t-loop. G-tail length is essential for chromosome capping (9). In addition, shelterin proteins TRF1, TRF2, POT1, TPP1, TIN2, and Rap1 also required for t-loop formation (9). Knockdown of these genes can affect G-tail length alterations and chromosome stability (10, 11). For example, when either TRF2 or POT1 is inhibited by dominant negative forms of the proteins, the overall amount of G-tail is diminished (12, 13). In the case of TRF2 inhibition, ERCC1/XPF-deficient cells retained the G-tail after TRF2 inhibition, suggesting that the loss of G-tail might involve ERCC1/XPF NER endonuclease which can remove 3′-overhang DNA (14). Taken together, G-tail alterations may be a good indicator for stability of the t-loop structure that protects chromosome ends.

Hybridization protection assay (HPA) utilizes probes that are labeled with a nonradioactive chemiluminescence compound – acridinium ester (AE) detector molecule that emits a chemiluminescent signal, and has been used for the detection of total telomere length and telomerase activity (15, 16). HPA reaction does not require physical separation of unhybridized vs. hybridized probe, therefore, HPA-based assays including G-tail telomere HPA can be applicable for high-throughput screening.

2. Materials

1. Hybridization buffer: 0.1 M succinic acid, 0.23 M lithium hydroxide monohydrate, 2% lithium lauryl sulfate, 1.2 M lithium chloride, 20 mM EDTA·2Na, 20 mM EGTA, 15 mM 2,20-dithiodipyridine, adjusted to pH 4.7 with HCl.
2. Hydrolysis buffer: 0.6 M boric acid, 182 mM NaOH, 1% Triton X-100, adjusted to pH 8.5 with NaOH.
3. Synthesized human telomere oligonucleotide: an 84mer of 5′-$(TTAGGG)_{14}$-3′ or a 35mer of 5′-$(TTAGGG)_{6}$-TTAGG-3′.
4. Acridinium ester (AE)-labeled telomere probe: 5′-CCCTAA CCCTAACC*CTAACCCTAACCCTA-3′ [*AE position, 8×10^{7} rlu/pmol probe DNA made by Fujirebio, Inc., (Tokyo, Japan)].
5. GEN-PROBE detection reagent kit: Catalog No. 1791, Kit contains Detection Reagent I (0.1% hydrogen peroxide in 0.001 N nitric acid) and Detection Reagent II (1 N sodium hydroxide).
6. Spectrophotometer such as NanoDrop (Thermo scientific).
7. Luminometer (Leader I, Gen-Probe, Inc., San Diego, CA).

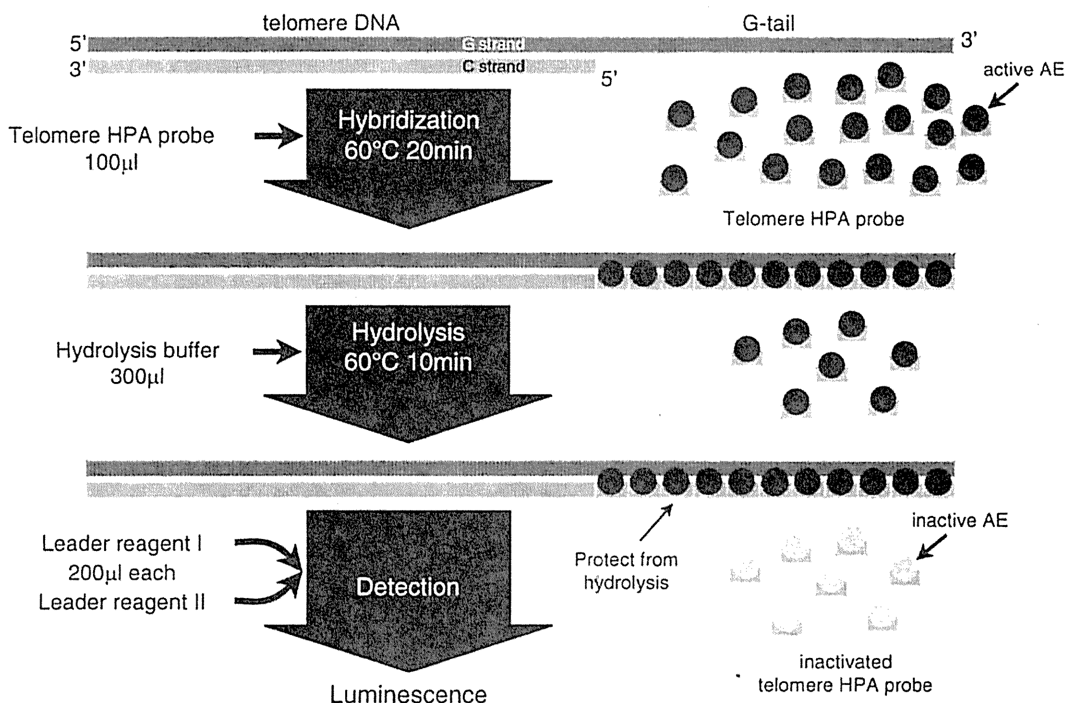

Fig. 1. The principle of G-tail telomere HPA. G-tail telomere HPA consists of three steps: "hybridization," "Hydrolysis," and "Detection" for the measurement of relative G-tail length from purified genomic DNA as well as nonpurified cell lysate using single tube. First step, "hybridization step": incubation of non-denatured genomic DNA with acridinium ester-labeled telomere HPA probe at 60°C for 20 min. Second step, "Hydrolysis step": incubation of reaction solution at 60°C for 10 min after adding hydrolysis buffer to inactivate unhybridized telomere HPA probe. Hybridized telomere HPA probe can be protected the inactivation of acridinium ester from hydrolysis. Last step, "Detection step": detection of luminescence from hybridized probe after adding Detection buffer I and Detection buffer II using autoinjector-equipped luminometer.

5. Dilute AE-labeled telomere HPA probe to 3×10^5 rlu (relative light units)/mL.
6. Add 100 μL of diluted telomere HPA probe to the bottom of each tube for detecting standard telomere oligo, G-tails, and total telomere (final 3×10^5 rlu/assay). Vortex at maximum speed for 5 s.
7. Incubate all the tubes simultaneously in a 60°C water bath for 20 min without agitation. Cover top of tubes with aluminum foil and protect from light (see Note 4).
8. Remove all tubes to room temperature from the water bath and let them sit for 10 min.
9. Add 100 μL of hydrolysis buffer. Vortex at maximum speed for 5 s (see Note 5).
10. Incubate all the tubes simultaneously in the 60°C water bath for 10 min.
11. Transfer all tubes to ice cold water and leave for over 1 min.

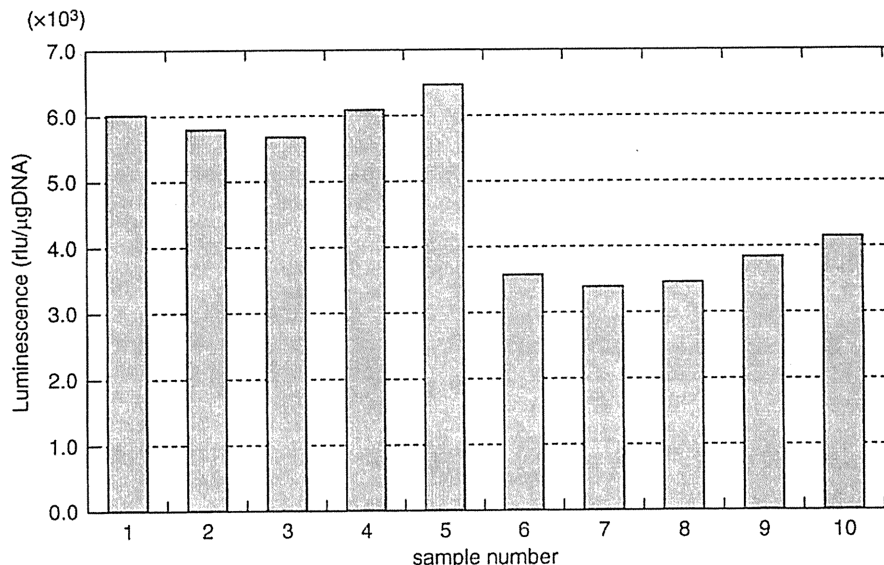

Fig. 3. The typical data of relative G-tail signals of human genomic DNA samples isolated from different persons using G-tail telomere HPA.

3. To normalize by the amount of genomic DNA, take 2 μL of DNA solution before starting following step. This step eliminate to measure DNA exact amount in each tube.
4. Do not use a heat block without water for this incubation step.
5. Make sure all reaction solution should be mixed with hydrolysis buffer, residual solution may cause nonspecific signals due to insufficient inactivation of unhybridized probe.
6. An autoinjector is essential for this assay. Luminescence should be counted from 0 to 2 s. Follow the instruction manual of the equipment.

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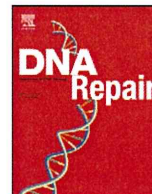

Two unrelated patients with *MRE11A* mutations and Nijmegen breakage syndrome-like severe microcephaly

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ABSTRACT

MRE11 and NBS1 function together as components of a MRE11/RAD50/NBS1 protein complex, however deficiency of either protein does not result in the same clinical features. Mutations in the *NBN* gene underlie Nijmegen breakage syndrome (NBS), a chromosomal instability syndrome characterized by microcephaly, bird-like faces, growth and mental retardation, and cellular radiosensitivity. Additionally, mutations in the *MRE11A* gene are known to lead to an ataxia–telangiectasia-like disorder (ATLD), a late-onset, slowly progressive variant of ataxia–telangiectasia without microcephaly. Here we describe two unrelated patients with NBS-like severe microcephaly (head circumference −10.2 SD and −12.8 SD) and mutations in the *MRE11A* gene. Both patients were compound heterozygotes for a truncating or missense mutation and carried a translationally silent mutation. The truncating and missense mutations were assumed to be functionally debilitating. The translationally silent mutation common to both patients had an effect on splicing efficiency resulting in reduced but normal MRE11 protein. Their levels of radiation-induced activation of ATM were higher than those in ATLD cells.

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1. Introduction

The development of the central nervous system is highly sensitive to DNA damaging agents and therefore several autosomal recessive disorders with defective DNA damage repair exhibit neurological abnormalities such as microcephaly and neurodegeneration [1,2].

The MRE11/RAD50/NBS1 (MRN) protein complex and the ataxia–telangiectasia mutated (ATM) protein, together play a central role in DNA double strand break repair [3]. Mutations in the *ATM* (MIM# 607585) and *MRE11A* (MIM# 600814) genes each give rise to a progressive cerebellar ataxia syndrome: *ATM* mutations to ataxia–telangiectasia (A–T [MIM# 208900]) [4], and *MRE11A* mutations to ataxia–telangiectasia-like disorder (ATLD [MIM# 604391]) [5,6]. A–T is an autosomal recessive disorder characterized by growth deficiency, progressive cerebellar ataxia, dysarthria, telangiectasia, frequent respiratory infections, and immunodeficiency. ATLD is also characterized by cerebellar ataxia, but its onset is later in life and its progression is slower than in A–T. In addition, there is no telangiectasia and immunoglobulin levels are normal.

Mutations in two different genes involved in the MRE11/RAD50/NBS1 complex are known to lead to a hereditary disorder with severe microcephaly: the *NBN* gene (MIM# 602667) to Nijmegen breakage syndrome (NBS [MIM# 251260]) [7–9], and the *RAD50* gene (MIM# 604040) to Nijmegen breakage syndrome-like disorder (NBSLD [MIM# 613078]) [10]. NBS is an autosomal recessive disorder characterized by microcephaly, growth and mental retardation, immunodeficiency, radiosensitivity, and cancer predisposition; NBSLD is a disorder with microcephaly, mental retardation, bird-like face, and short stature,

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PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). RT-PCR primers were synthesized to amplify the *MRE11A* cDNA spanning exons 1–8 (5′-CGAAAAGAAGACAGCCTTGG-3′ and 5′-TCCAAAAATTGTTCTGGAATGA-3′) (GenBank NM_005590.3). RT-PCR products were visualized following electrophoresis on a 2% NuSieve agarose gel. PCR primers for *RAD50* (GenBank Z75311.1) and *NBN* cDNAs (GenBank AF058696.2) were synthesized to amplify the open reading frame with several overlapping segments.

Transcript levels of the *MRE11A*-c.338A and *MRE11A*-c.338G alleles in LCLs from a normal individual, Patient 2 and the parents were determined by the cycleave quantitative real time PCR assay (Cycleave-qPCR, TaKaRa Co. Ltd.) carried out in triplicate. Transcripts from the *HPRT1* allele were used as a quantification control. RNaseH sensitive fluorescent probes that specifically recognize the *MRE11A*-c.338A and *MRE11A*-c.338G alleles were used for the assay. The qPCR results were analyzed by the $\Delta\Delta$CT method. qPCR primers and probes used for the assay are listed below.

MRE11A-F: 5′-ACGTTTGTAACACTCGATGAA-3′;
MRE11A-R: 5′-CTGGAATTGAAATGTTGAGG-3′;
MRE11Ac.338A: (Eclipse) 5′-dAdAdG(A)dTdGdGdCdAdA-3′ (FAM);
MRE11Ac.338G: (Eclipse) 5′-dAdAdG(G)dTdGdGdCdA-3′ (ROX);
HPRT1-F: 5′-CAGGCAGTATAATCCAAAGATG-3′;
HPRT1-R: 5′-ACTGGCGATGTCAATAGGA-3′;
HPRT1-probe: (Eclipse) 5′-dCdAdGdCdA(A)dGdCdT-3′ (FAM).

2.5. Western blot analysis

Western blotting was performed as described previously [17]. Primary antibodies used were: mouse anti-MRE11 monoclonal antibody (MRE11-12D7, 1:1000, GeneTex, Irvine, CA); mouse anti-RAD50 monoclonal antibody (13B3/2C6, 1:1000, GeneTex, Irvine, CA); rabbit anti-NBS1 polyclonal antibody (NB100-142, 1:500, Novus Biologicals, Littleton, CO); mouse anti-GAPDH monoclonal antibody (6C5, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); and mouse anti-β-tubulin monoclonal antibody (1:2000, Sigma–Aldrich, St. Louis, MO).

2.6. Radiation-sensitivity analysis

Clonogenic analysis was performed on fibroblast cell lines to learn of their radiosensitivity as previously described [12]. Chromosome breakage analysis of LCLs was carried out as follows. Cells were irradiated with 2 Gy X-ray and harvested 24 h after irradiation. Giemsa-stained chromosome slides were prepared, and chromatid or chromosome breaks and quadriradials were counted.

2.7. ATM autophosphorylation after γ-irradiation

Immortalized fibroblast cells or LCLs were irradiated with 0.5 Gy of γ ray. At 15 min and 30 min after irradiation, the cells were analyzed with Western blotting using rabbit anti-ATM-p1981 monoclonal antibody (1:1000, Epitomics Inc., Burlingame, CA) and mouse anti-ATM monoclonal antibody (2C1, 1:1000, GeneTex, Irvine, CA). Band intensities were estimated using a densitometer and are presented as means ± standard deviation. The statistical differences were analyzed with Student's *t*-test. Statistical significance was assumed for $p < 0.05$.

2.8. DNA damage response assay

ATM-dependent G2/M checkpoint arrest was performed according to the methods described previously [18].

2.9. p53 phosphorylation after γ-irradiation

Lymphoblastoid cells were irradiated with 0.5 Gy of γ ray. At 15 min and 30 min after irradiation, the cells were analyzed with Western blotting using rabbit anti-phosphorylated p53 (Ser15) polyclonal antibody (1:1000, Cell Signaling Technology, Beverly, MA) and mouse anti-p53 monoclonal antibody (1:1000, Oncogene Research Products, CA).

2.10. Caspase 3 activation after γ-irradiation

Immortalized fibroblast cells were irradiated with 0 or 10 Gy of γ ray. At 72 h after irradiation the cells were analyzed with Western blotting using rabbit anti-cleaved caspase 3 monoclonal antibody (#9664, 1:1000, Cell Signaling Technology, Beverly, MA).

3. Results

3.1. Identification of MRE11A mutations

Several studies have demonstrated that microcephaly, as was present in the two patients we described, is a common feature in a variety of DNA damage repair defective disorders [2]. Therefore, we examined DNA damage repair proteins including ATM, ATR, MRE11, RAD50, and NBS1 in the two patients. Western blot analysis showed normal levels of ATM and ATR (data not shown) and reduced levels of MRE11, RAD50, and NBS1 in both patients (Fig. 2a). We, therefore, sequenced all the *MRE11A*, *RAD50*, and *NBN* coding sequences in both patients and found only the *MRE11A* mutations c.658A>C and c.659+1G>A in Patient 1, and c.658A>C and c.338A>G in Patient 2 (Fig. 2b). In Patient 1, c.658A>C was derived from the father, and c.659+1G>A from the mother. Two brothers were a heterozygote for c.659+1G>A. RT-PCR and sequencing analysis demonstrated that c.659+1G>A resulted in exon 7 skipping leading to a premature termination codon (p.Ser183ValfsX31) (Fig. 2c). The c.658A>C substitution located within exon 7 did not alter amino acids but affected splicing efficiency that resulted in exon 7 skipping (Fig. 2c). RT-PCR analysis of exons 1–8 of *MRE11A* cDNA from Patient 1 detected a reduced but considerable amount of correctly spliced transcripts in addition to the exon-skipped transcript (Fig. 2c). Western blot analysis of fibroblasts from Patient 1 detected a reduced amount of normal-sized MRE11 protein (Fig. 2a). No smaller-sized protein corresponding to the predicted truncated form was detected in both lymphocytes and fibroblasts. The c.658A>C substitution was not found in 100 normal Japanese individuals. These results indicated that the c.658A>C mutation leads to exon 7 skipping but that some of the RNA is correctly spliced.

Patient 2 was another compound heterozygote with c.658A>C, the same single-base substitution as the one found in Patient 1, and c.338A>G, a single-base substitution in exon 5. The c.658A>C substitution was inherited from the mother while c.338A>G was derived from the father. A DNA sample from the older brother was not available. Cloning and sequencing of the RT-PCR products of Patient 2 revealed two kinds of mRNA from the c.338G allele; normal sized transcripts carrying the c.338A>G substitution and intermediately sized transcripts resulting from exon 5 skipping (Fig. 2c). The normal sized products lead to an amino acid substitution of Asp to Gly at the 113th residue (p.Asp113Gly). The 113th residue is located within the highly conserved phosphoesterase domain, which is essential for endonuclease activity [19]. On the other hand, the intermediately sized transcripts resulting from exon 5 skipping lead to a premature termination codon (p.Phe106GlnfsX10). We then examined the levels of the transcripts from the c.338A and c.338G alleles by quantitative RT-PCR analysis. The correctly spliced

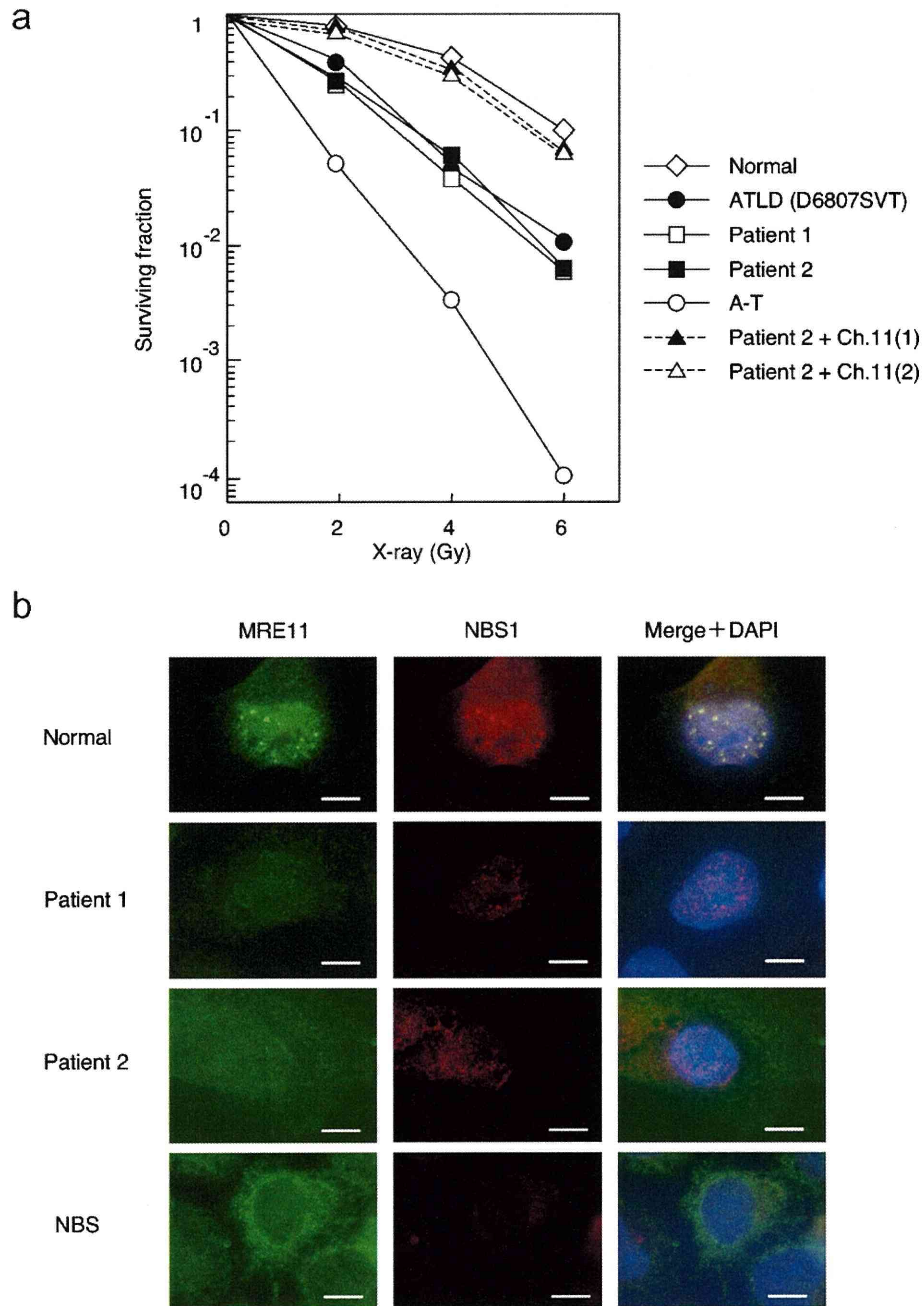

Fig. 3. Clonogenic survival curves for X-ray-irradiated fibroblasts. (a) Radiosensitivity was measured by counting colonies surviving radiation doses of 0–6 Gy. Colony survival was expressed as a logarithm. ATLD, ataxia-telangiectasia-like disorder; A–T, ataxia-telangiectasia. A–T cells were highly sensitive to radiation. Cells from Patients 1 and 2, and ATLD all showed intermediate levels of sensitivity. Microcell-mediated transfer of a human chromosome 11 (including the *MRE11A* locus) into the cells from Patient 2 restored radiation sensitivity. (b) Formation of MRE11 and NBS1 radiation-induced nuclear foci. Cells were analyzed by immuno-staining at 24 h after 6 Gy irradiation. Normal cells served as a control, and NBS cells served as an *NBN*-deficient reference. MRE11 and NBS1 formed nuclear foci after irradiation in normal cells. In contrast, cells from Patients 1 and 2 showed only very faint signals.

transcripts from the c.338G allele of Patient 2 showed 25% of the normal level (Fig. 2d). By contrast, the transcript levels from the c.338G allele of the father were not affected. The c.338A>G substitution was not detected in 100 normal Japanese individuals.

3.2. Cells from Patients 1 and 2 exhibit radiation-hypersensitivity

A clonogenic radiation sensitivity assay was performed on fibroblast cells from Patients 1 and 2 on ATLD and A–T cells as references, and on normal cells as a control. The cells from Patients 1 and 2, and ATLD were hypersensitive to X-ray irradiation, as measured by the share of surviving fractions after irradiation (Fig. 3a). A–T cells showed more marked radiation-hypersensitivity than in Patients 1 and 2, and ATLD. We introduced chromosome 11 (containing the *MRE11A* locus) into the cells from Patient 2 through microcell-mediated transfer [12]. Two microcell-hybrid clones obtained and both showed restoration of radiation-sensitivity (Fig. 3a).

Next, we studied MRE11 and NBS1 radiation-induced nuclear foci formation 24 h after exposure to 6 Gy. MRE11 and NBS1 formed nuclear foci after irradiation in normal cells. In contrast, cells from Patients 1 and 2 showed only very faint signals of NBS1 and MRE11. It is noteworthy that a few NBS1 foci were present in Patient 1. These findings are likely to be compatible with the level of the protein in the cells (Fig. 3b).

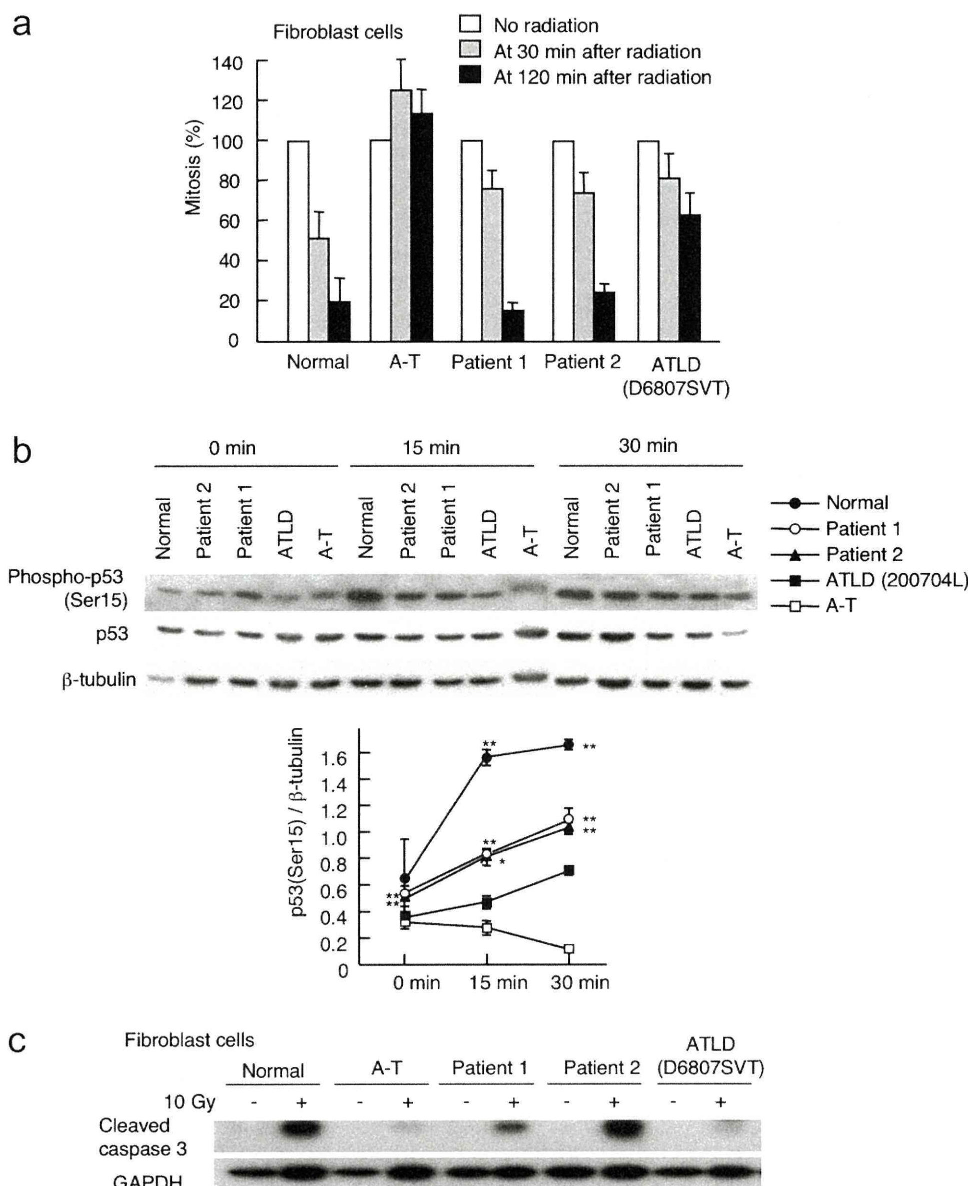

Fig. 5. Radiation-induced G2/M checkpoint and, p53 phosphorylation and caspase 3 activation as indicators of ATM activation deficiency. (a) Ataxia–telangiectasia (A–T) fibroblasts were used as an ATM-deficient reference, and ATLD fibroblasts (D6807SVT) harboring homozygous nonsense mutations were used as a control. Cultured fibroblast cells on a slide were irradiated with 2 Gy of γ ray, and stained 30 min or 2 h later with DAPI and rabbit anti-phospho-histone H3 polyclonal antibody. Mitotic index in each cell line without irradiation was estimated as 100%. Fibroblasts from Patients 1 and 2, and ATLD cells all showed a slight decrease in mitotic index at 30 min after irradiation, intermediate between normal and A–T cells. At 2 h after irradiation, cells from Patients 1 and 2 showed a decrease of mitotic index to the level of the normal cells, while ATLD cells showed a response intermediate between normal and A–T cells. Each column represents an average ± standard error from three separate experiments. (b) Western blots of phospohrylated p53 protein after irradiation. A–T and ATLD cells were used as controls. LCLs were irradiated with 0.5 Gy of γ ray. At 15 min and 30 min after irradiation, the cells were analyzed with Western blotting using rabbit anti-phosphorylated p53 (Ser15) polyclonal antibody (1:1000, Cell Signaling Technology, Beverly, MA) and mouse anti-p53 monoclonal antibody (1:1000, Oncogene Research Products, CA). Phosphorylated p53 in LCLs from Patients 1 and 2 increased after irradiation, whereas such an increase was smaller in ATLD cells. The statistical significance of the differences in phosphorylated p53/β-tubulin was tested by *t*-test. *$p < 0.05$; **$p < 0.01$ (normal, Patient 1, or Patient 2 versus ATLD). The data are shown as average ± standard error determined from three separate experiments. (c) Western blot analysis of cleaved caspase 3 protein after 10 Gy γ-irradiation of fibroblast cells. Cleaved caspase 3 bands were seen in normal, Patients 1 and 2 cells, but not in A–T and ATLD cells.

Patient 1 and the missense mutation in Patient 2 were assumed to be functionally debilitating.

MRE11A is known as the gene underlying ataxia–telangiectasia-like disorder (ATLD), a milder and slowly progressive variant of A–T without microcephaly [5]. To date, seven families with ATLD patients have been reported: two in the U.K., one in Italy, three in Saudi Arabia, and one in Japan. These patients with *MRE11A* mutations included: compound heterozygotes with a splicing mutation plus a missense mutation; homozygotes for a missense mutation; compound heterozygotes with a missense mutation plus a nonsense mutation; and homozygotes for a nonsense mutation.

It was reported that the MRE11/RAD50/NBS1 protein complex is involved in radiation-induced ATM activation [14]. We therefore analyzed level of radiation-induced ATM activation in cells from Patients 1 and 2, and those from ATLD patients. Cells from Patients 1 and 2 showed the level of ATM activation higher than those in ATLD cells. The cells from Patients 1 and 2 both showed the levels of p53 phosphorylation and caspase-3 activation higher than those in ATLD cells. The differences are likely to be attributable to the presence of normally functioning MRE11 protein in Patients 1 and 2.

Several explanations are conceivable for the unusual clinical features in the two patients. First, ATM-dependent neuronal apop-

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Total Numbers of Undiagnosed Carriers of Hepatitis C and B Viruses in Japan Estimated by Age- and Area-Specific Prevalence on the National Scale

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Key Words

Hepatitis C virus · Hepatitis B virus · Blood donors · Liver cirrhosis · Hepatocellular carcinoma · Healthcare · Japan

Abstract

Objective: To estimate total numbers of undiagnosed carriers of hepatitis C virus (HCV) and hepatitis B virus (HBV) in Japan. ***Methods:*** Area- and age-specific prevalence of HCV as well as HBV was determined in the first-time blood donors [20–39 years (n = 2,429,364)] and examinees of periodical health check-ups [40–74 years (6,204,968 for HCV and 6,228,967 for HBV)] in Japan. Prevalence in adolescents [5–19 years (79,256 for HCV and 68,792 for HBV)] was determined in a single prefecture, and that of HCV in the elderly (≥75 years) was estimated by the exponential model. HBV infection was determined by the detection of hepatitis B surface antigen, and HCV infection by either the algorithm or assuming persistent infection in 70% of the individuals with antibody to HCV. ***Results:*** Of the total population of 127,285,653 in 2005, 807,903 (95% CI 679,886–974,292) were estimated to be infected with HCV at a carrier rate of 0.63%, and 903,145 (837,189–969,572) with HBV at that of 0.71%. ***Conclusion:*** Accurate estimation of undiagnosed HCV and HBV carriers in the general population would help to predict the future burden of liver disease, and take appropriate measures for improving healthcare.

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Introduction

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are estimated to infect 170 and 350 million people over the world, respectively [1, 2]. Most infections with HCV or HBV do not induce clinical liver disease, while ~30% of them develop severe liver disease such as cirrhosis and hepatocellular carcinoma [3, 4]. Hence, there is a pressing need to identify the individuals who have undiagnosed HCV or HBV infection, and take effective measures for terminating viral infections and preventing the progression of liver disease.

For management of persistent HCV and HBV infections in a given country, it is necessary to know their exact numbers for assessing medical and financial needs in the foreseeable future. Prevalence of undiagnosed HCV or HBV

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infection has been estimated by survey of blood donors in Japan [5] and a representative population in the USA [6].

In the present study, area- and age-specific prevalence of HCV or HBV infection was determined in 8 jurisdiction areas of the Japanese Red Cross Blood Center. Then, the total numbers of undiagnosed HBV and HCV infections were estimated by compiling the results in the first-time blood donors and examinees of the periodical health check-up program. Of the 127,285,653 Japanese registered in 2005, 807,903 (0.63%) were estimated to be infected with HCV and 903,145 (0.71%) with HBV.

Materials and Methods

Japanese Population

Japan is divided into 8 areas, along its north-to-south axis, according to jurisdiction of the Japanese Red Cross Blood Center, into Hokkaido, Tohoku, Kanto, Hokuriku/Tokai, Kinki, Chugoku, Shikoku and Kyushu. Populations in 5-year age groups in each jurisdiction area were obtained from the registry at the National Census 2005.

First-Time Blood Donors

During 6 years from January 2001 to December 2006, 3,748,422 individuals (aged 16–64 years) donated whole blood or apheresis products for the first time, and their sera were tested for markers of HCV and HBV infections. Ongoing HCV infection was estimated by assuming the detection of HCV RNA in 70% of individuals with the antibody to HCV (anti-HCV), in accordance with a previous report [5].

Examinees of Hepatitis Virus Infections

Since the fiscal year 2002 in Japan, individuals who turned 40, 45, 50, 55, 60, 65 and 70 years were offered to take tests for hepatitis viruses at periodical health check-ups by a 5-year national project. During 5 years through 2006, 6,204,968 individuals received tests for HCV and 6,228,967 for HBV, corresponding to ~30% of the eligible Japanese, and their area- and age-specific prevalence of HCV or HBV infection was determined.

School Children and Adolescents

In the Iwate prefecture located in the north of Japan, biochemical markers of diseases dependent on the lifestyle were examined in children and adolescents at the entrance to schools. Their serum samples had been stored frozen, and were tested for markers of hepatitis virus infections. Carrier rates of HCV and HBV among them were calculated, with their ages adjusted to those in 2005; infants aged <5 were represented by the children aged from 5 to 9 years. Designs and procedures of this investigation were approved by the Ethics Committee of Hiroshima University.

Simulation of HCV and HBV Infections in the Elderly

By its age-specific profile, the prevalence of HCV was deduced to be an exponential function of the age. Accordingly, age-specific prevalence of HCV in the individuals aged ≥75 years was simulated by an exponential function model; it was constructed on the prevalence of HCV in each age group ≥50 years.

The formula was constructed as:

$$\log y(x) = a + bx$$

where x is the 5-year age code, $y(x)$ is an estimator of HCV prevalence in x, and a and b are coefficients.

The equation is transformed into:

$$y(x) = e^a e^{bx}$$

in which e^a represents the HCV prevalence when $x = 0$ (in the group aged 0–4 years), since $y(0)$ is equal to e^a. By replacing x for $x + 1$ in the above equation, it is converted to $y(x + 1) = e^a e^{b(x+1)}$.

Then, the following equation can be constructed:

$$y(x + 1) = e^b y(x)$$

where e^b is the slope of HCV prevalence increasing with age. Thus, the HCV prevalence is multiplied by a factor e^b for an increment of the age code by 1.

The simulation model was applied to estimate age-specific prevalence of HCV in each of 8 areas in the individuals ≥75 years.

Prevalence of HBV in the individuals ≥75 years was represented by that in those aged 70–74 years, since it stayed constant from 65 through 75 years.

Markers of Hepatitis Virus Infections

In blood donors, anti-HCV was determined by passive hemagglutination of the second generation with commercial assay kits (HCV PHA; Abbott Laboratories, North Chicago, Ill., USA) with a cutoff limit set at 2^5, as well as by particle agglutination with commercial assay kits (HCV PA Test-II; Fujirebio, Inc., Tokyo, Japan). HBsAg was determined by reversed passive hemagglutination with reagents prepared by the Japanese Red Cross.

In examinees of periodical health check-ups, ongoing HCV infection was determined by the algorithm with anti-HCV and HCV RNA [7]. Anti-HCV was determined by passive hemagglutination of the second generation with commercial assay kits (HCV PHA; Abbott Laboratories), and since 2002, it was determined by enzyme immunoassay with commercial assay kits (AxSYM HCV Dinapack-III; Abbott Laboratories). Samples with high anti-HCV titers contain HCV RNA, and therefore, only those with low and middle titers were examined for HCV RNA. HBsAg was determined by reversed-passive hemagglutination with commercial assay kits (Institute of Immunology Co., Ltd, Tokyo, Japan).

Statistical Analyses

Statistical analyses for the evaluation of R^2 values were performed with JMP 8.0 (SAS Institute, Inc., Cary, N.C., USA) and DeltaGraph 5.5 (RedRock Software, Inc., Salt Lake City, Utah, USA). A p value > 0.05 was considered significant.

Results

Age-Specific Prevalence of HCV in the First-Time Blood Donors and Examinees of Periodical Health Check-Ups

Figure 1 illustrates age-specific prevalence of HCV in the first-time blood donors (aged 15–69 years in 2005) and examinees of periodical health check-ups (39–73 years in 2005); 70% of individuals with anti-HCV were considered

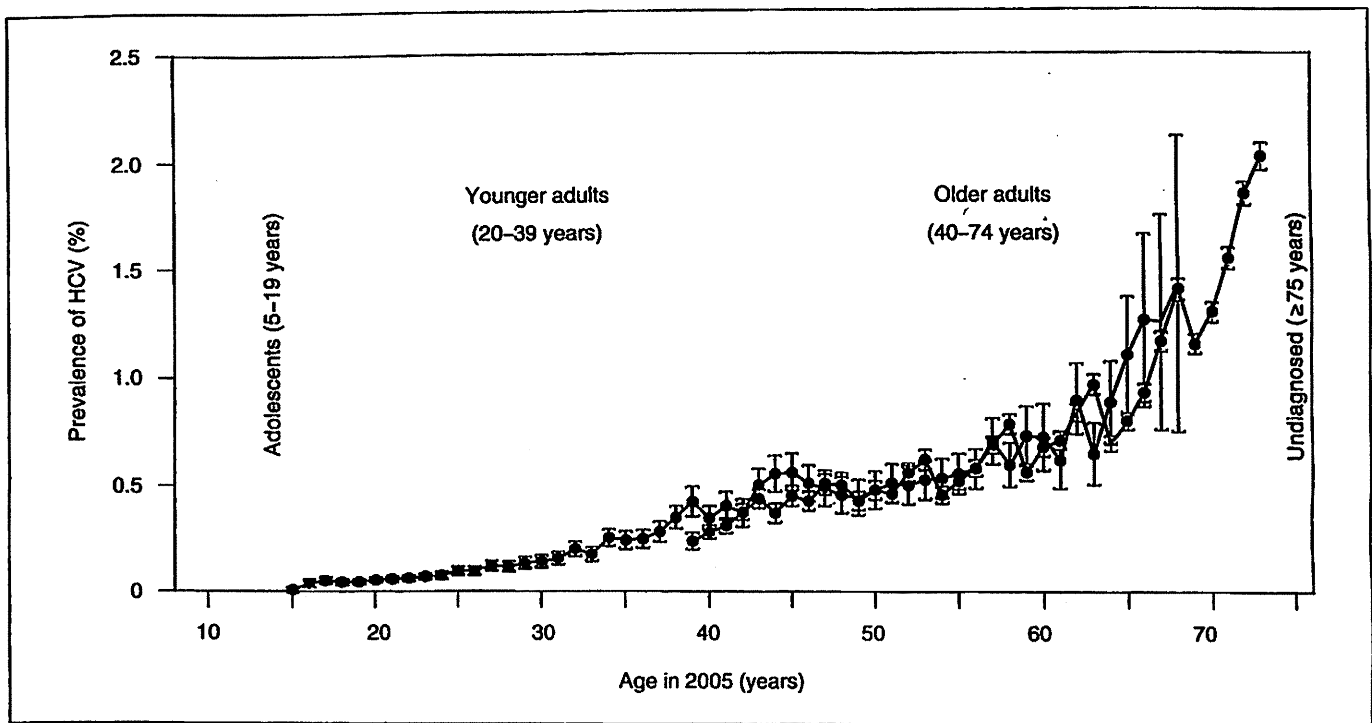

Fig. 1. Age-specific prevalence of HCV in Japan. The prevalence of HCV was determined in the first-time blood donors aged from 15 to 68 years (blue dots) and examinees of periodical health check-ups aged from 39 to 73 years (red dots). Their ages were adjusted to those in the year 2005. Bars indicate ranges of 95% CI.

to possess HCV RNA in serum [5]. Results of two distinct populations were well in accord. For the first-time blood donors, however, the variation (95% CI) widened increasingly with age. It would have reflected decreases in the first-time blood donors with age, since the majority of these (83.5%) were aged ≤39 years. As the prevalence of HCV in blood donors ≥40 years was unreliable in them, that in examinees of periodical check-ups was adopted for estimating the national prevalence of HCV.

Area-Specific Prevalence of HCV in Eight Jurisdiction Areas

In view of distinct geographic distribution of HCV, the prevalence of HCV in the general population would not be applicable to every area in Japan. Figure 2 compares results in the first-time blood donors and recipients of health check-ups among 8 jurisdiction areas spanning from north (Hokkaido) to south (Kyushu). They unfolded a wide variety in the age-specific prevalence of HCV. Although the prevalence of HCV increased with age in all areas, the slope of increase differed widely among them. Hence, it was necessary to employ a distinct age-specific prevalence in each of the 8 areas for estimating HCV carriers precisely.

Table 1. Age-specific prevalence of HCV in three different populations

Age in 2005	n	HCV-positive, n	Prevalence, % (95% CI)
School children			
5–9	17,390	2	0.012 (0.000–0.027)
10–14	29,817	3	0.010 (0.000–0.021)
15–19	32,049	7	0.022 (0.006–0.038)
Blood donors			
20–24	1,205,966	1,122	0.065 (0.061–0.070)[a]
25–29	536,560	874	0.114 (0.105–0.123)[a]
30–34	408,814	1,089	0.186 (0.173–0.200)[a]
35–39	278,024	1,190	0.300 (0.279–0.320)[a]
HCV screening			
40–44	611,146	2,127	0.348 (0.333–0.363)
45–49	495,032	2,292	0.463 (0.444–0.482)
50–54	675,350	3,485	0.516 (0.499–0.533)
55–59	947,438	5,974	0.631 (0.615–0.646)
60–64	1,081,854	8,423	0.779 (0.762–0.795)
65–69	1,264,496	13,722	1.085 (1.067–1.103)
70–74	1,054,472	17,649	1.674 (1.649–1.698)

[a] The prevalence in blood donors was based on an assumption of HCV infection persisting in 70% of those with anti-HCV [5].

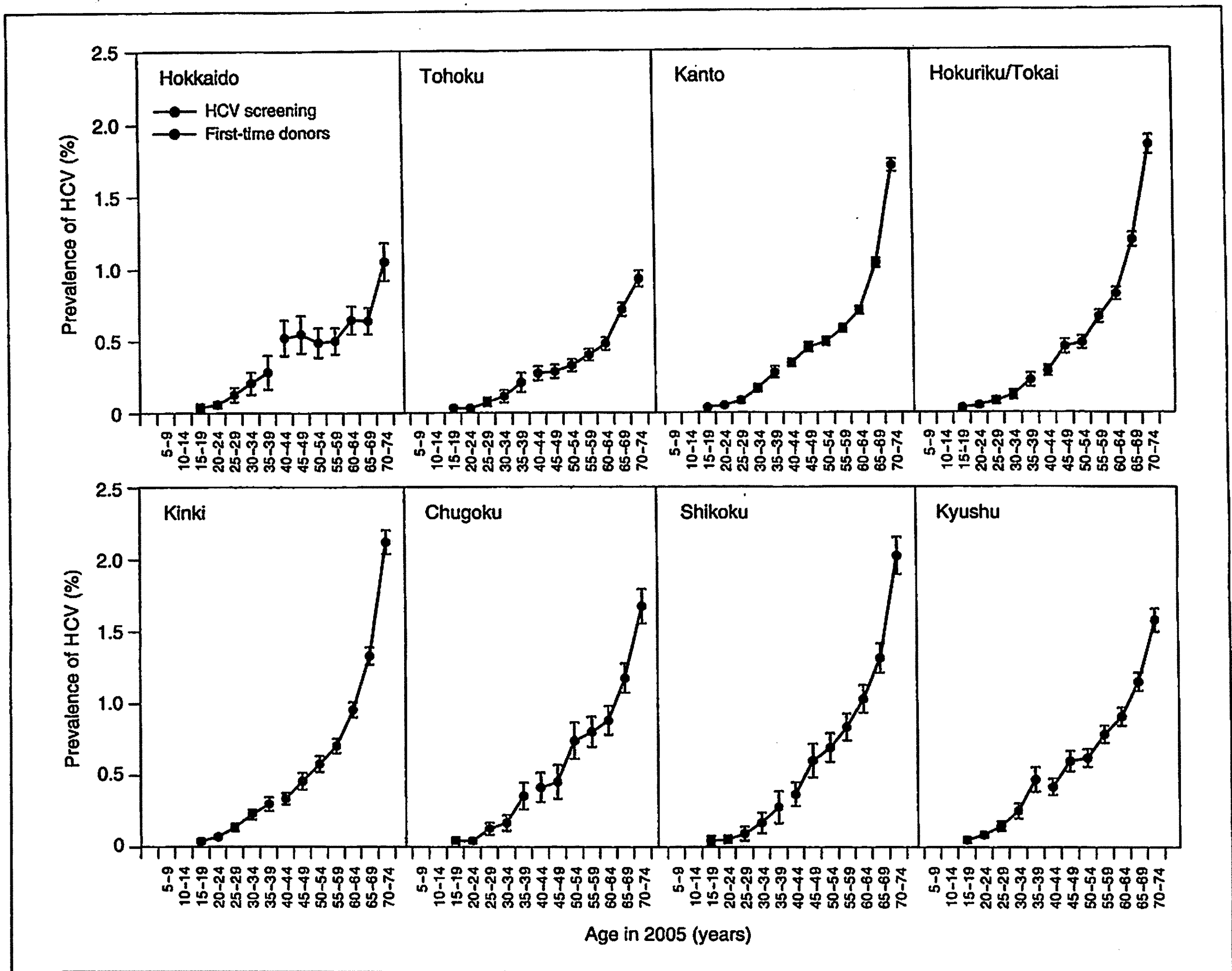

Fig. 2. Age-specific prevalence of HCV in 8 jurisdiction areas in Japan. The prevalence of HCV is calculated in each of twelve age groups notched by 5 years. The prevalence in five groups ≤39 years was represented by the first-time blood donors, and that in seven groups ≥40 years by recipients of HCV screening. Bars indicate ranges of 95% CI.

Prevalence of HCV in Adolescents

Since blood donors were restricted to 16–64 years of age, and health examinees were targeted on 40–70 years, they did not cover individuals aged ≤15 or ≥75 years in the year 2005. To fill in an opening on the younger side, the age-specific prevalence of HCV was determined in school children and adolescents in the Iwate prefecture (table 1). The prevalence in infants aged 0–4 years was assumed similar to that in the children aged 5–9 years; an extremely low prevalence of HCV (0.012%) would support such an assumption.

Simulating Prevalence of HCV in the Elderly

The prevalence of HCV appeared to be an exponential function of the age, according to its profiles in the first-time blood donors and examinees of health check-ups (fig. 1). Based on this assumption, a formula was constructed to simulate the prevalence of HCV in age groups ≥75 years for each of the 8 jurisdiction areas in Japan (see Materials and Methods).

Figure 3 compares actual (dots) and simulated data (red line) of five age groups from 50 to 74 years (corresponding to age codes 10–14) among the 8 areas. There was a high coefficient of determination between them,

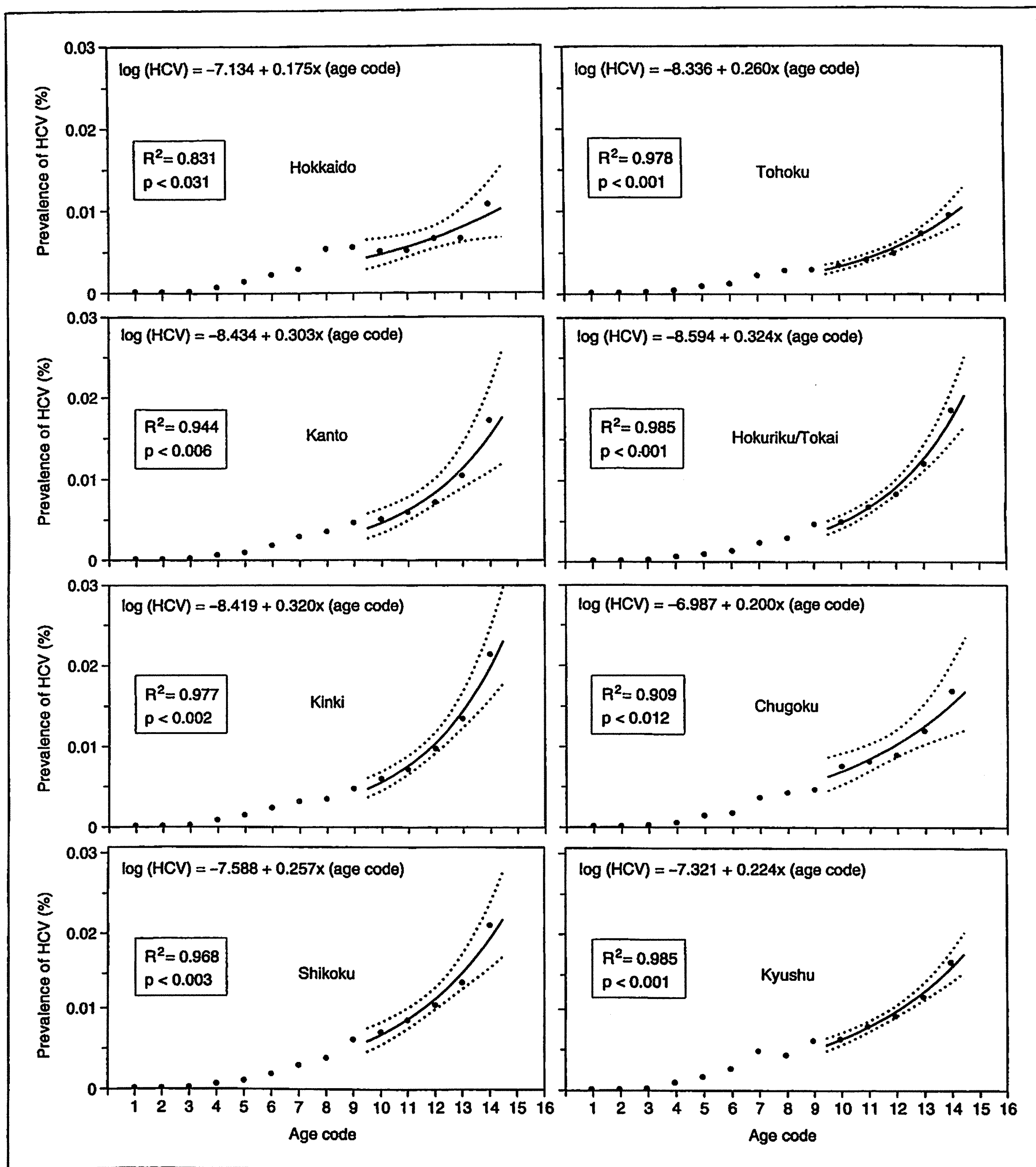

Fig. 3. Simulation of age-specific prevalence of HCV in the elderly. Prevalence of HCV in the first-time blood donors as well as examinees of periodical health check-ups (dots) and that simulated by formulation (red line with ranges of 95% CI in dotted line) are shown for 8 jurisdiction areas in Japan. Formula is shown at the top of each area. Age codes are: 1, 5–9 years; 2, 10–14 years; 3, 15–19 years; 4, 20–24 years; 5, 25–29 years; 6, 30–34 years; 7, 35–39 years; 8, 40–44 years; 9, 45–49 years; 10, 50–54 years; 11, 55–59 years; 12, 60–64 years; 13, 65–69 years; 14, 70–74 years, and 15, 75–79 years.

Table 2. Regional and total HCV carriers in Japan

Areas	Population	HCV carriers (95% CI)	Carrier rate
Hokkaido	5,620,813	26,097 (19,356–34,413)	0.46%
Tohoku	12,047,975	50,688 (42,754–59,953)	0.40%
Kanto	41,247,892	235,328 (195,408–293,611)	0.57%
Hokuriku/Tokai	19,294,443	132,434 (114,216–154,446)	0.69%
Kinki	22,657,542	173,808 (147,548–207,173)	0.52%
Chugoku	7,650,977	53,296 (42,299–67,698)	0.70%
Shikoku	4,083,698	35,159 (28,746–43,004)	0.86%
Kyushu	14,682,313	101,092 (89,379–113,993)	0.80%
Total	127,285,653	807,903 (679,886–974,292)	0.63%

Table 3. Age-specific prevalence of HBV in three different populations

Age in 2005	n	HBV-positive, n	Prevalence, % (95% CI)
School children			
5–9	17,363	3	0.017 (0.000–0.037)
10–14	29,817	14	0.047 (0.022–0.072)
15–19	32,049	12	0.037 (0.016–0.059)
Blood donors			
20–24	1,205,966	1,826	0.151 (0.144–0.158)
25–29	536,560	1,650	0.308 (0.293–0.322)
30–34	408,814	1,759	0.430 (0.410–0.450)
35–39	278,024	1,327	0.477 (0.452–0.503)
HBV screening			
40–44	613,960	5,491	0.894 (0.871–0.918)
45–49	497,589	5,373	1.080 (1.051–1.109)
50–54	679,893	8,700	1.280 (1.253–1.306)
55–59	950,508	12,891	1.356 (1.333–1.379)
60–64	1,085,119	13,282	1.224 (1.203–1.245)
65–69	1,268,304	12,406	0.978 (0.961–0.995)
70–74	1,057,469	9,545	0.903 (0.885–0.921)

with R^2 values ranging from 0.831 to 0.985 ($p < 0.031$ and $p < 0.001$, respectively), attesting to the validity of this simulation. Of note, the factor *b* in formula (by which age codes were multiplied) varied broadly among the 8 areas. Thus, it was the highest in Hokuriku/Tokai at 0.324 and lowest in Hokkaido at 0.175, with close to twofold differences between them.

Estimation of Undiagnosed HCV Carriers in Eight Areas and the Entire Nation

Based on age- and area-specific prevalence of HCV, numbers of undiagnosed HCV carriers were calculated for 8 jurisdiction areas, and they were compiled in the entire nation (table 2). The prevalence of HCV in each of three age groups (75–79, 80–84 and ≥85 years) was simulated by the formula, while that of HBV was represented by the prevalence in the group of 70–74 years. As of the year 2005, 127,285,653 were registered in the national census of Japan, and 807,903 of these are estimated to have undiagnosed HCV infection at an overall carrier rate of 0.63%. There was an increasing gradient in the prevalence of HCV along the north-to-south axis of Japan.

Age-Specific Prevalence of HBV

Figure 4 depicts age-specific prevalence of HBV in 2005. It was deduced from HBsAg in the first-time blood donors (15–69 years) and examinees of periodical health check-ups (39–73 years). Since the prevalence of HBV in the elderly did not increase with age so sharply as that of HCV (fig. 1), it was presumed not to increase further and stay around 1% in the individuals ≥75 years. The age-specific prevalence of HBV tabulated in three different

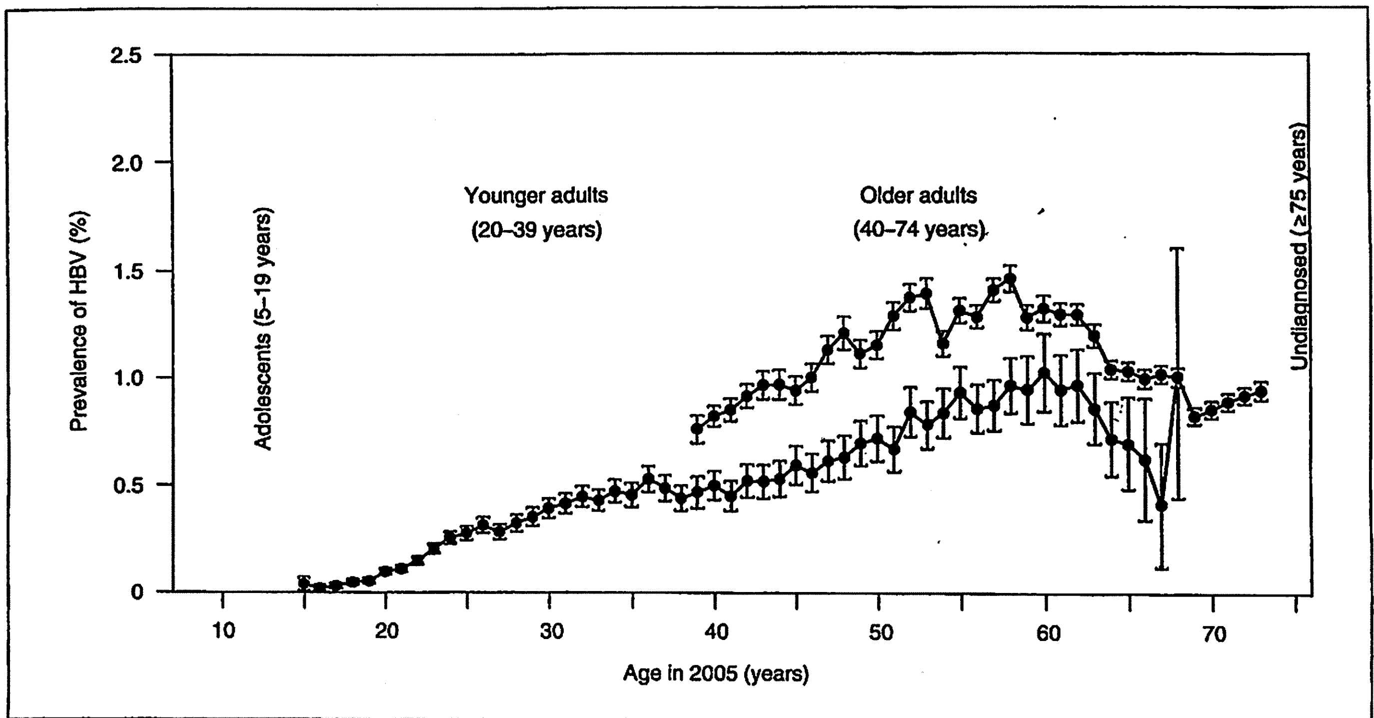

Fig. 4. Age-specific prevalence of HBV in Japan during 2002–2006. The prevalence of HBV was determined in the first-time blood donors aged from 15 to 68 years (blue dots) in the year 2005 and examinees of periodical health check-ups aged from 39 to 73 years (red dots) in the year 2005. Bars indicate ranges of 95% CI.

populations is listed in table 3. There was a constant decline with decreasing age in the frequency of HBV in individuals ≤39 years, and it was particularly low in children ≤9 years (0.017%).

In examinees of periodical health check-ups, the age-specific prevalence of HBV did not diverge and stayed within a narrow 95% CI (fig. 4). By contrast, that in the first-time blood donors dispersed widely. Such a variation in the age-specific prevalence of HBV would have been ascribed to the first-time blood donors who clustered in age groups ≤40 years.

Area-Specific Prevalence of HBV in Eight Jurisdiction Areas

The age-specific prevalence of HBsAg varied widely among 8 jurisdiction areas (fig. 5). HBsAg was most frequent in the age group of 55–59 years in every area, and reached 3.1% in the northern-most Hokkaido. The peak frequency decreased in central Japan (1.1% in Kanto and Hokuriku/Tokai), and increased towards the southern end (1.9% in Kyushu). Thus, the prevalence of HBsAg was determined individually along the axis of Japan in estimating the total number of HBV carriers in Japan.

Estimation of Undiagnosed HBV Carriers in Eight Areas and the Entire Nation

Numbers of undiagnosed HBV carriers were complied by multiplying age-specific prevalence of HBsAg by corresponding subpopulations in 8 jurisdiction areas (table 4). In total, 903,145 of the 127,285,653 (0.71%) individuals are estimated to have undiagnosed HBV infection in Japan in 2005.

Shift of Undiagnosed HCV and HBV Carriers during 5 Years (2000–2005) in Japan

Table 5 compares numbers of HCV and HBV carriers aged 15–69 years between 2000 and 2005 for 8 jurisdiction areas in Japan. Data for the year 2000 were extracted from a previous survey [5]. Data for the year 2005 were obtained in the first-time blood donors during 2001–2006 in this study by the same method as in the previous survey [5]. Undiagnosed HCV and HBV carriers decreased during 5 years by 55 and 47.5%, respectively. The overall carrier rate of HCV declined sharply from 0.95 to 0.44%, and that of HBV from 1.04 to 0.55% in Japan.

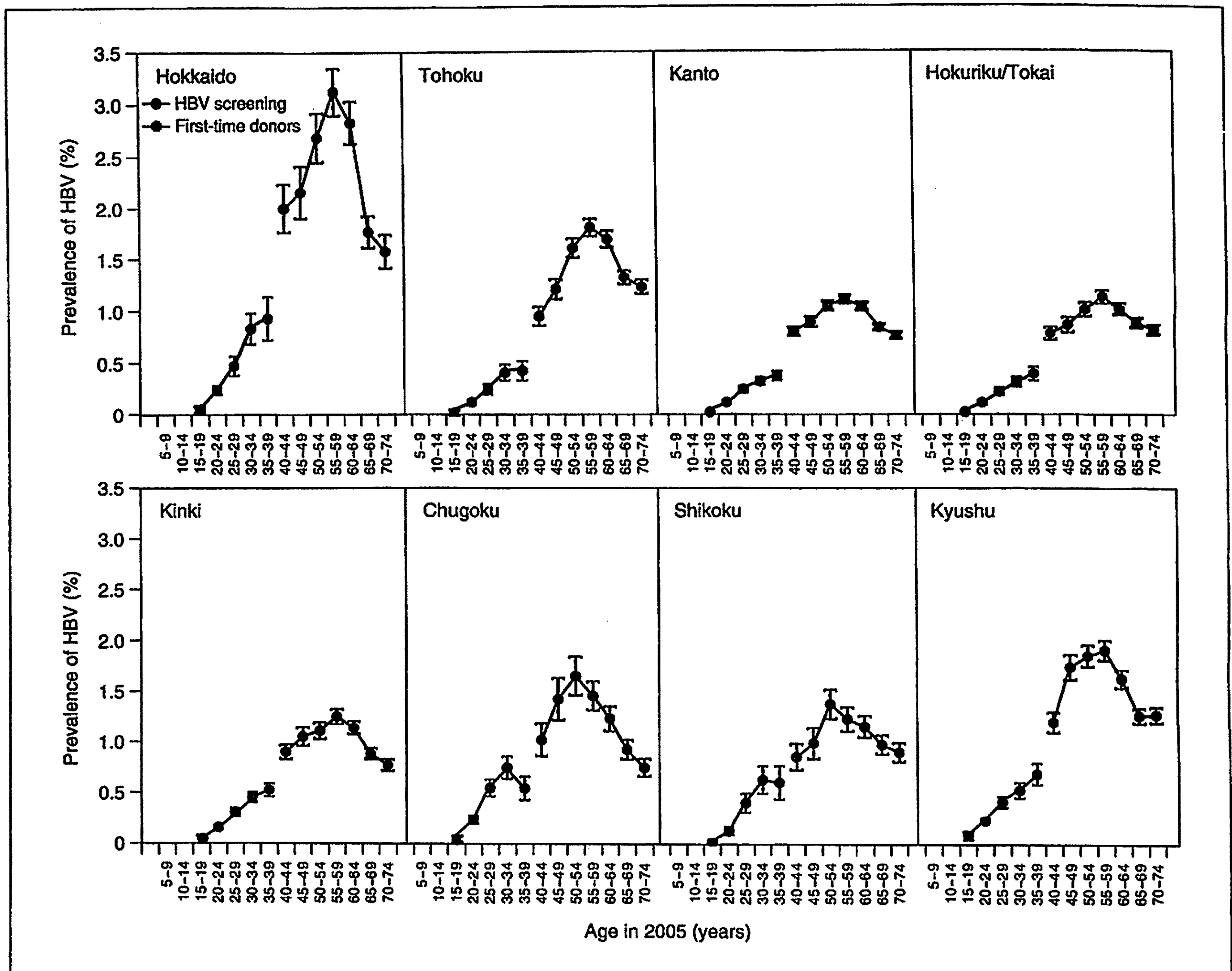

Fig. 5. Age-specific prevalence of HBV in 8 jurisdiction areas in Japan. The prevalence of HBV is calculated in each of twelve age groups notched by 5 years. The prevalence in five groups ≤39 years was represented by the first-time blood donors and that in seven groups ≥40 years by recipients of HCV screening. Bars indicate ranges of 95% CI.

Table 4. Regional and total HBV carriers in Japan

Areas	Population	HBV carriers (95% CI)	Carrier rate
Hokkaido	5,620,813	80,573 (72,314–88,765)	1.43%
Tohoku	12,047,975	104,736 (97,742–111,816)	0.87%
Kanto	41,247,892	231,799 (220,129–244,105)	0.56%
Hokuriku/Tokai	19,294,443	109,709 (101,722–117,581)	0.56%
Kinki	22,657,542	144,965 (134,387–155,464)	0.64%
Chugoku	7,650,977	59,948 (52,705–67,121)	0.78%
Shikoku	4,083,698	29,776 (26,080–33,437)	0.73%
Kyushu	14,682,313	141,639 (132,111–151,282)	0.96%
Total	127,285,653	903,145 (837,189–969,572)	0.71%

Table 5. Decrease of undiagnosed HCV and HBV carriers in the 15- to 69-year-old population in Japan

	Survey in 2000[a]		Survey in 2005		Difference	
	number estimated	carrier rate in area[b]	number estimated	carrier rate in area[b]	number estimated	balance
Shift of HCV carriers during 5 years from 2000 to 2005						
Hokkaido	41,139	0.99%	17,658	0.44%	-23,481	-57.1%
Tohoku	61,658	0.71%	30,525	0.37%	-31,133	-50.5%
Kanto	277,644	0.90%	126,283	0.41%	-151,361	-54.5%
Hokuriku/Tokai	88,724	0.64%	48,360	0.35%	-40,364	-45.5%
Kinki	178,871	1.06%	70,526	0.43%	-108,345	-60.6%
Chugoku	72,431	1.32%	24,595	0.47%	-47,836	-66.0%
Shikoku	43,497	1.49%	16,504	0.59%	-26,993	-62.1%
Kyushu	120,989	1.16%	64,115	0.63%	-56,874	-47.0%
Total	884,954	0.95%	398,567	0.44%	-486,387	-55.0%
Shift of HBV carriers during 5 years from 2000 to 2005						
Hokkaido	106,896	2.56%	54,557	1.35%	-52,339	-49.0%
Tohoku	104,923	1.21%	48,490	0.58%	-56,433	-53.8%
Kanto	255,207	0.83%	132,414	0.43%	-122,793	-48.1%
Hokuriku/Tokai	78,481	0.56%	51,477	0.37%	-27,004	-34.4%
Kinki	165,915	0.98%	85,083	0.52%	-80,832	-48.7%
Chugoku	90,041	1.64%	37,706	0.71%	-52,335	-58.1%
Shikoku	38,411	1.32%	19,162	0.69%	-19,249	-50.1%
Kyushu	127,879	1.23%	77,941	0.77%	-49,938	-39.1%
Total	967,753	1.04%	506,830	0.55%	-460,923	-47.6%

[a] Data for the year 2000 were extracted from a previous survey of hepatitis virus infections in Japan [5].
[b] The carrier rate specific for respective jurisdiction area was applied.

Discussion

There are many constraints in estimating total HCV and HBV infections in a given nation. Since it is not feasible to test every member for serological markers of hepatitis virus infection, populations representative of the entire nation have served for the estimation. Volunteer blood donors are recruited, but they have a restricted age range (16–64 years in Japan). Students attending schools and universities can close the opening in younger generations, but infants younger than the school age are not enrolled. Moreover, there are no means of estimating carrier rates of hepatitis virus infections in the individuals aged beyond the eligibility of blood donation. In addition, blood donors are selected individuals who are leading healthy lives above the average. In the survey of inhabitants in sentinel counties of the USA [6], who represent the average Americans, patients with liver disease and persons with restricted activities, such as those incarcerated or institutionalized, are not included.

Patients with clinical liver disease, as well as individuals found with HCV or HBV infection by health check-ups, can receive the medical care. However, many blood donors found with viral infections have developed severe liver disease already, and therefore, cannot receive efficient medical interventions [7, 8]. Hence, it is necessary to detect undiagnosed HCV and HBV infections hidden in the society. For this purpose, periodical health check-ups for screening hepatitis virus markers were started in April 2002 on the individuals, who turned 40, 45, 50, 55, 60 and 70 years, by a 5-year national project in Japan. The target age range (40–70 years) was selected due to a high incidence of hepatocellular carcinoma [9]. Since by far the majority of the first-time blood donors were younger than 40 years, the prevalence of HCV or HBV beyond that age dispersed widely (fig. 1, 4). In this study, therefore, the coverage by the first-time blood donors was confined to 20–39 years of age, and it was taken place by examinees of health check-ups aged 40–74 years; they left age groups ≤15 and ≥75 years uncovered, however.

The national prevalence of hepatitis virus infections in individuals ≤19 years was presumed to be similar to that in the Iwate prefecture situated in northern Japan. Since the prevalence of HCV or HBV infection in them was extremely low and stayed between 0.01 and 0.02%, such an assumption would not have affected the overall results to any significant extent. The prevalence of HCV in age groups ≥75 was simulated by a premise that it would be an exponential function of the age. Consequently, the formula based on profiles in five age groups from 50 to 74 years (at a 5-year notch) was extrapolated to three age groups ≥75 years. The simulation matched closely with the prevalence determined in corresponding age groups, with R^2 values ranging from 0.83 to 0.99 ($p < 0.05$ and $p < 0.01$, respectively) throughout 8 jurisdiction areas in Japan (fig. 3).

Japan has an axis spanning 2,000 kilometers from the north-east towards the south-west over the four major islands (Hokkaido, Honshu, Shikoku and Kyushu). Within a rather small land, the prevalence of HCV or HBV is not uniform all over Japan. The prevalence of HCV had an increasing gradient from north to south, and was the highest in Kyushu (table 2), while that of HBV was the highest in Hokkaido, decreased in between and then increased towards Kyushu (table 4). Reflecting such local differences, age-specific prevalence of HCV or HBV differed widely among 8 jurisdiction areas (fig. 2, 5).

Based on the results obtained on the area- and age-specific prevalence of HCV or HBV, carriers of these hepatitis viruses in 8 jurisdiction areas were tabulated separately over age groups from 20 to 74 years. Those in age groups ≤19 years were represented by the Iwate prefecture. The prevalence of HCV in age groups ≥75 years was simulated by the formula, and that of HBV was represented by individuals aged 70–74 years. Japan was populated by 127,767,994 people in 2005. Of these, 807,903 (95% CI 679,886–974,292) were estimated to have undiagnosed HCV infection at an overall prevalence of 0.63%, and 903,145 (837,189–969,572) to possess undiagnosed HBV infection at that of 0.71%. These estimates are much less than publically inferred numbers of HCV and HBV carriers in Japan at 1.5–2.0 million each. Leaving aside HCV and HBV carriers who have developed liver disease and stayed outside the scope of the present study, our estimates based on reasonable scientific grounds are much smaller; they add up barely half of generally referred figures around 1.5–2.0 million in Japan.

Based on the sex- and age-specific prevalence of hepatitis virus markers in the 3,478,422 first-time blood donors during 2001–2006, with the same criteria used in the previous study [5], we have estimated the number of undiagnosed HCV carriers aged 15–69 years in the year 2005 to be 398,567 (95% CI 295,410–501,453) and that of undiagnosed HBV carriers to be 506,830 (95% CI 398,115–616,113). In the previous study [5], undiagnosed HCV and HBV carriers aged 15–69 years in the year 2000 were assessed to be 884,954 (95% CI 725,082–1,044,826) and those with HBV to be 967,753 (95% CI 806,760–1,128,745). They decreased by 55.0 and 47.6%, respectively, during 5 years (table 5). In support of this view, the incidence of HCV or HBV infection during 10 years (1994–2000) in Japan is very low and estimated at 1.86 (95% CI 1.06–3.01) or 2.78 (1.87–4.145) per 100,000 person-years [10]. Decreases in undiagnosed HCV and HBV carriers in Japan would have been attributed to increased chances of receiving tests for hepatitis virus infections at health check-ups and medical institutions, as well as increased awareness due to educational programs or other healthcare campaigns or screening programs in high-risk individuals. Additionally, there would have been a cohort effect in individuals aged 15–69 years who have shifted by 5 years during the observation period.

The results of the Third National Health and Nutrition Survey (HANES III, 1988–1994) [11] and those of more recent HANES (2001–2002) [6] in the USA are essentially similar with respect to age-specific profiles of HCV infection, and shifted by 10 years. The incidence of de novo HCV and HBV infections may have decreased substantially both in the USA and Japan, driven partly by the introduction of the nucleic acid amplification test and a more stringent questionnaire on donors to exclude blood donations in the window period of infection [12–17]. The national burden of HCV infection has been reported in Great Britain [18], where the prevalence of anti-HCV in hospitalized patients was 3.4% and that in the first-time blood donors was 0.03% in the year 2008.

In spite of many improvements in the control of hepatitis virus infections, there are many HCV and HBV carriers buried in the society who need immediate identification for receiving timely and efficient medical interventions. Treatment of viral hepatitis keeps improving, especially for liver disease induced by HCV. The sustained virological response in the patients infected with HCV of genotype 1, who have received triple therapy with pegylated interferon, ribavirin and protease inhibitors, has increased to 70% or higher, from 50% with the state-of-care therapy with pegylated interferon and ribavirin [19, 20]. With the advent of new antiviral drugs that will enter the scene in the foreseeable future, the virological response is expected to increase further. There would be

nothing like early detection of HCV and HBV infections for appropriate and timely medical care to prevent the progression of liver disease. Such a rational strategy will benefit not only patients themselves, but also merit the society and government, which are going to be burdened with ever-increasing morbidity and mortality along with skyrocketing costs.

Acknowledgements

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Interferon Alone or Combined with Ribavirin for Acute Prolonged Infection with Hepatitis C Virus in Chimpanzees

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Key Words
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Abstract
Infection with hepatitis C virus (HCV) persisted for longer than 29 weeks in 2 chimpanzees after they had been inoculated with it experimentally. One of them (C-210) received short-term subcutaneous interferon-α (IFN-α) 6 million units (MU) daily for 7 days at week 29. He cleared HCV RNA from the serum and remained negative for it during 25 weeks after the withdrawal of IFN. The other (C-224) did not respond to 2 courses of a short-term IFN monotherapy at weeks 20 and 23. Twelve weeks thereafter, he received IFN-α 3 MU daily for 2 weeks and then 3 times a week for 14 weeks combined with oral ribavirin 600 mg daily during 16 weeks. HCV RNA disappeared from the serum and stayed negative until the last follow-up 24 weeks after the completion of combination therapy.
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Due to a very narrow species-specificity of hepatitis C virus (HCV), chimpanzees remain the only animal that can be infected with it. Once they served as the sole means of identifying the infection with HCV that had been referred to as non-A, non-B hepatitis virus until its discovery in 1989 [1]. HCV infection can persist in chimps at rates ranging from 30 to 60%, depending on the age and gender as well as viral strains in inocula they have received [2, 3]; the persistence rate is comparable to that of 55–85% in humans [4, 5]. The long-term outcome of chimpanzees infected with HCV is not known, nor have there been any attempts to treat them with either interferon (IFN) alone or IFN in combination with ribavirin.

Two chimps with acute prolonged HCV infection received antiviral treatment. They were chimps No. 210 (male, 14 years old and weighing 62.8 kg) and No. 224 (male, 14 years old and weighing 59.1 kg). Both of them were kept in individual cages and received humane care, in accordance with all relevant requirements for the use of primates in an approved facility. Chimp No. 210 participated in the experimental transmission study for determining the minimum infectious dose of HCV [6]. He received 1 ml of fresh-frozen plasma from a donor in the window period of HCV infection with mixed genotypes (1b plus 2a) containing 7.0×10^6 copies/ml of HCV RNA. Chimp No. 224 was inoculated with 1 ml of fresh-frozen plasma from another donor in the window period of HCV infection with genotype 1b containing 8.4×10^6

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Fig. 1. Clinical course of chimpanzee No. 210 (C-210). The duration of IFN monotherapy is indicated at the top. HCV RNA was determined qualitatively by Amplinat MPX. Anti-HCV was determined by passive hemagglutination. Fluctuating levels of ALT and AST in the serum are shown below.

Fig. 2. Clinical course of chimpanzee No. 224 (C-224). The duration of 2 courses of IFN monotherapy (1 week each) as well as IFN (daily for 2 weeks and then 3 times a week for 14 weeks) combined with ribavirin (16 weeks) is indicated at the top.

copies/ml of HCV RNA; his preacute plasma has been included in the panel for standardization of polymerase chain reaction (PCR). They both received IFN therapy for evaluating the efficacy in treatment of acute prolonged HCV infection. The study design was approved by the Committee of Ethics for Handling of Primates in the institutions.

The 2 chimps were bled under anesthesia with ketamine hydrochloride weekly for the initial 21–24 weeks, and then at intervals until the completion of this study. HCV RNA was determined qualitatively by Amplinat MPX (Roche Diagnostics K.K., Tokyo, Japan). Antibody to HCV (anti-HCV) was determined semiquantitatively using a commercially available hemagglutination assay system (the second-generation HCV PHA, Abbott Japan, Co. Ltd., Tokyo, Japan), and the results were expressed by the highest twofold dilution of serum (2^N) that induced hemagglutination. They received IFN-α (Sumiferon®, Sumitomo Pharmaceutical Co. Ltd., Tokyo, Japan) alone or in combination with ribavirin.

Figure 1 illustrates the clinical course of chimp No. 210 who had been inoculated with 7.0×10^6 copies/ml of HCV of mixed genotypes (1b and 2a) and developed viremia during the first 24 weeks. He developed anti-HCV, 11 weeks after inoculation, along with sharp increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Anti-HCV increased, reached 2^6 hemagglutination titers and remained positive through the observation period of 55 weeks. Upon the confirmation of HCV RNA 29 weeks after inoculation, he received IFN-α 6 MU daily for 1 week. HCV RNA was not detectable by qualitative assay in his sera at the next week,