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# Hepatitis C Virus Core Protein Modulates Fatty Acid Metabolism and Thereby Causes Lipid Accumulation in the Liver

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We studied the roles of hepatitis C virus (HCV) core protein in hepatic steatosis and changes in hepatic lipid metabolism. HCV core protein expression plasmid was transfected in HepG2. Triacylglyceride (TG) and mRNA level associated with lipid metabolism were measured. Male C57BL/6 mice were infected with HCV core recombinant adenovirus and used for lipids and mRNA studies. In HCV core protein-expressing cells, peroxisome proliferator-activated receptor (PPAR) $\alpha$ , multidrug resistance protein (MDR) 3, and microsomal triglyceride transfer protein (MTP) were down-regulated 48 hr after transfection. In HCV core protein-expressing mice, hepatic TG content and hepatic thio-barbituric acid-reactive substances increased. PPAR $\alpha$ , MDR2, acyl-CoA oxidase (AOX), and carnitine palmitoyl transferase-1 (CPT-1) were down-regulated. HCV core protein down-regulated lipid metabolism-associated gene expression, Mdr2, CPT, and AOX, accompanied by down-regulation of PPAR $\alpha$ . These findings may contribute to the understanding of HCV-related steatosis, induction of reactive oxygen species, and carcinogenesis.

**KEY WORDS:** HCV core protein; steatosis; nuclear receptor; ABC transporter.

Chronic hepatitis C virus (HCV) infection results in necroinflammatory liver disease that is characterized by the insidious progression of hepatic fibrosis and the loss of functioning hepatocytes (1–3). Little is known about the molecular mechanisms underlying liver injury due to infection with this virus, but a cell-mediated immune response associated with prominent lymphocytic infiltration of hepatic tissues is thought to play a major role (4, 5). In addition, various observations have suggested that nonimmune mechanisms may also play an important role. These

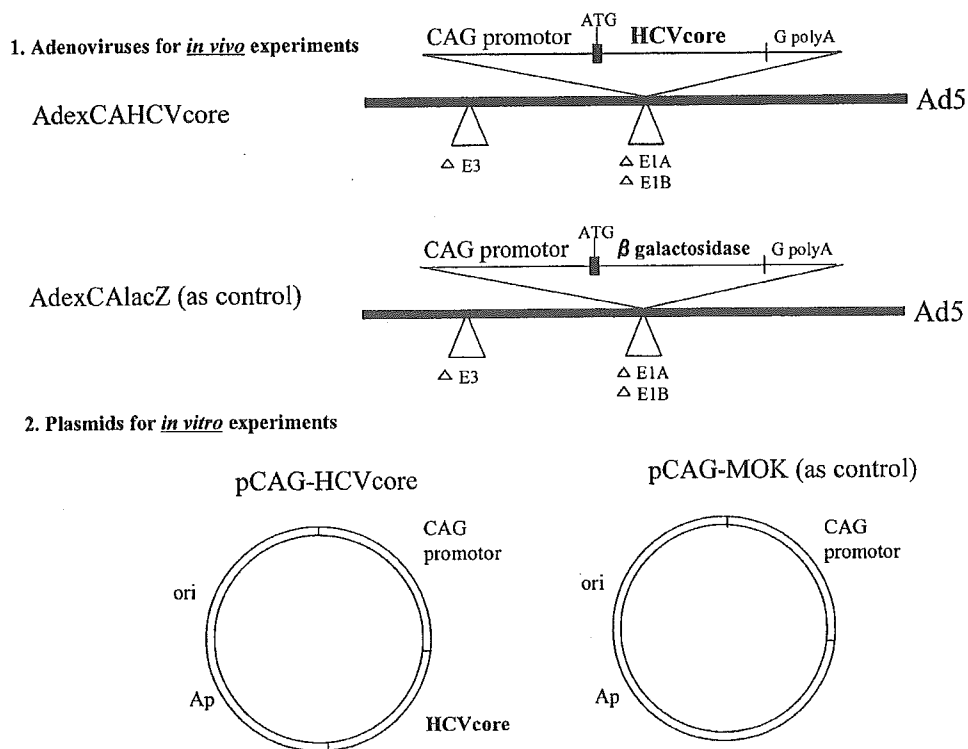
findings include the frequent presence of hepatic steatosis in patients with chronic hepatitis C, an abnormality that is not often observed in other inflammatory conditions such as autoimmune hepatitis and chronic hepatitis B (6–9). Also, a considerable number of *in vitro* studies have suggested that expression of various HCV proteins may lead to alterations of lipid metabolism and transport, cell cycle dysregulation, increased or decreased susceptibility to apoptosis, and cellular transformation (10–17). In particular, HCV core protein has been suggested to contribute to hepatic steatosis (18–20), induction of reactive oxygen species (ROS) (19–21), and hepatic carcinogenesis (22).

Regarding HCV core protein-induced steatosis, the following findings have been reported: (a) HCV core protein interacts with apoA2, a major component of high-density lipoprotein (10, 23), (b) HCV core protein interferes with the assembly of very low-density lipoprotein (VLDL) by reducing the level of microsomal triglyceride transfer

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**Fig 1.** Constructs of recombinant adenoviruses and plasmids employed in this study. See Materials and Methods. ATG, start codon; G poly(A), rabbit  $\beta$ -globin poly(A); CAG promoter; cytomegalovirus enhancer, chicken  $\beta$ -actin promoter, and rabbit  $\beta$ -globin poly(A); Ad5, adenovirus type 5 genome lacking E1A, E1B, and E3.

protein (24), and (c) HCV core protein causes steatosis due to mitochondrial toxicity and production of ROS (19, 20). However, the details of the interaction between HCV and lipid metabolism remain unclear. Hepatocytes represent the crossroads of various metabolic pathways, so HCV may interfere with lipid metabolism via one or several pathways. To investigate the role of HCV core protein in steatosis and the accompanying changes in hepatic lipid metabolism, we focused on fatty acid metabolism-associated proteins, including those involved in fatty acid oxidation and lipid transport into blood and bile, as well as nuclear receptors.

## MATERIALS AND METHODS

**Plasmid and Recombinant Adenovirus.** The complementary DNA clone of the full-length HCV core protein (amino acids [aa] 1–191) was derived from the serum of a patient with HCV 1b by reverse transcription and nested polymerase chain reaction. First-strand primers were 5'-CTGCTAGCCGAGTAGTGTG-3' and 5'-CATTGAGGACCACCAGGTTCT-3', while second-strand primers were 5'-CGGGAATTCTCGTAGACCGTGACCATGAGC-3' and 5'-GTTGGGATCCTCTAAGCGGAAGCTGGAT-3'. The gene was inserted into pBluescript (Stratagene,

La Jolla, CA, USA) and cloned. Then it was made to correspond with HCV 132996 (GenBank) using a QuikChange Site-Directed Mutagenesis kit (Stratagene). The HCV core protein expression plasmid (pCAG-HCVcore), a control plasmid (MOK), and a  $\beta$ -galactosidase expression plasmid (pCAG-LacZ) were prepared using an adenovirus expression vector kit (Takara Biotechnology, Tokyo) (25, 26). The HCV core gene was inserted into the *Swa*I site in cosmid vector pAxCawt, which is a 44.741-kilobase cosmid containing a 31-kilobase adenovirus type 5 genome lacking the E1A, E1B, and E3 genes, but including the cytomegalovirus enhancer, chicken  $\beta$ -actin promoter, and rabbit  $\beta$ -globin poly(A) signal (pAxCaiHCVcore). The cosmid vector pAxCaiLacZ, with the  $\beta$ -galactosidase gene inserted into pAxCawt, was included in the adenovirus expression vector kit. These three vectors (pAxCawt, pAxCaiHCVcore, and pAxCaiLacZ) were digested at the *Sa*I site and ligated, yielding the pCAG-MOK, pCAG-HCVcore, and pCAG-LacZ expression plasmids for cell transfection experiments. The cosmid pAxCaiHCVcore or pAxCaiLacZ was cotransfected into 293 cells with adenovirus DNA by calcium phosphate precipitation. Incorporation of the expression cassette was confirmed by digestion with *Cla*I. Recombinant adenovirus (AdexCAHCVcore or AdexCALacZ) was propagated in 293 cells and the viral titer was determined as the 50% tissue culture infectious dose using 293 cells. These viruses were used for animal experiments (Figure 1).

**Cell Culture.** HepG2 cells were seeded into 56-cm<sup>2</sup> tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM)

(Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and an antibiotic/antimycotic mixture (100 U/ml each) (Gibco) and were cultured in a humidified incubator (5% CO<sub>2</sub>) at 37°C. The medium was replaced with fresh medium every 3–4 days. Prior to each experiment, the cells were seeded into 6- or 12-well plates and allowed to attach for at least 24 hr (6-well for triglyceride [TG] assay and 12-well for RNA extraction).

**Transfection.** Using SuperFect Transfection Reagent (Qiagen, Tokyo), cells were transfected with 4 or 3 µg of pCAG-HCVcore or pCAG-MOK (4 µg for 6-well plates and 3 µg for 12-well plates) and were cultured in DMEM with 10% FBS. After 24 or 48 hr, the cells were harvested for analysis. The efficiency of transfection was investigated using pCAG-LacZ. Cells were washed with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS. Then the cells were stained with X-gal using a β-Gal Staining Set (Roche, Tokyo).

**Animals.** Adult male C57BL/6 mice (Charles River Laboratories, Yokohama, Japan), which were over 8 weeks old and weighed 21–24 g, were used in this study. All animals were housed in an environmentally controlled facility with a 12-hr lighting time (lights on from 0700 until 1900 hr). They were given free access to standard chow and water. Experiments (intravenous injection and sacrifice) were performed from 0900 to 21 hr. The animals received humane care according to the institutional guidelines for handling experimental animals.

**HCV Core Protein Expression in Mice.** The animals received an intravenous injection of  $1 \times 10^9$  pfu (plaque-forming units) of AdexCAHCVcore or AdexCALacZ and were sacrificed 3 days later. Mice were anesthetized with pentobarbital (100 mg/kg intraperitoneally). Blood was collected by cardiac puncture with a heparinized syringe, after which the liver was rapidly removed, weighed, and perfused with ice-cold PBS (pH 7.4). Part of the liver was fixed in 10% neutral buffered formalin and embedded in paraffin for histologic analysis. Another part was stored in RNA later reagent (Qiagen, Tokyo) at 4°C for extraction of RNA, and the remaining liver tissue was snap-frozen in liquid nitrogen and stored at –80°C until required. Plasma was immediately separated by centrifugation (10,000 rpm at 4°C) and stored at –20°C.

**Liver Histology and Serum ALT Level.** Sections of liver tissue (4 µm thick) were stained with hematoxylin and eosin for analysis. The serum alanine aminotransferase (ALT) level was measured using an automated technique by SRL Co. (Hiroshima, Japan).

**HCV Core Protein Expression in Cells.** Proteins were extracted from cells using PRO-PREP protein extraction solution (containing 1.0 mM PMSF, 1.0 mM EDTA, 1 µM pepstatin, 1 µM leupeptin, and 1 µM aprotinin) (Intron Biotechnology, Kyungki-Do, Korea). HCV core antigen levels were measured in cells using an HCV core antigen enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics K.K., Tokyo).

**HCV Core Protein Expression in Mice.** We confirmed HCV core protein expression in liver tissue by Western blot analysis. Proteins were extracted using PRO-PREP protein extraction solution. Then 50 µg of protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a tank blotting system according to the manufacturer's instructions (Bio-Rad Laboratories). After transfer, the membrane was blocked for 2 hr at room temperature with 5%

powdered skim milk dissolved in Tris-buffered saline containing 0.05% between 20 and then incubated overnight at 4°C with a monoclonal mouse antibody to HCV core protein (kindly provided by Ortho-Clinical Diagnostics K.K.). Immune complexes were detected using alkaline phosphatase-conjugated anti-mouse IgG (Cosmo Bio, Tokyo) according to the manufacturer's instructions (Bio-Rad Laboratories). Detection of HCV core protein was performed by comparison with the following standards: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

**Measurement of Triglyceride Content.** After the medium was removed, the cells were washed three times with PBS and resuspended in 200 µl of PBS. Then lipids were extracted from 100 µl of PBS by the method of Bligh and Dyer (27) and resuspended in 100 µL of 10% Triton X. The cellular content of TG was measured using enzyme reagents and standards from Wako (Osaka, Japan). The remainder of the PBS suspension was used for the protein assay. In mice experiments, livers were homogenized in PBS and 100 µl of the homogenate was used for extraction of lipids. Total protein was measured with protein assay reagents from Bio-Rad (Richmond, CA, USA).

**Hepatic Level of Thiobarbituric Acid-Reactive Substances (TBARS).** The hepatic level of TBARS was measured using an OXI-TEK TBARS Assay Kit (Zeptometrix Corporation, New York, USA). Briefly, 100 mg of liver tissue was homogenized in 10 vol of normal saline. Then 100 µl of SDS and 2.5 ml of TBA/buffer reagent were added to 100 µl of this homogenate or the malondialdehyde standard. Samples were incubated at 95°C for 60 min, cooled in an ice bath for 10 min, and centrifuged at 3000 rpm for 15 min, after which the supernatant was analyzed by spectrophotometry (532 nm).

**Extraction of RNA and RT-PCR.** The medium was removed and the cells were washed twice with PBS. After centrifugation, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Tokyo). From mouse, 20 mg of liver tissue was used for RNA extraction. Then 2 µg of total RNA was employed for reverse transcription using random hexamers (final concentration: 2.5 µM) and murine leukemia virus reverse transcriptase (final concentration: 2.5 U/µl) (Roche, Tokyo). Specific primer sets were synthesized for performance of the PCR (Table 1) and were used for assessment of liver-predominant mitochondrial carnitine palmitoyl transferase-1 (CPT1A in humans and CPT1 in mice; the rate-limiting enzyme of mitochondrial β-oxidation), acyl-CoA oxidase (ACO1 in humans and AOX in mice; the rate-limiting enzyme of peroxisomal β-oxidation), cytochrome P-450 4A11 (CYP4A11; involved in microsomal ω-oxidation), multidrug resistance protein 3 (MDR3 in humans and Mdr2 in mice; an ABC transporter and phospholipid flippase), microsomal TG transfer protein (MTP: a vital protein for TG incorporation into VLDL), and two nuclear receptors (peroxisome proliferator-activated receptor α [PPARα]) and peroxisome proliferator-activated receptor γ (PPARγ). Roles of these genes are summarized in Table 2. Amplification involved 30 cycles of denaturation at 95°C for 60 sec, annealing at each specified temperature (Table 1) for 30 sec, and extension at 72°C for 60 sec. The reaction products were analyzed on a 2% agarose gel and were visualized by ethidium bromide staining. The PCR products were excised from the gel, purified using a gel purification kit (Qiagen), and quantified by spectrophotometry. Dilutions

TABLE 1. PRIMER SETS IN THE EXPERIMENTS

|               | Forward                  | Reverse                      | Annealing temp. (°C) |
|---------------|--------------------------|------------------------------|----------------------|
| <b>Human</b>  |                          |                              |                      |
| GAPDH         | GAACGGGAAGCTCACTGGCATGGC | TGAGGTCCACCCCTGTTGCTG        | 65                   |
| PPAR $\alpha$ | GGAAAGCCCACTCTGCCCCCT    | AGTCACCGAGGAGGGGCTCGA        | 63                   |
| PPAR $\gamma$ | CAITCTGGCCACCAACTTTGG    | TGGAGATGCAGGCTCCACTTTG       | 63                   |
| MDR3 (ABCB4)  | GATGAAAAGGCTGCCACTAG     | TTGCACTTCTGTGCTTCAC          | 62                   |
| MTP           | GGCTAGCCTATTTTCAGACACA   | GATGAGCCTGGTAGGTCAC          | 60                   |
| CPT1A         | AGACGGTGGAACAGAGGCTGAAG  | TGAGACCAAACAAAGTGATGATGTCAG  | 67                   |
| ACO1          | GGGCATGGCTATTTCTCATTCG   | CGAACAAGGTCAACAGAAGTTAGGTTTC | 60                   |
| CYP4A11       | GTGGCCCAACCCAGAGGT       | TCCCAATGCAGTTCCTTGATC        | 55                   |
| <b>Mouse</b>  |                          |                              |                      |
| GAPDH         | AGAACATCCCTGCATCC        | TTGTCATTGAGAGCAATGCC         | 56                   |
| PPAR $\alpha$ | TGCAGAGCAACCATCCAG       | TAATGGCGAATTATAAAC           | 50                   |
| PPAR $\gamma$ | GGTGAAACTCTGGGAGATTC     | CAACCATTGGGTCAGCTCTT         | 59                   |
| Mdr2 (Abcb4)  | TATCCGCTATGGCCGTGGGAA    | ATCGGTGAGCTATCACAATGG        | 56                   |
| MTP           | TGAGCGGCTATACAAGCTCAC    | CTGGAAGATGCTCTTCTCGC         | 60                   |
| LCPT          | CGCACGGAAGGAAAATGG       | TGTGCCCAATATTCCTGG           | 52                   |
| AOX           | CTTGTTTCGCGCAAGTGAGG     | CAGGATCCGACTGTTTACC          | 56                   |

ranging from  $3 \times 10^{-5}$  to  $3 \times 10^2$  pg were prepared in water and used as the standards.

**Quantitative PCR.** Quantitative PCR was performed using the Light-Cycler Fast-Start DNA Master SYBR Green system (Roche Molecular Biochemicals, Tokyo). PCR was carried out in a final reaction volume of 20  $\mu$ l using 1  $\mu$ l of each primer at 10  $\mu$ M (final concentration: 0.5  $\mu$ M), 1.6  $\mu$ l of 25 mM MgCl<sub>2</sub> (final concentration: 3 mM), 2  $\mu$ l of the enzyme mix supplied, 12.4  $\mu$ l of H<sub>2</sub>O, and 2  $\mu$ l of the template. The enzyme mix contained the reaction buffer, Fast-Start Taq DNA polymerase, and DNA double strand-specific SYBR Green I dye for detection of PCR products. PCR was performed in a Light-Cycler (Roche) with preincubation for 10 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 5 sec at each specified temperature (see Table 1), and extension for 25 sec at 72°C, with fluorescent detection at the end of extension. Next, the PCR products were subjected to melting curve analysis to exclude the amplification of primer dimmers or other nonspecific products. If primer dimmers and nonspecific bands were detected, fluorescence detection was repeated after extension at each specified temperature for 1 sec. Analysis was carried

out with Light-Cycler 3.5 software (Roche). Quantification was done using the "point fitting" mode and baseline adjustment. The standard curve for each gene was created using five different dilutions. The plot of the number of PCR cycles versus log concentration was considered reliable when the error was <0.2.

**Statistical Analysis.** Results are expressed as the mean  $\pm$  SE. Statistical analysis was performed using Student's *t*-test, and *P* < 0.05 was defined as indicating significance.

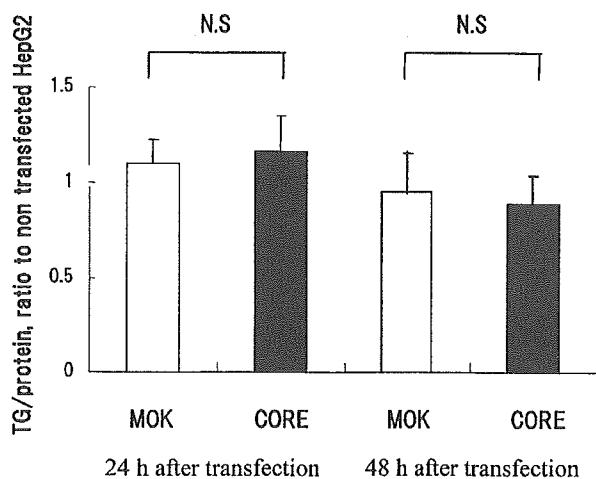
## RESULTS

**HCV Core Protein Expression in HepG2 Cells.** The transfection efficiency of pCAG-LacZ was about 20%. HCV core protein expression by the cells was confirmed using the HCV core antigen ELISA. No HCV core antigen was detected in mock-transfected and nontransfected cells. The level of HCV core protein expression showed no difference between 24 and 48 hr after transfection (24 hr,

TABLE 2. ROLES OF ANALYZED GENES IN FATTY ACID METABOLISM

|               |  |
|---------------|--|
| MDR3          | Multidrug resistance protein 3<br>An ABC transporter and phospholipid flippase<br><i>Role: Phospholipid secretion into bile</i>  |
| MTP           | Microsomal triglyceride transfer protein<br>A vital protein for TG incorporation into VLDL<br><i>Role: triglyceride secretion into blood</i>   |
| CPT1A         | Liver-predominant mitochondrial carnitine palmitoyl transferase-1<br>The rate-limiting enzyme of mitochondrial $\beta$ -oxidation<br><i>Role: Fatty acid <math>\beta</math>-oxidation in the liver</i> |
| ACO1          | Acyl-CoA oxidase<br>The rate-limiting enzyme of peroxisomal $\beta$ -oxidation<br><i>Role: Fatty acid <math>\beta</math>-oxidation in the liver</i>  |
| PPAR $\alpha$ | Peroxisome proliferator-activated receptor $\alpha$<br>A nuclear receptor<br><i>Role: A nuclear receptor controlling lipid metabolism-associated genes</i>   |
| PPAR $\gamma$ | Peroxisome proliferator-activated receptor $\gamma$<br>A nuclear receptor<br><i>Role: A nuclear receptor controlling lipid metabolism-associated genes</i>   |

HCV CORE PROTEIN MODULATES FATTY ACID METABOLISM

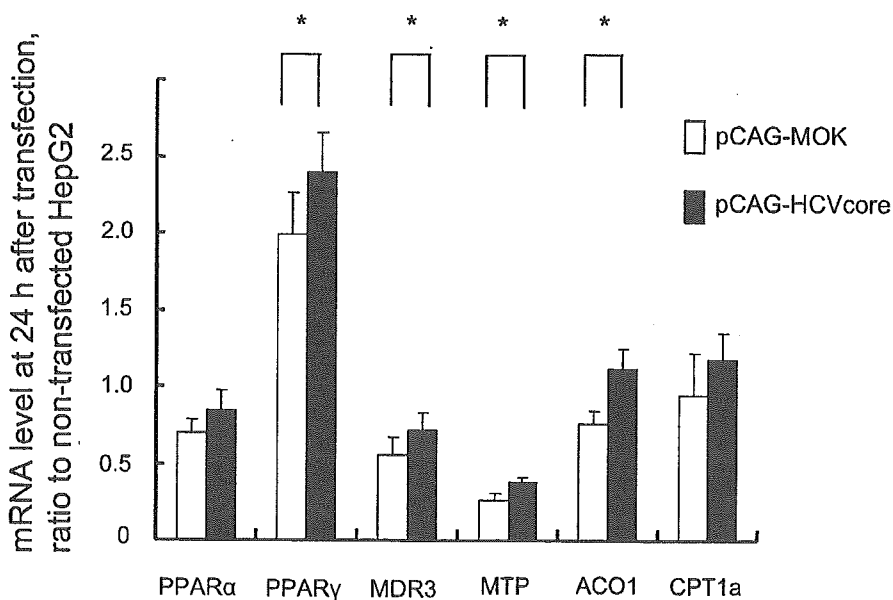


**Fig 2.** Effect of HCV core protein expression on cellular triglyceride (TG) content. Four micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in six-well plates by the lipofection method. At 24 or 48 hr after transfection, cells were collected for protein assay and lipid extraction. TG content was measured and expressed as the ratio to the protein content. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean  $\pm$  SD of six individual experiments.  $P = NS$  compared with pCAG-MOK (Student's  $t$ -test).

1.31  $\pm$  0.20 nmol/mg protein; 48 hr, 1.25  $\pm$  0.16 nmol/mg protein).

**TG Content of HepG2 Cells.** The cellular TG content at 24 hr after transfection showed no difference between HCV core transfectants (CORE) and mock transfectants (MOK) as control (CORE, 1.16  $\pm$  0.19; MOK, 1.10  $\pm$  0.13;  $P = 0.57$ ). At 48 hr after transfection, the TG content also showed no difference between the groups (CORE, 0.88  $\pm$  0.16; MOK, 0.95  $\pm$  0.18;  $P = 0.55$ ). Data are expressed as the ratio to nontransfected cells (Figure 2).

**Expression of Target Genes by HepG2 Cells.** At 24 hr after transfection, HCV CORE showed increased expression of mRNA for PPAR  $\gamma$  (CORE, 2.39  $\pm$  0.26; MOK, 1.98  $\pm$  0.28;  $P = 0.025$ ), MDR3 (CORE, 1.30  $\pm$  0.21; MOK, 1.02  $\pm$  0.20;  $P = 0.030$ ), MTP (CORE, 0.37  $\pm$  0.04; MOK, 0.26  $\pm$  0.05;  $P < 0.01$ ), and ACO1 (CORE, 1.11  $\pm$  0.14; MOK, 0.76  $\pm$  0.08;  $P < 0.01$ ) compared to MOK, while CPT (CORE, 1.18  $\pm$  0.16; MOK, 0.94  $\pm$  0.28;  $P = 0.102$ ) and PPAR $\alpha$  (CORE, 0.84  $\pm$  0.14; MOK, 0.69  $\pm$  0.10;  $P = 0.055$ ) expression was normal (Figure 3). At 48 hr after transfection, HCV CORE showed lower expression of mRNA for PPAR  $\alpha$  (CORE, 0.89  $\pm$  0.02; MOK, 0.96  $\pm$  0.08;



**Fig 3.** Effect of HCV core protein expression on mRNA levels at 24 hr after transfection. Three micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in 12-well plates by the lipofection method. At 24 hr after transfection, cells were collected for extraction of RNA. Complementary DNA was synthesized from 2  $\mu$ g of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH level was measured as the internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean  $\pm$  SD of 6 individual experiments. \* $P < 0.05$  compared with pCAG-MOK (Student's  $t$ -test).

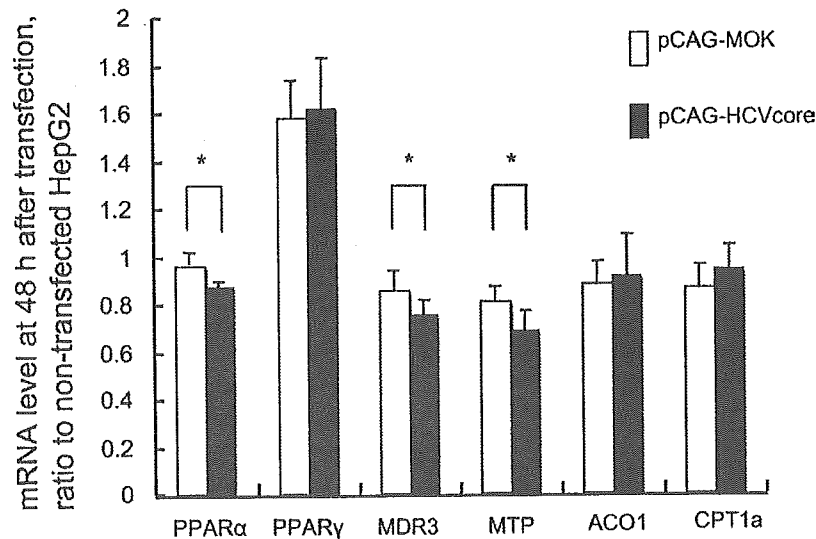


Fig 4. Effect of HCV core protein on mRNA expression at 48 hr after transfection. Three micrograms of pCAG-MOK (control) or pCAG-HCVcore was transfected into HepG2 cells cultured in 12-well plates by the lipofection method. At 48 hr after transfection, cells were collected and used for RNA extraction. Complementary DNA was synthesized from 2  $\mu$ g of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH was measured as an internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as values relative to those for nontransfected HepG2 cells. Each data point represents the mean  $\pm$  SD of 6 individual experiments. \* $P < 0.05$  compared with pCAG-MOK (Student's  $t$ -test).

$P = 0.048$ ), MDR3 (CORE,  $0.75 \pm 0.06$ ; MOK,  $0.86 \pm 0.08$ ;  $P = 0.031$ ), and MTP (CORE,  $0.69 \pm 0.08$ ; MOK,  $0.81 \pm 0.07$ ;  $P = 0.016$ ) compared with MOK, while ACO1 returned to the control level (CORE,  $0.91 \pm 0.18$ ; MOK,  $0.88 \pm 0.09$ ;  $P = 0.70$ ) and the CPT level was normal (CORE,  $0.94 \pm 0.13$ ; MOK,  $0.86 \pm 0.10$ ;  $P = 0.27$ ). Data are expressed as the ratio to nontransfected cells (Figure 4). Experiments were repeated three times and similar results were obtained, with statistical significance. CYP4A11 was not detected by RT-PCR, so we could not make a standard for the Light-Cycler.

**HCV Core Protein Expression in Mice.** HCV core protein-expressing mice looked healthy and their body weight (BW) and liver weight remained within the normal range (BW [g]: PBS,  $22.5 \pm 0.816$ ; AdexCAHCVcore (CORE),  $21.7 \pm 0.84$ ; AdexCALacZ (LacZ), as control,  $21.5 \pm 0.71$ ). Similar mild elevation of ALT and mild hepatic lymphocyte infiltration were observed in both groups of adenovirus-infected mice, showing no differences between Core and LacZ (GPT [IU/ml]: PBS,  $65 \pm 17.8$ ; CORE,  $170 \pm 59.4$ ; LacZ,  $142.5 \pm 82.2$ ). Lipid drops were not observed in either group (data not shown). Western blot analysis revealed the HCV core protein of about 19–20 kDa (Figure 5). In preliminary experiments, animals receiving an intravenous injection of  $1 \times 10^9$  pfu developed severe hepatitis after 7 days, while animals re-

ceiving  $1 \times 10^8$  pfu showed a mild elevation of ALT, but their HCV core protein expression (based on quantification of mRNA and HCV core antigen) was significantly lower at 7 days after injection. Thus, we selected injection of  $1 \times 10^9$  pfu and sacrifice at 3 days for the study protocol.

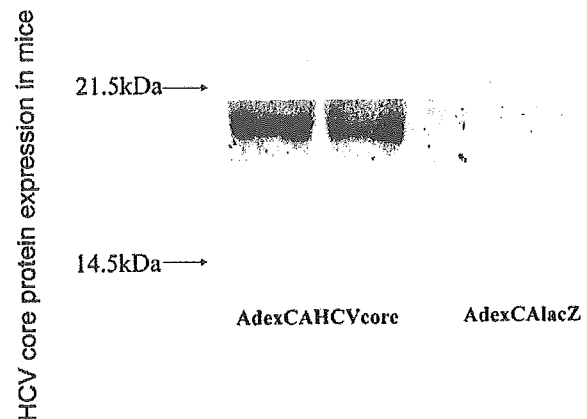
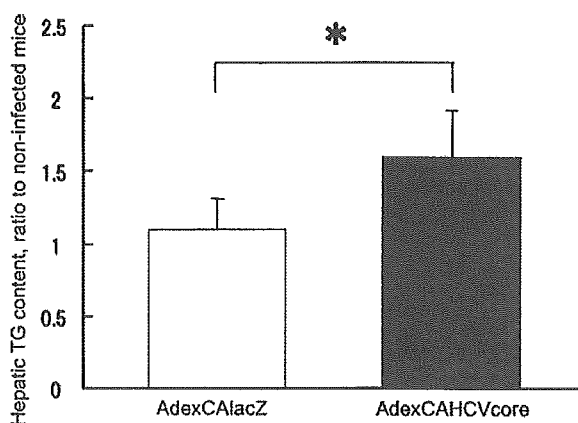


Fig 5. HCV core protein expression in mice. AdexCALacZ (control recombinant adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration ( $1 \times 10^9$  pfu). Three days after infection, livers were collected for protein assay. Using 50  $\mu$ g of protein, HCV core protein expression was confirmed by Western blotting with a mouse monoclonal antibody for HCV core protein (19–20 kDa).

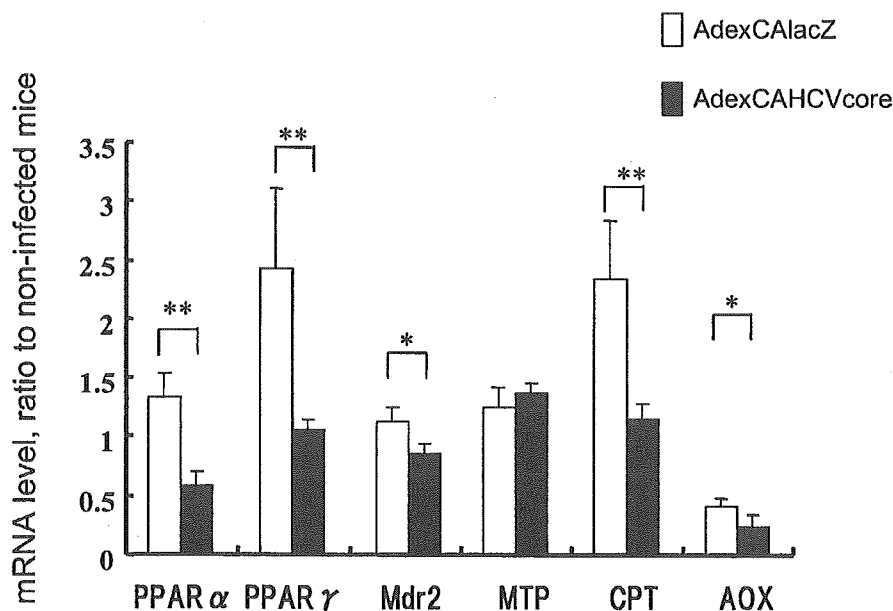


**Fig 6.** Effect of HCV core protein expression on the hepatic triglyceride content in mice. AdexCALacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration ( $1 \times 10^9$  pfu). At 3 days after infection, the livers were collected and 100  $\mu$ l of liver homogenate was used for lipid extraction and for the protein assay. The TG content was measured and expressed as the ratio to the protein content. Data are shown as values relative to those for noninfected mice. Each data point represents the mean  $\pm$  SD of four individual mice. \* $P < 0.05$  compared with AdexCALacZ (control adenovirus) by Student's *t*-test.

**Hepatic TG Level in Mice.** Animals injected with AdexCAHCVcore showed a 1.45-fold increase in hepatic TG content compared to animals injected with AdexCALacZ (CORE,  $1.60 \pm 0.33$ ; LacZ,  $1.10 \pm 0.21$ ;  $P = 0.044$ ;  $N = 4$ ). Data are expressed as the ratio to noninfected mice (Figure 6).

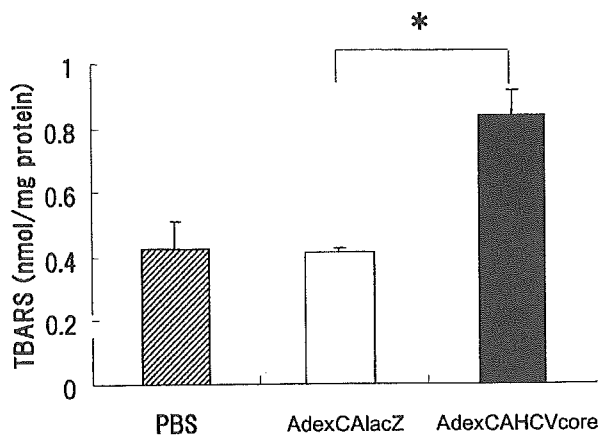
**Expression of Target Genes in Mice.** In the livers of HCV core protein-expressing mice, PPAR  $\alpha$  (CORE,  $0.59 \pm 0.11$ ; LacZ,  $1.33 \pm 0.21$ ;  $P < 0.01$ ), PPAR  $\gamma$  (CORE,  $1.05 \pm 0.10$ ; LacZ,  $2.43 \pm 0.69$ ;  $P < 0.01$ ), Mdr2 (CORE,  $0.85 \pm 0.08$ ; LacZ,  $1.12 \pm 0.12$ ;  $P = 0.011$ ), AOX (CORE,  $0.235 \pm 0.08$ ; LacZ,  $0.401 \pm 0.07$ ;  $P = 0.02$ ), and CPT (CORE,  $1.14 \pm 0.14$ ; LacZ  $2.34 \pm 0.51$ ;  $P < 0.01$ ) were all down-regulated, while the level of MTP mRNA was unchanged (CORE,  $1.37 \pm 0.08$ ; LacZ,  $1.24 \pm 0.17$ ;  $P = 0.22$ ;  $N = 4$ ). Data are expressed as the ratio to noninfected mice (Figure 7).

**Hepatic TBARS Level.** In the livers of HCV core protein-expressing mice, the TBARS level was increased compared with that in the control group (CORE,  $0.84 \pm 0.08$ ; LacZ,  $0.41 \pm 0.01$ ;  $P < 0.01$ ;  $N = 4$ ) (Figure 8).



**Fig 7.** Effect of HCV core protein expression on mRNA levels in mice. AdexCALacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration ( $1 \times 10^9$  pfu). At 3 days after infection, livers were collected for RNA extraction. Complementary DNA was synthesized from 2  $\mu$ g of RNA and used for quantified PCR with the Light-Cycler Fast-Start DNA Master SYBR Green system. GAPDH was measured as an internal control, and the ratio to GAPDH was calculated for each sample. Data are shown as relative values to those for noninfected mice. Each data point represents the mean  $\pm$  SD of four individual mice. \* $P < 0.05$  and \*\* $P < 0.01$  compared with AdexCALacZ (control adenovirus) by Student's *t*-test.





**Fig 8.** Effect of HCV core protein expression on TBARS in the mouse liver. AdexCALacZ (control adenovirus) or AdexCAHCVcore was used to infect male C57BL/6 mice (8–10 weeks old) by intravenous administration ( $1 \times 10^9$  pfu). At 3 days after infection, livers were homogenized in 10 vol of normal saline. TBARS and total protein (TP) levels were measured as described under Materials and Methods. Data are expressed as the ratio to the TP level. Each data point represents the mean  $\pm$  SD of four individual mice. \* $P < 0.01$  compared with AdexCALacZ (control adenovirus) by Student's *t*-test.

## DISCUSSION

HCV core protein was recently reported to cause hepatic steatosis and induction of reactive oxygen species (ROS) in an HCV core protein transgenic mouse model (18–20). In the transgenic mouse model, it was also shown that a decrease in MTP activity contributes to HCV core protein-related steatosis, while  $\beta$ -oxidation is unchanged (24), but the mechanism involved is still unclear. This study was the first investigation of the effect of HCV core protein on the expression of fatty acid metabolism-associated molecules in the acute expression mice model.

Hepatic accumulation of TG is principally driven by the following factors: (a) fatty acid overload (28, 29), (b) inhibition of fatty acid  $\beta$ -oxidation (28, 29), (c) decreased secretion of TG-rich very low density lipoprotein (VLDL) (28, 29), (d) increased de novo fatty acid synthesis, (e) decreased transformation to phospholipids, and (f) a combination of these mechanisms.

In the present study, we initially tested the effect of HCV core protein on a human cell line (HepG2). At 24 hr after transfection, the cellular TG level was unchanged, but the expression of several genes that are thought to promote fatty acid consumption (MTP, ACO1, and MDR3) was up-regulated. At 48 hr after transfection, there was either normal gene expression (ACO1) or a decrease in expression (PPAR $\alpha$ , MDR3, and MTP). At 48 hr after transfection, the level of HCV core antigen was still the same as at 24 hr, so it seems possible that HCV core protein may

act to down-regulate these genes over a longer period. To further evaluate the effects of HCV core protein, we performed in vivo experiments using transient expression of HCV core protein in mice. Although fatty change of the liver was not seen histologically, the hepatic TG level was increased by transient HCV core protein expression. In addition, expression of mRNA for all of the molecules investigated, except MTP, was down-regulated by HCV core protein expression. The mechanism involved is not understood at present, but reduced expression of these genes might contribute to hepatic TG accumulation.

CPT is the rate-limiting enzyme for mitochondrial  $\beta$ -oxidation (30), which is the main pathway of fatty acid consumption and ROS production. There was a recent report (20) that localization of HCV core protein in the mitochondria led to the increased production of ROS, decreased mitochondrial membrane permeability, and impairment of mitochondrial function. It remains unclear whether ROS induces fat accumulation or whether the accumulation of fat causes an increase in ROS, as well as whether decreased expression of CPT-1 is the first response to HCV core protein expression or follows other earlier changes. However, HCV core expression seems to contribute to hepatic accumulation of lipids and an increase in ROS in mice, along with reduced expression of various fatty acid metabolism-associated genes. AOX is vital for peroxisomal  $\beta$ -oxidation (30) and it has been reported that AOX knockout mice develop steatohepatitis, up-regulation of CYP4A gene expression, and increased production of ROS (31). We were unable to evaluate CYP4A11 in the present study, but the association of HCV-related steatosis with microsomal  $\omega$ -oxidation is interesting. Mdr2 (Abcb4) is a member of the Abcb subfamily of adenosine triphosphate-binding cassette (ABC) transporter proteins. Mdr2 Pgp is exclusively localized to the canalicular membrane and controls the secretion of phospholipids into the bile (32). We thought that impaired biliary phospholipid secretion might have a role in HCV-related steatosis, based on the fact that phospholipid-associated fatty acid secretion into bile (about 25  $\mu$ mol per day) is substantial in relation to the hepatic amount of triglyceride-associated fatty acids (about 75  $\mu$ mol) (33). We found that the expression of MDR3 and Mdr2 was down-regulated, suggesting that reduced expression of these genes could have a causative role in HCV-related steatosis.

Interestingly, down-regulation of Mdr2, AOX, and CPT in the mice was accompanied by down-regulation of PPAR $\alpha$ . In mice, the other three genes are thought to undergo transcriptional regulation by PPAR $\alpha$  (33, 34), so their expression might be down-regulated secondary to the down-regulation of PPAR $\alpha$ . HCV core protein is mainly

localized in the cytosol, but also exists in the nucleus (35, 36), so it is possible that this protein could influence gene transcription. Tsutsumi *et al.* (37) used a luciferase assay to show that transcriptional activation of ACO-1 via PPRE is promoted at 24 hr after HCV core protein expression (23). However, we found down-regulation of target gene expression accompanied by decreased PPAR $\alpha$  expression after 3 days of HCV core protein expression in mice, as well as at 48 hr after transfection of cells. The expression of PPAR $\alpha$  was reported to be under transcriptional regulation by glucocorticoids (38), but the mechanism remains unclear. Accordingly, the mechanism leading to down-regulation of PPAR $\alpha$  after HCV core protein expression is also unclear. The lower expression of PPAR $\alpha$  and the genes it regulates in human hepatocytes than in mouse hepatocytes (39) could be a reason for the lack of an increase in TG and the small decline in gene expression in our cell experiment. Fibrates that bind with PPAR $\alpha$  and increase its activity (although not its expression) might be useful for controlling HCV-related steatosis by increasing the  $\beta$ -oxidation and biliary secretion of fatty acids.

PPAR $\gamma$  improves insulin resistance and is also reported to improve hepatic fibrosis and nonalcoholic steatohepatitis (40, 41). Because PPAR $\gamma$  gene expression also showed down-regulation by HCV core protein expression in this study, it may be necessary to examine the role of glucose metabolism, de novo synthesis of fatty acids from glucose, and fatty acid flux through hepatocytes in HCV-related steatosis.

In this study, the increase in TBARS level was found in mice with transient expression of HCV core protein. This suggests that ROS production might be induced by HCV core protein expression, although no mechanistic information for this was provided in this study. It also remains unclear whether intrahepatic fat accumulation enhances ROS production as reflected by an increase in TBARS or, inversely, whether ROS production induces fatty liver change through ROS-associated mitochondrial dysfunction. Certainly, further investigations are needed to clarify this uncertainty, but the fact that HCV core protein expression in mice contributes to the increase in TBARS level may partially characterize the pathogenesis of HCV-related hepatic damage.

In summary, transient expression of HCV core protein in mice down-regulated the expression of various lipid metabolism-associated genes (Mdr2, CPT, and AOX). It also caused down-regulation of PPAR $\alpha$  expression and led to the accumulation of TG and the induction of oxidative stress. These findings may provide some clues to the understanding of HCV-related steatosis and to the induction of ROS production and carcinogenesis by infection with this virus.

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## HCV CORE PROTEIN MODULATES FATTY ACID METABOLISM

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# 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<sup>+</sup>αβ T cells, γδ 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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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 <sup>51</sup>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

**Table 1. Baseline characteristics of study subjects**

| Gender  | Cases                   |                           | Controls selected from cohort members with trisected natural cytotoxic activity |                           |                          |                           |                          |                           |
|---|-------------------------|---------------------------|---|---------------------------|--------------------------|---------------------------|--------------------------|---------------------------|
|   |                         |                           | High  |                           | Medium                   |                           | Low                      |                           |
|   | Men<br>( <i>n</i> = 54) | Women<br>( <i>n</i> = 48) | Men<br>( <i>n</i> = 108)  | Women<br>( <i>n</i> = 96) | Men<br>( <i>n</i> = 108) | Women<br>( <i>n</i> = 96) | Men<br>( <i>n</i> = 108) | Women<br>( <i>n</i> = 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     | TGGGCAAAACACAATGTTCCAGAATT                  | T/G        | GGGCGTCAACAAACGAATCTTG              |
| 2   | rs2537752     | TCTGGAGTCTATAAAATGTTTTAAACAGTGTC            | A/T        | TCTCAAATGTAGGTGAACGAATTCATCA        |
| 3   | rs1049174     | CTGCCCATGAGGCAATTTCC                        | C/G        | GGATCAGTGAAGGAAGAGAAGGC             |
| 4   | rs2255336     | CTGTAGCCATGGGAATCCGTTT                      | A/G        | GCAATCTACTTCTCTGTGTGCACCTACA        |
| 5   | rs2294148     | AGAAACTAAACTAAACTACACAGAGGTTGC              | A/G        | GATGTGGAGTCAGACTTGAATTTTACTCA       |
| 6   | rs2049796     | AAGCATCTAAGAAAACAATTAGAATTACCTTATAGTGAAATAT | C/A        | CAGGTGTGTGTATGTGTGTATGTGT           |
| 7   | rs2617160     | ATGACTAATGTAAAAGTAAAAAGTCTGCAAACA           | A/T        | GCCTTGAGTTCATATAATTACAATACACCAGT    |
| 8   | rs7972757     | TGATTGCCATTAAACCTTCCATTTCCT                 | A/G        | GTCGTAAAGGCATCGTTCCATCTA            |
| 9   | rs2246809     | ACCCTTAAGAGAAAAGGCTTTCATGTAC                | A/G        | ACTGGTCATTCTGTATTGCTGTGTT           |
| 10  | rs2617169     | GGGATGCAAAATGATAATAAAATGTTTTGGG             | A/T        | GGAGAAAAGGACATGCCCTCATAT            |
| 11  | rs2617170     | TGACAATCATAATGTACCTTCTGCATTCT               | C/T        | CACFTTAATTTTTCTAGGTATTGGAGTACTGGA   |
| 12  | rs2617171     | CCCAAGATAATATGCTGCTTCTGAAC                  | C/G        | TCTCTTAAACATGTCTTTGAGTCATGAAATCA    |
| 13  | rs1971939     | TCATTGCATATACCTAATGATACAAGTTCAACA           | C/G        | GGCTCACTGGCCTGTCTT                  |
| 14  | rs1915319     | GTATTCTGTATTTGACATAAATATTACTAGTGGGAACAAT    | A/G        | CTATTGGTGTAAACATTTTGAAGAATCTAACCTTA |
| 15  | rs4763525     | AGACATGCCTTTTCATGTAAGCATAAAGA               | A/G        | CCTGGGAGTGGGATTGCT                  |
| 16  | rs3003        | TGTACTTTAGTAATTTGTGTGCATCCTATTTC            | 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. 1A. 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.

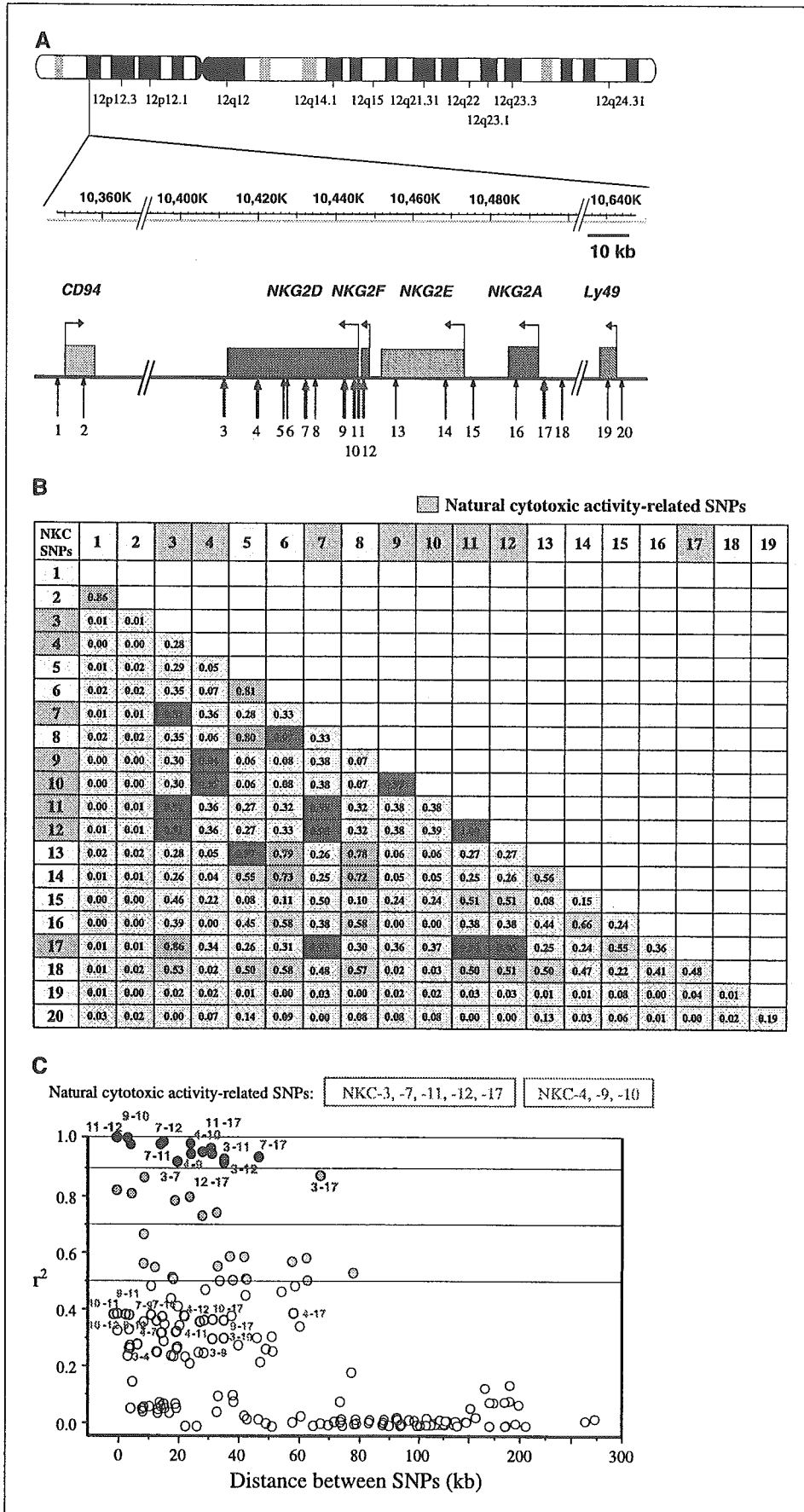
**Haplotype analysis and risk estimation.** The linkage disequilibrium was estimated by relative linkage disequilibrium coefficients ( $D'$ ),  $r^2$  values, and the  $\chi^2$  values. Haplotype allele frequencies and haplotype distributions were estimated on the basis of multiple SNPs by the expectation-maximization algorithm, using SNPalyze (DYNACOM, Yokohama, Japan, <http://www.dynacom.co.jp/>). Statistical significance was examined by the  $\chi^2$  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. 1A).

**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. 1B). On the basis of the linkage disequilibrium analysis, Fig. 1C 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. 1C 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  $r^2$



**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).



values <0.5 (Fig. 1C), 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. 2A).

**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. 2B). 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 ( $\pm$ SE) natural cytotoxic activity of *LNK1/LNK1*, *LNK1/HNK1*, and *HNK1/HNK1* haplotypes among a total of 612 controls: The results were  $42.1 \pm 1.2$  ( $n = 196$ ),  $47.8 \pm 1.1$  (260), and  $50.1 \pm 1.7$  (109), respectively ( $P_{\text{trend}} < 0.001$ ); 47 controls having heterozygous haplotypes other than *LNK1/HNK1* showed mean natural cytotoxic activity of  $45.1 \pm 2.7$ .

**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        | $P = 0.0002$        |

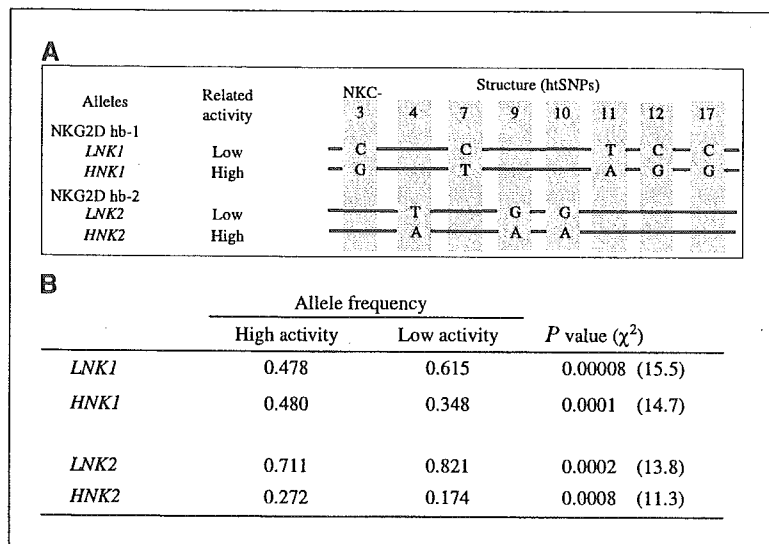


Figure 2. Natural cytotoxic activity-related haplotype alleles. A, *LNK1* and *HNK1* are generated from haplotype block NKG2D hb-1, and *LNK2* and *HNK2* from NKG2D hb-2. B, allele frequencies are estimated for groups (*n* = 408 chromosomes for each group) with high and low natural cytotoxic activity.

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 <i>n</i> (%) | Controls <i>n</i> (%) | Crude OR (95% CI)   | Adjusted OR* (95% CI) |
| <i>LNK1/LNK1</i>   | 42 (41)            | 196 (32)              | 1.00                | 1.00                  |
| <i>LNK1/HNK1</i> † | 42 (41)            | 260 (42)              | 0.754 (0.473-1.20)  | 0.694 (0.430-1.12)    |
| <i>HNK1/HNK1</i>   | 11 (11)            | 109 (18)              | 0.471 (0.233-0.952) | 0.482 (0.237-0.982)   |
|                    | Allele frequency   |                       | <i>P</i>            |                       |
| <i>LNK1</i>        | 0.632              | 0.554                 | 0.036               |                       |
| <i>HNK1</i>        | 0.333              | 0.406                 | 0.049               |                       |
| NKG2D hb-2         |                    |                       |                     |                       |
| Haplotype          | Cases <i>n</i> (%) | Controls <i>n</i> (%) | Crude OR (95% CI)   | Adjusted OR (95% CI)  |
| <i>LNK2/LNK2</i>   | 67 (65)            | 371 (61)              | 1.00                | 1.00                  |
| <i>LNK2/HNK2</i> ‡ | 26 (25)            | 203 (33)              | 0.709 (0.437-1.15)  | 0.701 (0.425-1.16)    |
| <i>HNK2/HNK2</i>   | 3 (3)              | 27 (4)                | 0.615 (0.181-2.09)  | 0.642 (0.188-2.19)    |
|                    | Allele frequency   |                       | <i>P</i>            |                       |
| <i>LNK2</i>        | 0.789              | 0.778                 | 0.7                 |                       |
| <i>HNK2</i>        | 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.

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.

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