Research Article

Identification of the *NKG2D* Haplotypes Associated with Natural Cytotoxic Activity of Peripheral Blood Lymphocytes and Cancer Immunosurveillance

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Abstract

We have previously shown that natural cytotoxic activity of peripheral blood lymphocytes was inversely related to cancer development based on a prospective cohort study. The genetic fraction of cytotoxic activity needs to be clarified, identifying individuals immunogenetically susceptible to cancer. A case-control study within the cohort members was designed: 102 cancer cases with peripheral lymphocyte DNA available and three control groups, each of which consisted of 204 subjects with each tertile level of cytotoxic activity. We first compared two control groups with high and low cytotoxic activity in terms of the single nucleotide polymorphisms in the natural killer complex gene region on chromosome 12p, identifying the haplotype alleles that were associated with the activity. Next, cancer risks were assessed for these haplotypes. We found two haplotype blocks, each of which generated two major haplotype alleles: low-activity-related *LNK1* (frequency 0.478 and 0.615 in groups with high and low activity, respectively; $P < 0.00008$) and high-activity-related *HNK1* (0.480 and 0.348; $P < 0.0001$), *LNK2* (0.711 and 0.821; $P < 0.0002$), and *HNK2* (0.272 and 0.174; $P < 0.0008$). These *NKG2D* haplotype alleles showed a significant difference between cases (0.632 for *LNK1* and 0.333 for *HNK1*) and controls (0.554 for *LNK1* and 0.406 for *HNK1*). The haplotype *HNK1/HNK1* revealed a decreased risk of cancer (odds ratio, 0.471; 95% confidence interval, 0.233-0.952) compared with *LNK1/LNK1*. Individuals who are genetically predisposed to have low or high natural cytotoxic activity can in part be determined by *NKG2D* haplotyping, which in turn reveals an increased or decreased risk of cancer development. (Cancer Res 2006; 66(1): 563-70)

Introduction

The initial mechanism of cancer immunosurveillance is thought to be a tumor-associated antigen nonspecific cytotoxicity that involves natural killer (NK) cells. In numerous past laboratory studies on cancer immunosurveillance, there were clear indications of significant roles played by the natural cytotoxicity of various lymphocytes in preventing the development of cancer (1–5) but it was a difficult task to extrapolate these results to yield an estimation of human cancer risk. One of the most critical questions in immunosurveillance against cancer has been whether interindividual differences of natural immunologic host defense could predict future development of common cancers, including those with no known viral etiology, among healthy individuals. To answer this question, we began a prospective cohort study among a Japanese general population in 1986 using various immunologic and biochemical markers measured at baseline. Using an 11-year follow-up of this cohort study—where the cytotoxic activity (measured at baseline by the isotope release method using K567 as target cells) was categorized into high, medium, and low levels by tertiles—we previously reported that individuals with high or medium levels of natural cytotoxic activity of peripheral blood lymphocytes had a decreased risk of cancer development compared with those with low cytotoxic activity (6). This was the first evidence of the vital role played by natural immunologic defense in the occurrence of common cancers among the general population (who do not have obvious defects in their immune systems), indicating the possible feasibility of cancer immunoprevention and the usefulness of natural cytotoxic activity as a surrogate biomarker for this prevention (7).

It seems unlikely that the wide variations of natural cytotoxic activity among healthy individuals observed in this cohort study can be fully explained by environmental or lifestyle factors alone. A cross-sectional analysis of cohort members estimates the contribution of usual lifestyle to interindividual variations of natural cytotoxic activity to be ∼30% and selected healthy lifestyle factors (e.g., not smoking, regular diet and sleep, proper body weight, moderate physical activity, and less mental stress) are in part associated with increased cytotoxic activity (8, 9). Given the important implications of our previous findings, we feel it is warranted to examine the genetic background underlying individual variations in natural cytotoxic activity, if such exists.

This study thus aims to identify the genetic factors associated with natural cytotoxic activity and then to assess the cancer risk of individuals who are predisposed to have low natural cytotoxic activity based on a phenotype-genotype association analysis and a case-control study within the cohort study. In this phenotype-genotype association analysis, we focused on a 270 kb region within an annotated region of ∼2 Mb called the natural killer complex (NKC) gene region 12p13.2-p12.3 because this 270 kb region contains important NK receptor gene loci, such as *CD94* gene and killer cell lectin-like receptor family genes (10). Of these, we found that the *NKG2D* haplotypes revealed a significant association with the natural cytotoxic activity of individuals. The *NKG2D* gene encodes an activating homodimeric C-type lectin receptor, which is expressed on NK cells, $CD8^{+}\alpha\beta$ T cells, $\gamma\delta$ T cells, and activated macrophages, and is located at the NK complex gene locus (11, 12). The NKG2D triggers cell-mediated cytotoxicity in NK cells via the

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doi:10.1158/0008-5472.CAN-05-2776

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DAP10-phosphoinositol 3-kinase signaling pathway, upon the recognition of their self-ligands, such as MICA, MICB, ULBP1, and ULBP2, which are distantly related to MHC class I (11–16). MICA and MICB are not usually expressed in normal cells but are found at low levels on intestinal epithelial cells; they are induced by cellular stress, typically in tumor or virus-infected cells (17, 18). Recently, NKG2D was reported to be a key factor in priming T-cell immunity as well as a primary cytotoxicity receptor (19).

Next, a case-control study was conducted within the cohort to assess the risk of cancer development on the basis of the *NKG2D* haplotypes: Results indicated that these haplotypes may be associated with immunogenetic susceptibility to cancer development. Along with these findings, our results also show an advantage of molecular epidemiology cohort studies (i.e., they make possible the measurement of phenotype biomarkers that would potentially be influenced by cancer and the subsequent genetic association analyses for both phenotype biomarkers and cancer risk).

Materials and Methods

Study population. We conducted a case-control study within the Saitama prospective cohort study, which began in 1986, with measurement of natural cytotoxic activity of peripheral blood lymphocytes and other immunologic markers among self-selected 3,625 individuals ages over 40 years living in a town in Saitama Prefecture, Japan, who participated in yearly health checks during 1986 to 1990 (accounting for ∼40% of all residents of this age group). We did a follow-up survey on cancer incidence and death from all causes up to 2000: Cancer cases were identified primarily by death certificate and national health insurance receipts, followed by confirmation of primary site, histology, and date of diagnosis through inquiry at the hospitals. This study is described in detail elsewhere (6, 9, 20, 21). Briefly, the cytotoxic activity of peripheral lymphocytes was determined by ^{51}Cr-release assay with an effector-to-target ratio of 20 and incubation of effector and target cells for 3 hours 30 minutes, by using K562, a human myeloid leukemia cell line, as target cells. On the basis of a follow-up study from 1986 to 1997, we previously reported that individuals with high or medium cytotoxic activity revealed a decreased risk of cancer development, with a relative risk of 0.59 [95% confidence interval (CI), 0.40-0.87, estimated for both sexes] or 0.63 (95% CI, 0.43-0.92), respectively, when the cytotoxic activity (percent specific lysis) was categorized by tertiles: ≤42%, 43% to 58%, and >58% for low, medium, and high, respectively, among men; ≤34%, 35% to 51%, and >51% for low, medium, and high among women (corresponding tertiles for men and women were combined for the analysis of both sexes). Of 3,625 participants, a total of 2,063 individuals gave additional peripheral blood samples for DNA extraction.

In an extended follow-up study from 1986 to 2000, we identified 259 cancer incidence cases in all sites, 115 of whom have lymphocyte DNAs available. Of 115 cancer cases with their DNAs available, we further excluded 13 cancer cases who were ages over 75 years at the time of the assay of cytotoxic activity or who were diagnosed within 2 years after the assay of cytotoxic activity, as we had done in our previous analysis (6). The final total was 102 cancer cases (54 men and 48 women) in all sites, with the most frequent cancers being stomach (n = 19), lung (n = 8), and colorectum (n = 5) for men, and stomach (n = 10), colorectum (n = 6), and lung (n = 5) for women.

Assays for immunologic measurements and DNA extraction were done at the health screening checks. DNA was obtained from the participants at their second visit to the health screening checks during the baseline survey because all blood samples at the first visit had to be used for immunologic and biochemical assays. We compared cancer risk (based on tertile levels of the cytotoxic activity) and natural cytotoxic activity between the groups with and without DNA available. No significant differences were found between them (data not shown). Epidemiologic variables (smoking, alcohol consumption, physical activity, body mass index, etc.) in cancer cases and noncancer cohort members also showed no significant differences by the status of DNA extraction. Therefore, we think that a selection bias, even if it exists, did not significantly influence our results.

Two controls, who were individually matched to one case with respect to gender and age (±5 years), were randomly selected from each of the trisected groups with low, medium, and high cytotoxic activity. The final total was 612 controls comprising three groups (204 controls each) with low, medium, and high cytotoxic activity, who showed median 31% (range 5-42%), 51% (43-58%), and 68% (59-90%) among men; 26% (8-34%), 43% (35-51%), and 59% (52-85%) among women.

This case-control study has two purposes: (*a*) identification of genetic factors involved in individually differing cytotoxic activity and (*b*) estimation of cancer risk for these cytotoxic activity–related genetic factors. The former approach was undertaken by comparing the two control groups with low and high cytotoxic activity in terms of frequencies of single nucleotide polymorphisms (SNPs) in a 270 kb region within the NKC gene region on chromosome 12p, called the phenotype-genotype association analysis. The latter was undertaken by comparing cases and entire control groups (with low, medium, and high cytotoxic activity) in terms of odds ratios (OR). The baseline characteristics of cases and controls are shown in Table 1.

This study was approved by the Genome Ethical Committee at the Radiation Effects Research Foundation.

Identification and genotyping of SNPs. The Celera Genomic database (22, 23) was used to screen marker SNPs in the NKC gene region, along with

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Table 1. Baseline characteristics of study subjects

Gender	Cases		Controls selected from cohort members with trisected natural cytotoxic activity					
			High		Medium		Low	
	Men (n = 54)	Women (n = 48)	Men (n = 108)	Women (n = 96)	Men (n = 108)	Women (n = 96)	Men (n = 108)	Women (n = 96)
Age at entry (y)								
40-49	3	9	6	18	6	18	6	18
50-59	15	20	32	40	34	40	32	40
60-69	31	17	63	34	60	34	62	34
70-74	5	2	7	4	8	4	8	4
Mean (SE)	61.6 (0.9)	57.1 (1.2)	60.6 (0.7)	57.3 (0.8)	60.7 (0.6)	57.3 (0.8)	60.8 (0.6)	57.4 (0.9)
Natural cytotoxic activity (percent specific lysis)								
Mean (SE)	48.6 (2.3)	41.4 (2.5)	68.5 (0.7)	60.1 (0.7)	51.2 (0.4)	42.5 (0.6)	29.6 (0.9)	23.8 (0.8)
Range	83-18	89-18	90-59	85-52	58-43	51-35	42-5	34-8
Smokers (%)	32 (60.4)	2 (4.2)	62 (57.4)	4 (4.2)	70 (64.8)	2 (2.1)	72 (67.3)	6 (6.3)

Table 2. Primers used for 20 SNPs

NKC	SNP ID (NCBI)	Forward primer	Variations	Reverse primer
1	rs3759272	TGGGCAAAACACAATGTTCAGAATT	T/G	GGGCGTCAACAAACGAATCTTG
2	rs2537752	TCTGGAGTCTATAAAATGTTTTTAAACAGTGTCA	A/T	TCTCAAATGTAGGTGAACGAATTTCATCA
3	rs1049174	CTGCCCATGAGGCAATTTCC	C/G	GGATCAGTGAAGGAAGAGAAGGC
4	rs2255336	CTGTAGCCATGGGAATCCGTTT	A/G	GCAATCTACTTCTCTGTTGTCACTTACA
5	rs2294148	AGAAACTAAACTAAACTACACAGAGGTTGC	A/G	GATGTGGAGTCAGACTTGAATTTTACTCA
6	rs2049796	AAGCATCTAAGAAACAATTAGAATTACCTTATAGTGTAAATAT	C/A	CAGGTGTGTGTATGTGTGTATGTGT
7	rs2617160	ATGACTAATGTAAAAGTAAAAAAGTCTGCAAACA	A/T	GCCTTGAGTTCATATAATTACAATACACCAGT
8	rs7972757	TGATTGCCATTAAACCTTCCATTTCCT	A/G	GTCGTTAAAGGCATCGTTCCATCTA
9	rs2246809	ACCCTTAAGAGAAAAGGCTTTCATGTAC	A/G	ACTGGTCATTCTGTATTGCCTGTTT
10	rs2617169	GGGATGCAAAATGATAATAAAATGTTTTGGG	A/T	GGAGAAAAGGACATGCCCTCATAT
11	rs2617170	TGACAATCATAATGTACCTTTCTGCATTCT	C/T	CACTTTAATTTTTCTAGGTATTGGAGTACTGGA
12	rs2617171	CCCAAGATAATATGCTGCTTCTGAAC	C/G	TCTCTTAAAACATGTCTTTGAGTCATGAAATCA
13	rs1971939	TCATTGCATATACCTAATGATACAAGTTCAACA	C/G	GGCTCACTGGCCTGTCTT
14	rs1915319	GTATTCTGTATTTGACATAATATTACTAGTGGGAACAAT	A/G	CTATTGGTGTTAAACATTTTTGAAGAATCTAACCTTA
15	rs4763525	AGACATGCCTTTCATGTAAGCATAAAGA	A/G	CCTGGGAGTGGGATTGCT
16	rs3003	TGTACTTTAGTAATTGTGTGCATCCTATTTCA	C/T	GCCCAGTGTGGATCTTCAATGATAT
17	rs1983526	GGCCCTCTGAGGCACTAAATAG	C/G	CAGAGTGGGATCTTTGGTTCATGAT
18	rs10772285	AGCCTCAGTAATGGCAGATGC	C/G	ACTGCCAGCAGAGCATTCT
19	rs1915325	TCACTGGTAAGTAAAGTGTAGTGTATCTGA	A/G	TGTTTATCATTTAGCCACACAAAAGAGC
20	rs2607893	CACCTTATCCCAAGTGCATCAACT	T/C	ACCAATGTAAAACCCATAGCACAGT

the detection of novel SNPs over the region using National Center for Biotechnology Information (NCBI) database: In this region, over 1,300 SNPs have been registered in the Celera Genomic database and NCBI database. We selected the 25 SNPs with allele frequency >10% among either Caucasian or Japanese. After examining allele frequency in the study population, we found that 20 of 25 SNPs actually showed a frequency >10%. We then selected these 20 SNP loci, named NKC-1 to NKC-20, which revealed variant allele frequencies >10% among our study population. The sequences of the primers used for 20 SNPs are listed in Table 2; the SNPs from NKC-1 to NKC-20 cover *CD94, NKG2D, NKG2F, NKG2E, NKG2A*, and *Ly49* genes, and the localization is shown in Fig. 1*A*. Primers and probes for these SNPs were designed using Primer Express software, version 2.1 (Applied Biosystems, Foster City, CA). The TaqMan-Allelic Discrimination method was used for the detection of SNPs. All of the assays were conducted in 384-well PCR plates. The principle of TaqMan Real-Time PCR assay system using fluorogenic probes and the 5′ nuclease is described by Livak (24). Amplification reactions (5 μL) were done in duplicate with 10 ng of template DNA, 1 × TaqMan Universal Master Mix buffer (Applied Biosystems), 300 nmol/L of each primer, and 200 nmol/L of each fluorogenic probe. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then by 40 cycles of 15 seconds at 95°C and of 1 minute at 60°C. After PCR was completed, plates were brought to room temperature, read in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems), and results were analyzed using the Allelic Discrimination software.

Haplotype analysis and risk estimation. The linkage disequilibrium was estimated by relative linkage disequilibrium coefficients (D'), r^2 values, and the χ^2 values. Haplotype allele frequencies and haplotype distributions were estimated on the basis of multiple SNPs by the expectation-maximization algorism, using SNPAlyze (DYNACOM, Yokohama, Japan, http://www.dynacom.co.jp/). Statistical significance was examined by the χ^2 test. ORs were calculated along with 95% CI values using SPSS software program (version 11.1).

Results

Association between SNPs in the NKC region and natural cytotoxic activity. A genome approach was undertaken in the Saitama cohort study. Before case-control comparison, we did a phenotype-genotype association analysis done to identify the genetic factors involved in the natural cytotoxic activity of peripheral blood lymphocytes of individuals. Specifically, we examined the association between the 20 SNPs on the annotated 270 kb region within the NKC gene region and natural cytotoxic activity by comparing the allele frequency of the two control groups with high and low natural cytotoxic activity, together with ORs estimated for low natural cytotoxic activity versus high activity. Among these 20 SNPs, we found, in Table 3, that eight SNPs were closely associated with natural cytotoxic activity, having P values <0.001: NKC-3 (P = 0.00004), NKC-4 (0.0002), NKC-7 (0.00004), NKC-9 (0.0006), NKC-10 (0.0005), NKC-11 (0.00003), NKC-12 (0.00004), and NKC-17 (0.0002). It is notable that these natural cytotoxic activity–related SNPs are mostly located in the *NKG2D* gene region, except for NKC-17 that is located in the promoter region of the *NKG2A* gene (Fig. 1*A*).

Identification of haplotype blocks. We did linkage disequilibrium analysis on the basis of the 20 SNPs listed in Table 2. When looking at natural cytotoxic activity–related SNPs, many of these are closely linked to each other, with r^2 values >0.9, and this kind of close linkage is hardly ever found among other activity-nonrelated SNPs, except NKC-6 and NKC-8 (Fig. 1*B*). On the basis of the linkage disequilibrium analysis, Fig. 1*C* shows the relation between linkage disequilibrium (r^2) and the physical distance between the SNPs. All combinations of each pair of SNPs are plotted. An abrupt drop of r^2 values in the distance >80 kb in Fig. 1*C* implies that there are no haplotype blocks longer than 80 kb in this region. It is of much interest that most combinations of the natural cytotoxic activity–related SNPs revealed relatively strong linkage disequilibrium, whereas those of nonrelated SNPs showed weak or no linkage disequilibrium. When we divided the natural cytotoxic activity–related SNPs into two groups colored blue and orange, all combinations of blue-blue and orange-orange revealed a strong linkage disequilibrium, with r^2 values >0.9, whereas blue-orange combinations showed much weaker linkage disequilibrium, with r^2

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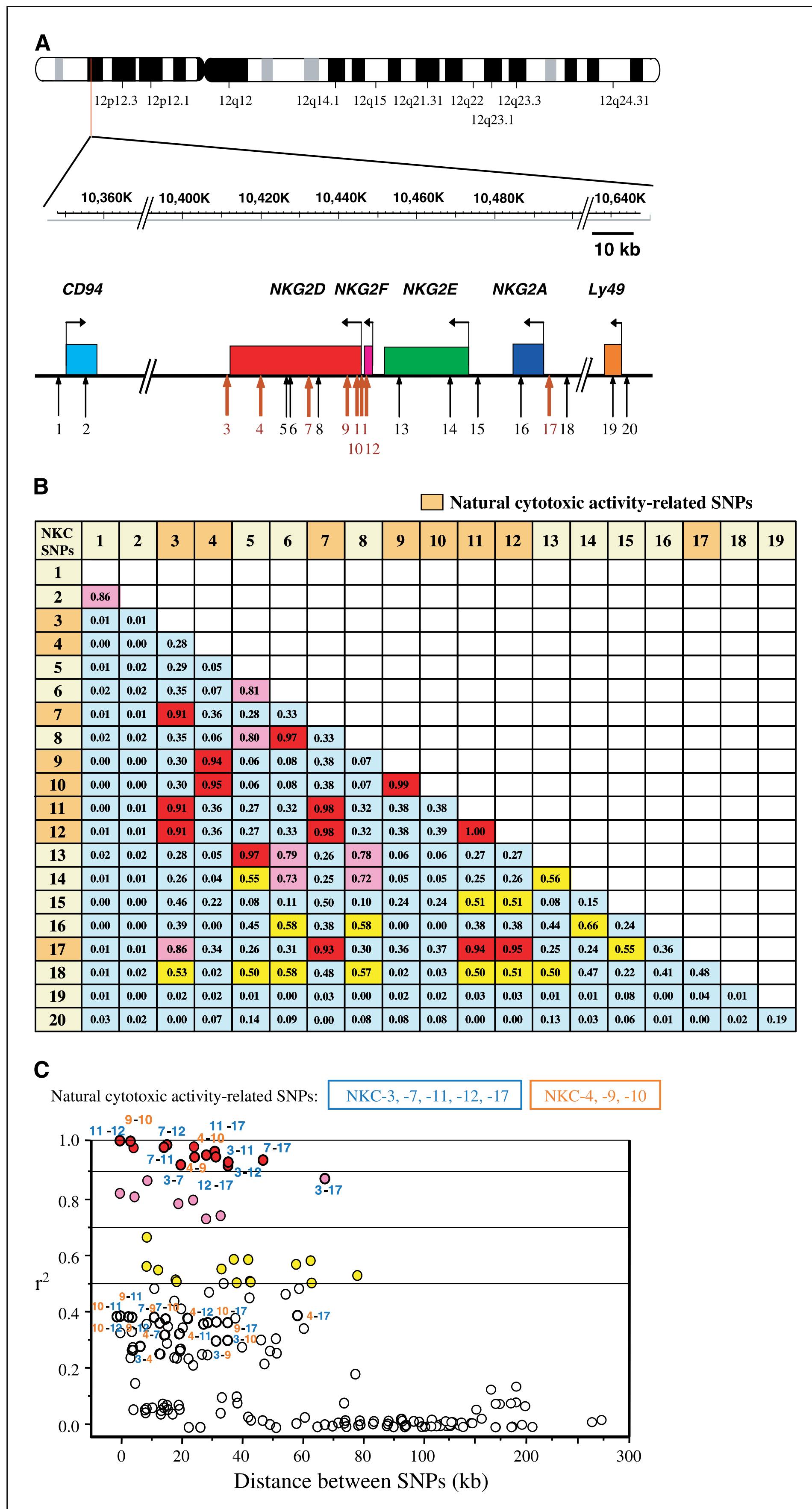

NKC SNPs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	0.86																		
3	0.01	0.01																	
4	0.00	0.00	0.28																
5	0.01	0.02	0.29	0.05															
6	0.02	0.02	0.35	0.07	0.81														
7	0.01	0.01	0.91	0.36	0.28	0.33													
8	0.02	0.02	0.35	0.06	0.80	0.97	0.33												
9	0.00	0.00	0.30	0.94	0.06	0.08	0.38	0.07											
10	0.00	0.00	0.30	0.95	0.06	0.08	0.38	0.07	0.99										
11	0.00	0.01	0.91	0.36	0.27	0.32	0.98	0.32	0.38	0.38									
12	0.01	0.01	0.91	0.36	0.27	0.33	0.98	0.32	0.38	0.39	1.00								
13	0.02	0.02	0.28	0.05	0.97	0.79	0.26	0.78	0.06	0.06	0.27	0.27							
14	0.01	0.01	0.26	0.04	0.55	0.73	0.25	0.72	0.05	0.05	0.25	0.26	0.56						
15	0.00	0.00	0.46	0.22	0.08	0.11	0.50	0.10	0.24	0.24	0.51	0.51	0.08	0.15					
16	0.00	0.00	0.39	0.00	0.45	0.58	0.38	0.58	0.00	0.00	0.38	0.38	0.44	0.66	0.24				
17	0.01	0.01	0.86	0.34	0.26	0.31	0.93	0.30	0.36	0.37	0.94	0.95	0.25	0.24	0.55	0.36			
18	0.01	0.02	0.53	0.02	0.50	0.58	0.48	0.57	0.02	0.03	0.50	0.51	0.50	0.47	0.22	0.41	0.48		
19	0.01	0.00	0.02	0.02	0.01	0.00	0.03	0.00	0.02	0.02	0.03	0.03	0.01	0.01	0.08	0.00	0.04	0.01	
20	0.03	0.02	0.00	0.07	0.14	0.09	0.00	0.08	0.08	0.08	0.00	0.00	0.13	0.03	0.06	0.01	0.00	0.02	0.19

Figure 1. Identification of haplotype blocks. *A,* 20 SNPs examined in the 270 kb region within the NKC gene region (*arrows with numbers from 1 to 20*). *Red arrows,* eight SNPs closely associated with natural cytotoxic activity with $P < 0.001$. *B,* linkage disequilibrium analysis. Brown SNPs (*3, 4, 7, 9, 10, 11, 12,* and *17*) are natural cytotoxic activity-related SNPs with $P < 0.001$; *red elements in the lower triangle,* close linkage disequilibrium with $r^2 \geq 0.9$; *pink,* $0.9 > r^2 \geq 0.7$; *yellow,* $0.7 > r^2 \geq 0.5$. *C,* relation between linkage disequilibrium (r^2) and the physical distance between the SNPs. All combinations of every pair of SNPs among the 20 are plotted. *Redplot,* $r^2 \geq 0.9$; *pinkplot* $0.9 > r^2 \geq 0.7$; *yellow plot,* $0.7 > r^2 \geq 0.5$. *Numbers in plots,* the combined two NKC SNPs belonging to natural cytotoxic activity–related SNPs (*blue* or *orange*).

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values <0.5 (Fig. 1*C*), indicating that five blue SNP sites belong to one haplotype block and three orange SNP sites to a different haplotype block. We finally identified the two haplotype blocks and named them NKG2D hb-1 and hb-2, each of which generated two major haplotype alleles related to low and high natural cytotoxic activity phenotypes (Fig. 2*A*).

Association between *NKG2D* haplotypes and natural cytotoxic activity. We estimated the haplotype allele frequencies in the groups with high and low natural cytotoxic activity and compared between groups (Fig. 2*B*). Respective low and high cytotoxic activity–related alleles *LNK1* and *HNK1* on NKG2D hb-1 revealed a close association with natural cytotoxic activity (P = 0.00008 and 0.0001, respectively) and this was also the case with *LNK2* and *HNK2* on NKG2D hb-2 (P = 0.0002 and 0.0008, respectively). To confirm the close association between natural cytotoxic activity and *NKG2D* haplotypes, we compared mean (±SE) natural cytotoxic activity of *LNK1/LNK1, LNK1/HNK1*, and *HNK1/HNK1* haplotypes among a total of 612 controls: The results were 42.1 ± 1.2 (n = 196), 47.8 ± 1.1 (260), and 50.1 ± 1.7 (109), respectively ($P_{trend} < 0.001$); 47 controls having heterozygous haplotypes other than *LNK1/HNK1* showed mean natural cytotoxic activity of 45.1 ± 2.7.

***NKG2D* haplotypes and cancer risk.** Finally, we estimated the risk of cancer development for the *NKG2D* haplotypes: *LNK1/LNK1, LNK1/HNK1*, and *HNK1/HNK1* from NKG2D hb-1 along with *LNK2/LNK2, LNK2/HNK2*, and *HNK2/HNK2* from NKG2D hb-2. A case-control study within the Saitama cohort study was done among those cohort members whose DNA of peripheral lymphocytes were available for this study. In Table 4, cases revealed increased and decreased frequencies (0.632 and 0.333, respectively) of *LNK1* and *HNK1* alleles, compared with those (0.554 and 0.406, respectively) in controls (Table 4). Individuals carrying *HNK1/HNK1* have a significantly reduced risk of cancer with an OR of 0.471 (crude, 95% CI, 0.233-0.952) or 0.482 (adjusted, 0.237-0.982), indicating that those with *LNK1/LNK1*, one third of the general population, have an enhanced risk of cancer development (Table 4). On the other hand, *LNK2* and *HNK2* alleles did not show any statistically significant differences between cases and controls because of the small number of subjects with *HNK2/HNK2*.

Discussion

Natural immunologic host defense plays the key role in occurrence of common cancers found in a general population, as we previously reported on the basis of an 11-year follow-up of the Saitama cohort study (6). This finding could lead us to a new field, cancer immunoprevention, which would aim to enhance the ability of the immune system to recognize and

Table 3. Eight SNPs closely associated with natural cytotoxic activity

NKC (reference SNP ID)	Genotype	No. subjects (%)		OR (95% CI)
		High activity	Low activity	
NKC-3 (rs1049174)	*C/C*	53 (26)	89 (44)	1.00
	C/G	102 (50)	88 (43)	0.514 (0.330-0.801)
	G/G	49 (24)	27 (13)	0.328 (0.184-0.568)
	Fr. of *C*-allele	0.510	0.652	P = 0.00004
NKC-4 (rs2255336)	*G/G*	107 (52)	139 (68)	1.00
	G/A	79 (39)	59 (29)	0.575 (0.377-0.876)
	A/A	18 (9)	6 (3)	0.257 (0.098-0.669)
	Fr. of *G*-allele	0.718	0.826	P = 0.0002
NKC-7 (rs2617160)	*T/T*	51 (25)	84 (41)	1.00
	T/A	101 (50)	93 (46)	0.559 (0.357-0.875)
	A/A	52 (25)	27 (13)	0.315 (0.176-0.563)
	Fr. of *T*-allele	0.498	0.640	P = 0.00004
NKC-9 (rs2246809)	*G/G*	107 (53)	137 (67)	1.00
	G/A	80 (39)	61 (30)	0.596 (0.392-0.905)
	A/A	17 (8)	6 (3)	0.276 (0.105-0.723)
	Fr. of *G*-allele	0.721	0.821	P = 0.0006
NKC-10 (rs2617169)	*T/T*	106 (52)	137 (67)	1.00
	T/A	81 (40)	61 (30)	0.583 (0.384-0.885)
	A/A	17 (8)	6 (3)	0.273 (0.104-0.717)
	Fr. of *T*-allele	0.718	0.821	P = 0.0005
NKC-11 (rs2617170)	*C/C*	49 (24)	83 (41)	1.00
	C/T	102 (50)	93 (45)	0.538 (0.343-0.845)
	T/T	53 (26)	28 (14)	0.312 (0.175-0.556)
	Fr. of *C*-allele	0.490	0.635	P = 0.00003
NKC-12 (rs2617171)	*C/C*	49 (24)	83 (41)	1.00
	C/G	103 (50)	93 (45)	0.533 (0.340-0.837)
	G/G	52 (26)	28 (14)	0.318 (0.178-0.567)
	Fr. of *C*-allele	0.493	0.635	P = 0.00004
NKC-17 (rs1983526)	*G/G*	47 (23)	78 (38)	1.00
	G/C	104 (51)	95 (47)	0.550 (0.349-0.869)
	C/C	53 (26)	31 (15)	0.352 (0.199-0.625)
	Fr. of *C*-allele	0.485	0.615	P = 0.0002

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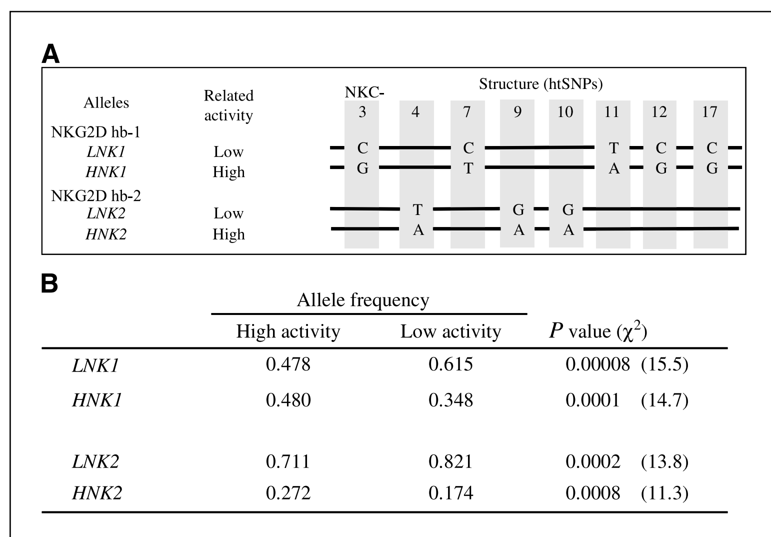

	Allele frequency		
	High activity	Low activity	P value (χ^2)
LNK1	0.478	0.615	0.00008 (15.5)
HNK1	0.480	0.348	0.0001 (14.7)
LNK2	0.711	0.821	0.0002 (13.8)
HNK2	0.272	0.174	0.0008 (11.3)

Figure 2. Natural cytotoxic activity–related haplotype alleles. *A, LNK1* and *HNK1* are generated from haplotype block NKG2D hb-1, and *LNK2* and *HNK2* from NKG2D hb-2. *B,* allele frequencies are estimated for groups (n = 408 chromosomes for each group) with high and low natural cytotoxic activity.

eliminate nascent transformed cells in the body (7). The innate immune system, in its initial response to a pathogen, may also be involved in determining how long and how strongly inflammation will continue after pathogen infection, in some cases leading to a sequential process of infection to inflammation to cancer (25, 26).

The natural cytotoxic activity measured in the Saitama cohort study revealed wide variations among individuals, only a part of which can be explained by environmental factors. We thus investigated genetic determinants of this cytotoxic activity, where NK cells work as a major effector. Given that the varying cancer risk of individuals can be in part ascribed to natural cytotoxic activity, it is necessary to clearly assess the genetic/invariable fraction of the cytotoxic activity so that we can look at the variable fraction of the activity, which would be a surrogate marker for cancer immunoprevention. In this study, we succeeded in identifying haplotype alleles, which were constructed from five or three SNPs mostly located in the *NKG2D* gene region and closely associated with high and low natural cytotoxic activity of individuals. This was the first identification of

Table 4. Risk of cancer incidence for the *NKG2D* haplotypes

NKG2D hb-1

Haplotype	Cases n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted OR* (95% CI)
LNK1/LNK1	42 (41)	196 (32)	1.00	1.00
LNK1/HNK1 †	42 (41)	260 (42)	0.754 (0.473-1.20)	0.694 (0.430-1.12)
HNK1/HNK1	11 (11)	109 (18)	0.471 (0.233-0.952)	0.482 (0.237-0.982)
	Allele frequency		P	
LNK1	0.632	0.554	0.036	
HNK1	0.333	0.406	0.049	

NKG2D hb-2

Haplotype	Cases n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
LNK2/LNK2	67 (65)	371 (61)	1.00	1.00
LNK2/HNK2 ‡	26 (25)	203 (33)	0.709 (0.437-1.15)	0.701 (0.425-1.16)
HNK2/HNK2	3 (3)	27 (4)	0.615 (0.181-2.09)	0.642 (0.188-2.19)
	Allele frequency		P	
LNK2	0.789	0.778	0.7	
HNK2	0.171	0.212	0.2	

*Adjusted for relative body weight, cigarette smoking, alcohol consumption, and intake of green vegetables.
†Seven cases and 47 controls with heterozygous haplotypes other than *LNK1/HNK1* were excluded.
‡Six cases and 11 controls with heterozygous haplotypes other than *LNK2/HNK2* were excluded.

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individuals who are genetically predisposed to have low natural cytotoxic activity and consequent high risk of cancer development: It is they who will, therefore, be the logical targets for immunoprevention of cancer and virus-related diseases. Our preliminary analysis implied that the influence of lifestyle factors on the cytotoxic activity of individuals might depend on their haplotypes, e.g., cigarette smokers with *HNK1/HNK1* showed lower activity than nonsmokers with the same haplotype, although this decrease was not obvious in other haplotypes; increased intake of green vegetables was associated with increased cytotoxic activity among those with *LNK1/LNK1* but not *HNK1/HNK1* (data not shown). Although an intervention study is needed to confirm the influence of lifestyle factors, this preliminary finding suggests the possibility of individualized cancer prevention based on gene-environment interactions.

Because no strong linkage disequilibrium spanning over 80 kb was found in the 270 kb region, the five or three cytotoxic activity–related SNPs located on NKG2D hb-1 or hb-2, respectively, apparently include the SNP(s) carrying functional significance, although all these SNPs showed high significance levels of association. These five or three SNPs (Table 3) are located in the noncoding regions of the genes and it is likely that some of these SNPs may be involved in transcription regulation of the *NKG2D* or *NKG2A* gene; we excluded the possibility of as-yet-undiscovered SNPs in the coding region closely linked to the five or three SNPs by scanning the *NKG2D* gene region with denaturing high-performance liquid chromatography (data not shown). Further investigation is needed to identify which SNP(s) carries functional significance and to clarify the molecular mechanisms of individually differing cytotoxic activity.

Further investigation will also be needed of the genetic factors, other than the *NKG2D* haplotypes, involved in individual natural cytotoxic activity, specifically the genetic polymorphisms of killer immunoglobulin-like receptor (*KIR*) genes and human histocompatibility leukocyte antigen (*HLA*) class I genotypes (10, 27, 28). The involvement of HLA class I in NK cell repertoire selection leads to the hypothesis that HLA class I may play a role in determining individual NK cell activity, so we examined this hypothesis using the same cohort groups (with high and low natural cytotoxic activity) by comparing the frequency of *HLA class I* (*HLA-A, HLA-B*, and *HLA-C*) genotypes between the groups: Specific *HLA* genotypes of *B*1301, B*4403, B*5401, Cw*0401*, and *Cw*0702* showed significant association with cytotoxic activity (29). This implies that the polymorphisms of other immunorelated genes may also be associated with natural cytotoxic activity—immunogenetic susceptibility to cancer and other diseases. In the future, the combination of these genetic polymorphisms with the *NKG2D* haplotypes will provide more precisely defined, individually based descriptions of innate immune responses.

Our findings in this study show the advantage of molecular epidemiology cohort studies—a combination of phenotype and genotype markers. One possible combination would be to assess the cancer risk of genetic factors, which is modified by environment or other host factors described by phenotype markers, as was typically shown in the Shanghai prospective cohort study (30). This Saitama cohort study reveals another possibility: a phenotype-genotype association analysis combined with subsequent genome association analysis (risk estimation) done within the same cohort study. In a case-control study design within the cohort, we may be able to identify the genetic factors involved in a particular phenotype marker with a high degree of reliability by comparing the genome characteristics of two control groups who are matched to each other with major confounding factors (e.g., gender and age) and who show contrasting high and low values of this phenotype marker. We anticipate that this approach will provide useful information for future cancer prevention based on gene-environment interactions.

Acknowledgments

Received 8/9/2005; revised 10/11/2005; accepted 10/17/2005.

Grant support: Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

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