt-NKG2CII) are present, which are equally diverged from human NKG2C. In contrast with other genes in the NKC, NKG2C and NKG2E have the highest interspecies divergence, and are also polymorphic within each species (29,30). These data suggest that NKG2C has evolved under relatively weak selection pressures. Our present data indicating the frequent occurrence of the NKG2C deletion in the general population support this possibility. On the other hand, unlike killer cell Ig-like receptors or leukocyte Ig-like receptors (also referred as Ig-like transcripts), NKG2C is conserved among mammalian species from mice to humans, suggesting an essential role throughout mammalian evolution. It should be noted, however, that there may be differences in the expression pattern of the NKG2 receptors between human and mice (31–33), and therefore, although the orthologous genes exist in both species, their functional roles may differ.

Further studies on population genetics, especially on possible associations with susceptibility to viral infections or cancers, as well as on functional consequences are necessary to gain insight into the significance of NKG2C deletion.

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Abbreviations

DAP DNA-activation protein
KLRC killer cell lectin-like receptor, subfamily C
LD linkage disequilibrium
NKC NK gene complex
NKG2 NK cell group 2 subfamily
RA rheumatoid arthritis
SLE systemic lupus erythematosus
UTR untranslated region

References


