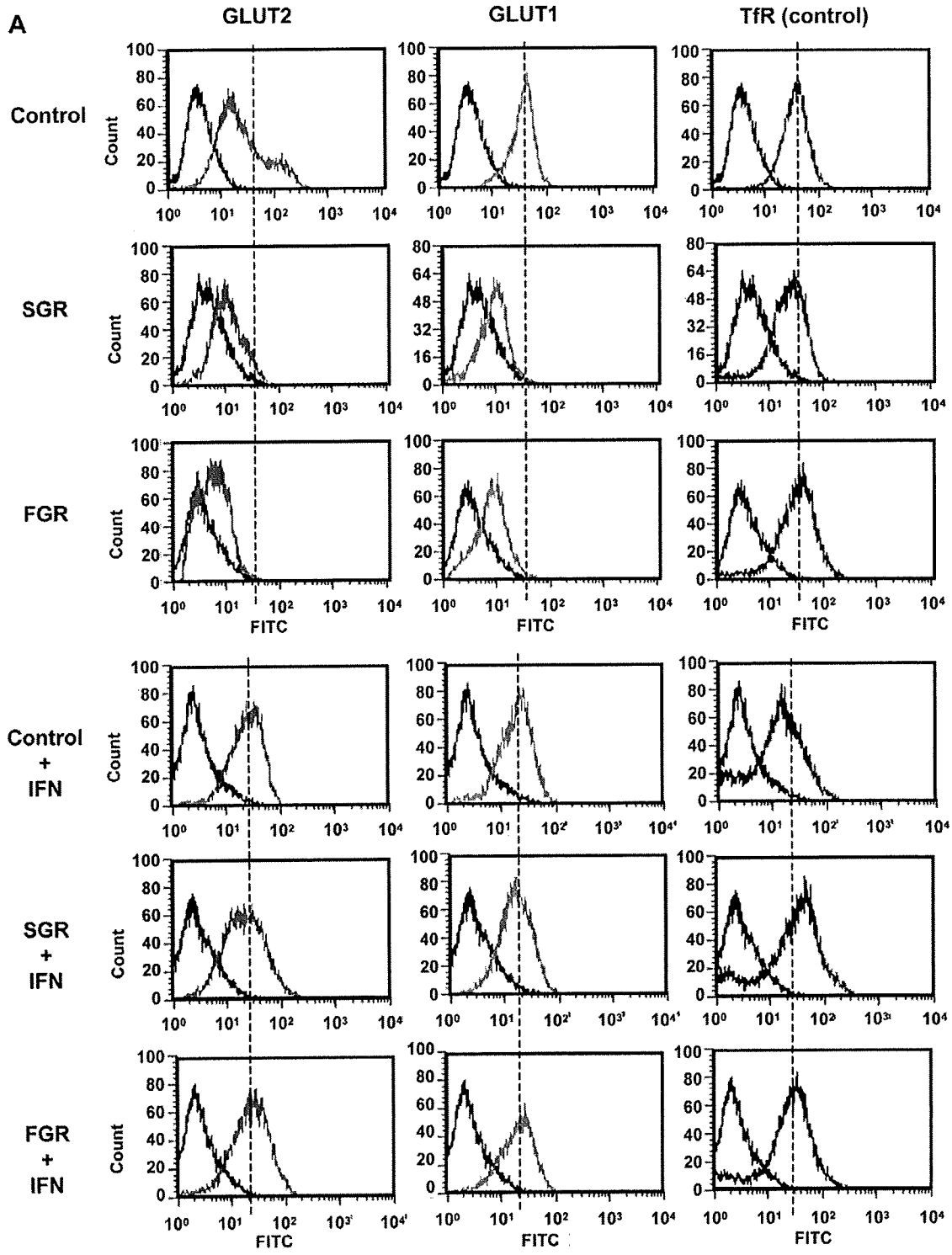

Fig. 4. Down-regulation of cell surface expressions of GLUT2 and GLUT1 by HCV replication. (A) SGR, FGR, the HCV-negative control cells were stained with specific antibodies, followed by FITC-conjugated second antibody (GLUT2, red line; GLUT1, green line) or stained with FITC-conjugated antibody alone (black line). Transferrin receptor (TfR) served as a control (blue line). In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry. (B) HCV-infected cells and the uninfected control were analyzed by flow cytometry as in (A). In parallel, cells at 5 days after infection were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to flow cytometry analysis.

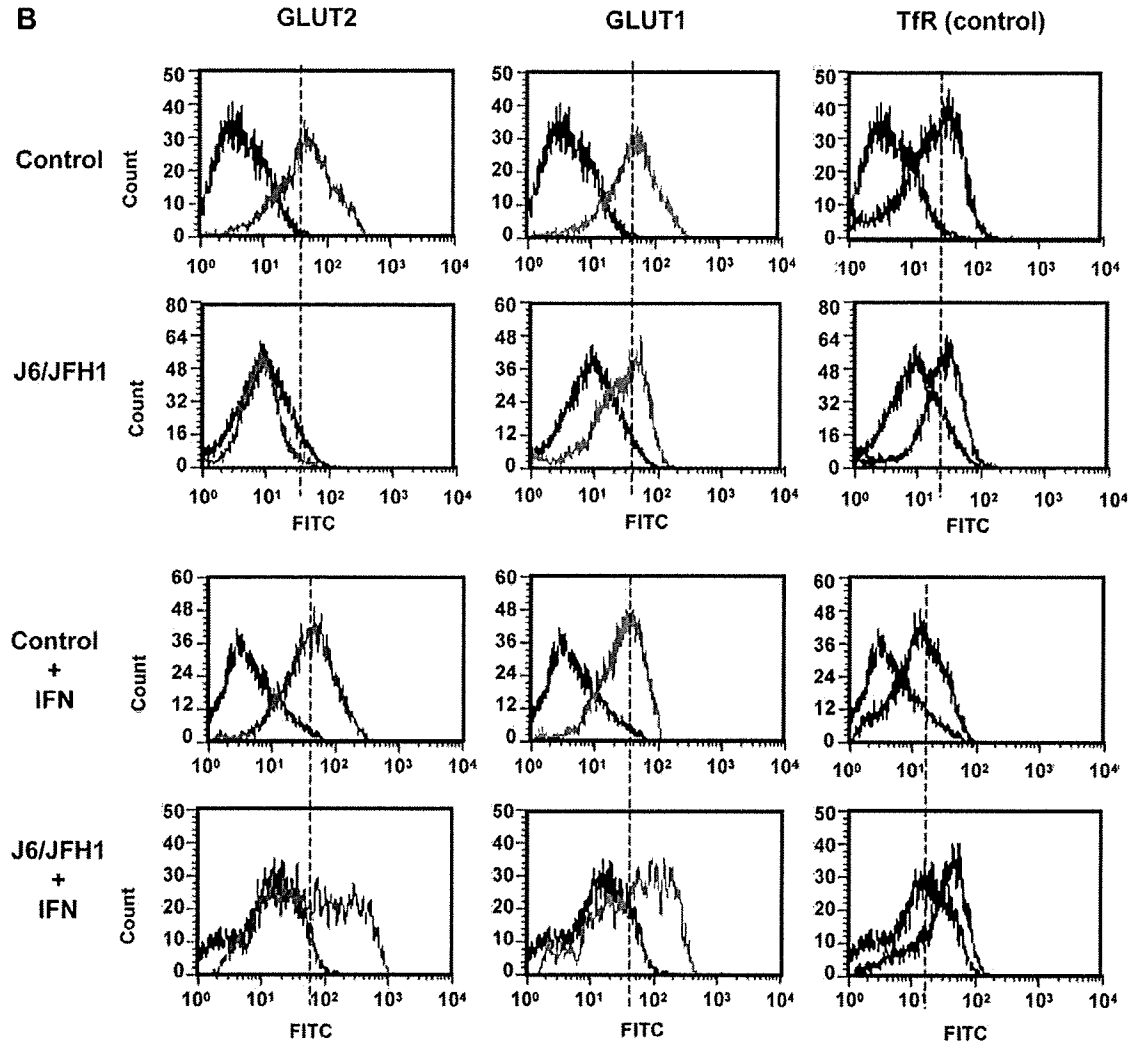

Fig. 4 (*continued*)

than in SGR cells. On the other hand, GLUT1 mRNA levels were not affected by HCV RNA replication (SGR and FGR) or HCV infection (Fig. 6B).

We also confirmed that GLUT2 mRNA expression levels in SGR, FGR and HCV-infected cells were restored by IFN treatment (Fig. 6A).

3.6. Suppression of GLUT2 promoter activity by HCV replication

Next, we performed luciferase reporter assay to examine the possible effect of HCV replication on GLUT2 promoter activities. The result obtained demonstrated that GLUT2 promoter activities were significantly suppressed in SGR, FGR and HCV-infected cells, compared to the control cells (Fig. 6C). Furthermore, GLUT2 promoter activities in SGR, FGR and HCV-infected cells were restored by IFN treatment. It is thus likely that HCV replication suppresses GLUT2 promoter activity, thereby decreasing GLUT2 mRNA levels.

3.7. Ectopically expressed GLUT1 or GLUT2 mediates increased glucose uptake in SGR, FGR and HCV-infected cells

We examined the possible effects of ectopically expressed GLUT1 and GLUT2 on glucose uptake in SGR, FGR and HCV-infected cells. Glucose uptake was significantly increased by ectopically expressed GLUT1 or GLUT2 in SGR, FGR and HCV-infected cells as well as in the control Huh-7.5 cells (Fig. 6D). It should be noted that, in this series of transient transfection experiments, only ca. 20% of the cells were ectopically overexpressing GLUT1 or GLUT2. These results collectively suggest the possibility that down-regulation

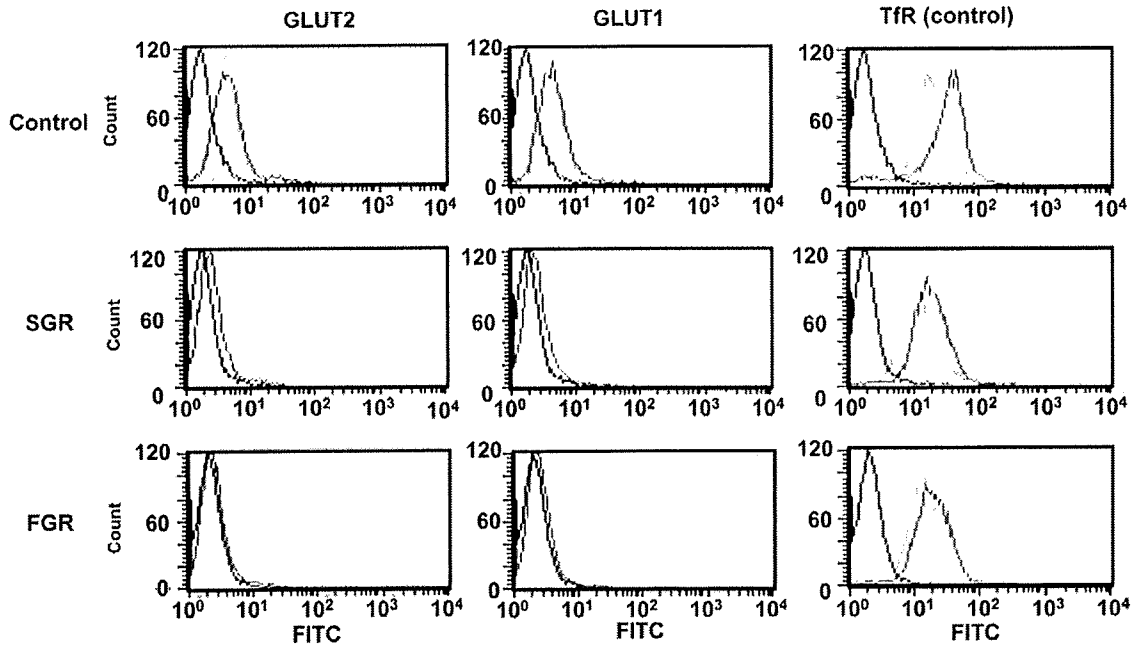

Fig. 5. Effects of lactacystin treatment on cell surface expression of GLUT2, GLUT1 and transferrin receptor (TfR). Cells were treated with lactacystin (10 μM) overnight to inhibit proteasomal degradation, and analyzed by flow cytometry. Cells treated with lactacystin are shown in red line and those left untreated in blue line. The negative controls stained with FITC-conjugated antibody alone are shown in black line.

of GLUT1 and GLUT2 expression is primarily involved in the decreased glucose uptake in SGR, FGR and HCV-infected cells.

3.8. Decreased GLUT2 expression in hepatocytes obtained from HCV-infected patients

GLUT2 is the principal glucose transporter expressed in hepatocytes *in vivo*. As shown in Fig. 7B, practically all hepatocytes obtained from patients without HCV infection showed positive staining for GLUT2, which was most evidently observed near the plasma membrane. On the other hand, hepatocytes obtained from HCV-infected patients showed markedly reduced GLUT2 staining in most, if not the entire, areas of the section, compared with the uninfected control (Fig. 7D). This heterogeneous staining pattern might reflect concomitant presence of areas comprising either virus-infected or uninfected hepatocytes in a tissue sample. Whereas all the sections obtained from 8 patients without HCV infection showed evenly positive staining for GLUT2, sections from 8 (89%) of 9 HCV-infected patients showed moderately to markedly reduced GLUT2 staining (Table 2). Reduced GLUT2 staining was observed also with hepatocytes in the liver tissues obtained from HBV-infected patients. However, the areas of reduced GLUT2 staining appeared to be more restricted in sections obtained from HBV-infected patients than in those from HCV-infected ones.

4. Discussion

HCV infection is known as an initiation and precipitating factor of type 2 diabetes [7–10,26,27]. Progression of liver fibrosis induced by persistent viral infection may induce diabetes [28]. Furthermore, it has been reported that the prevalence of diabetes is higher among patients with HCV-associated liver cirrhosis than in those with HBV-associated cirrhosis [7]. It is likely, therefore, that HCV infection itself is a risk factor of diabetes. Previous reports suggest that HCV infection directly causes insulin resistance that would cause the progression of diabetes [29–31]. However, the underlying mechanism(s) is not yet completely elucidated. In this study, we analyzed the effect of HCV infection on cellular glucose uptake and expression of glucose transporters.

We observed that glucose uptake was suppressed in cells harboring HCV RNA replicons (SGR and FGR) and those infected with HCV than in the control cells (Fig. 3). It has been reported that glucose disposal *in vivo* occurs through both insulin-dependent and insulin-independent mechanism [32]. We observed that treatment of SGR, FGR and the control Huh-7.5 cells with insulin (10^{-4} M to 10^{-9} M) increased glucose uptake by only about 50% from their basal levels (data not shown). Nevertheless, decreased glucose uptake by HCV-infected hepatocytes is a potential cause of hyperglycemia as the liver is a big organ accounting for 2% of the total body weight.

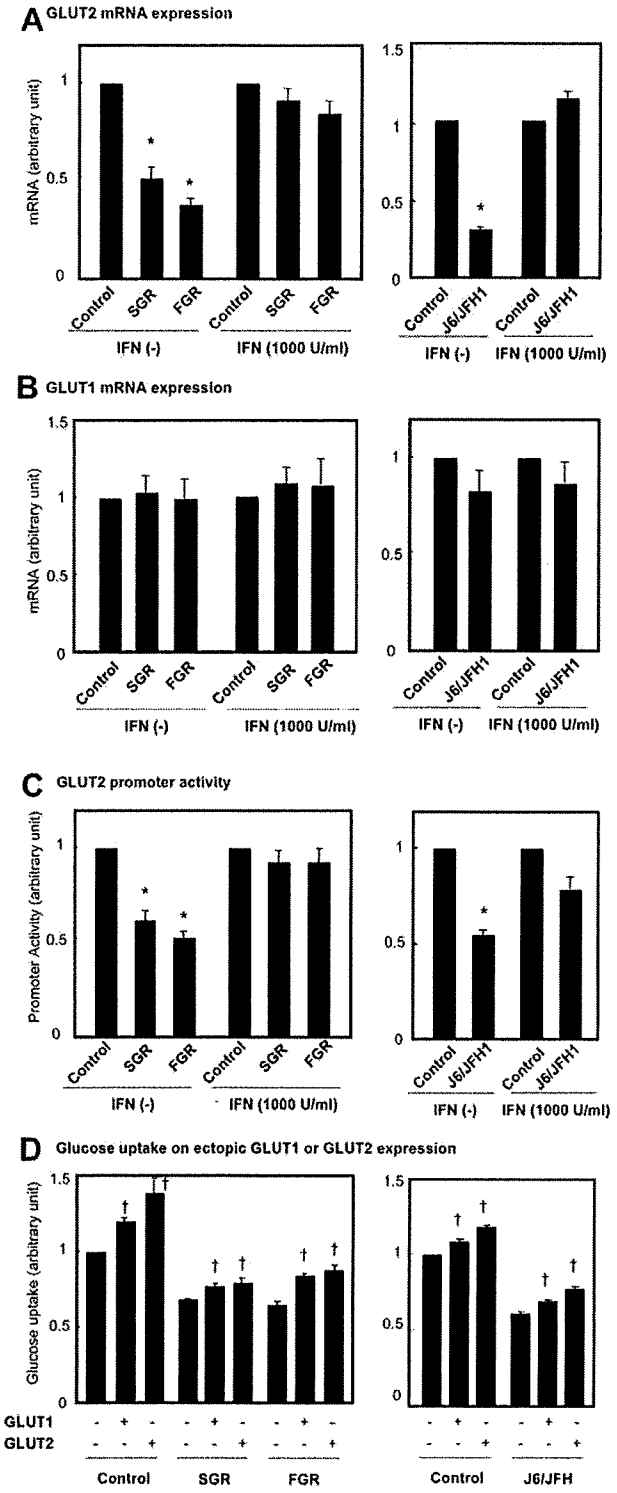

Fig. 6. Differential suppression of GLUT2 and GLUT1 mRNAs by HCV replication. (A and B) Quantitative RT-PCR analysis of mRNA for GLUT2 (A) and GLUT1 (B). mRNA expression levels of GLUT2 and GLUT1 in SGR, FGR and HCV-infected cells were determined and normalized with β-glucuronidase mRNA levels. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to quantitative RT-PCR analysis. Data represent mean ± SEM of three independent experiments. $^{*}P < 0.01$, compared with the control. (C) GLUT2 promoter activities in SGR and FGR, HCV-infected cells were analyzed using luciferase reporter assay. In parallel, cells were treated with IFN (1000 IU/ml) for 10 days to eliminate HCV replication before being subjected to luciferase reporter assay. Data represent mean ± SEM of five independent experiments. $^{*}P < 0.01$, compared with the control. (D) Glucose uptake in cells ectopically expressing GLUT1 or GLUT2. Data represent mean ± SEM of two independent experiments. $^{\dagger}P < 0.01$, compared with mock transfected control.

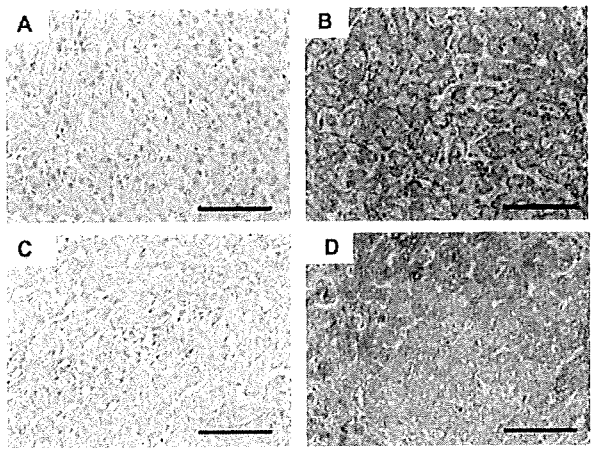

Fig. 7. Down-regulation of GLUT2 expression in HCV-infected human liver tissues *in vivo*. Normal human adult liver tissues (A and B) and HCV-infected, non-cancerous liver tissues (C and D) were fixed with formalin, sectioned and stained with normal rabbit IgG (A and C) or polyclonal anti-GLUT2 antibody (B and D). Scale bar = 100 μm.

Any proliferating cell requires energy sources, including glucose, and GLUTs play an important role in glucose uptake into the cell. In the liver, GLUT2 is the predominant glucose transporter, which regulates glucose metabolism by mediating a bidirectional transport, both entry and exit, of glucose into and from hepatocytes [13]. GLUT1, on the other hand, is known to be expressed in malignant cells including hepatocellular carcinoma [12,13] and a wide variety of cultured cells. In the present study we found that cell surface expression of GLUT2 and GLUT1 was markedly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 4A and B).

Table 2
Reduction of GLUT2 expression in hepatocytes of HCV-infected and HBV-infected human liver tissues.

Liver tissues	Sample No.	Reduction of GLUT2 expression
Uninfected	1	– *
	2	–
	3	–
	4	–
	5	–
	6	–
	7	–
	8	–
HCV-infected	9	1+ (Focal)[a]
	10	1+ (Focal)
	11	3+ (Diffuse)
	12	3+ (Diffuse)
	13	3+ (Diffuse)
	14	3+ (Focal)
	15	–
	16	2+ (Focal)
	17	3+ (Diffuse)
HBV-infected	18	–
	19	3+ (Diffuse)
	20	1+ (Focal)
	21	–
	22	2+ (Focal)
	23	1+ (Focal)
	24	2+ (Focal)

* -, no reduction; 1+, weak reduction; 2+, moderate reduction; 3+, strong reduction.

[a] Parentheses indicate either focal or diffuse appearance of the areas with reduced GLUT2 expression in each liver tissue sample.

GLUT2 expression is regulated at the transcriptional level, at least partly, by glucose [33]. It has been reported that hyperglycemia increases the GLUT2 mRNA and protein expression in an *in vivo* study [34]. Our present study demonstrated that GLUT2 mRNA expression was significantly suppressed in SGR, FGR and HCV-infected cells compared to the control (Fig. 6A). Consistent with this result, GLUT2 promoter activities, as measured by luciferase reporter assay, were suppressed in SGR, FGR and HCV-infected cells (Fig. 6C). In this connection, it was reported that GLUT2 promoter activities were up-regulated by sterol response element-binding protein (SREBP)-1c [35,36]. We confirmed in our study that GLUT2 promoter activities were up-regulated by over-expression of human SREBP-1c, and that the SREBP-1c-mediated GLUT2 promoter activities were suppressed significantly in SGR, FGR and HCV-infected cells (data not shown).

Unlike GLUT2 mRNA, GLUT1 mRNA was not suppressed by HCV RNA replication or HCV infection (Fig. 6B). Nevertheless, cell surface expression of GLUT1 was markedly down-regulated in SGR and FGR cells (Fig. 4A). As GLUT1 surface expression was not restored by treatment with lactacystin, a potent proteasome inhibitor (Fig. 5), it was unlikely that HCV-mediated suppression of GLUT1 surface expression was mediated through increased degradation by the ubiquitin-proteasome system. We assume that intracellular trafficking of GLUT1 (and possibly GLUT2 as well) is impaired by HCV RNA replication although we could not precisely prove it due mainly to the lack of an appropriate antibody that enables us to monitor GLUT1 trafficking. Further study is needed to elucidate the issue.

By means of immunohistochemical analysis, we confirmed that GLUT2 was strongly expressed in hepatocytes of the liver tissues obtained from all of 8 individuals without HCV infection (Fig. 7B and Table 2). More importantly, we demonstrated that GLUT2 expression was significantly down-regulated in hepatocytes obtained from 8 of 9 HCV-infected patients (Fig. 7D and Table 2). Interestingly, the areas where GLUT2 down-regulation was observed appeared to be scattered across the liver tissue sections. This may reflect the general observation that a group of hepatocytes in limited areas of the hepatic lobules, but not all the hepatocytes, are infected with HCV *in vivo*. By means of real-time quantitative PCR analysis, we found a tendency that levels of GLUT2 mRNA expression in liver tissues obtained from HCV-infected patients were lower than that obtained from uninfected controls although the dif-

ference was not statistically significant (data not shown). As stated above, not all the hepatocytes in the liver were infected with HCV and, therefore, the possible reduction of GLUT2 mRNA expression in HCV-infected hepatocytes might have been masked by the normal levels of expression in uninfected hepatocytes concomitantly present in the same tissue samples.

It should also be noted that GLUT2 staining was also reduced in hepatocytes obtained from HBV-infected patients, though to a lesser extent than that from HCV-infected ones (Table 2). We assume that inflammatory responses in the liver may trigger some intracellular event that leads to decreased GLUT2 expression in hepatocytes *in vivo*.

In conclusion, we have demonstrated for the first time that HCV replication inhibits cellular glucose uptake through down-regulation of cell surface expression of GLUT2 and possibly GLUT1. It is conceivable that the decreased glucose uptake by hepatocytes causes impaired glucose metabolism, leading eventually to the initiation and progression of diabetes mellitus during a prolonged period of HCV persistence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.029.

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ORIGINAL ARTICLE

Effect of selective vaccination on a decrease in the rate of hepatitis B virus-positive Japanese first-time blood donors

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SUMMARY. The government of Japan started a selective vaccination programme to prevent mother-to-infant infection by hepatitis B virus (HBV) since January 1986. The effect of the programme on first-time blood donors has not been examined in detail. Data of first-time blood donors aged 16–25 years from 1996 to 2007 were extracted from the Japanese Red Cross (JRC) donors' database. Principal component analysis (PCA) was used to visualize the birth-year-dependent group of rate of HBV-positive donors. According to the birth of year, donors were divided into four groups by PCA. After the start of the programme, donors born in 1986–1989 comprised a single group. Before the start of the programme, three groups (1980, 1981–1984 and 1985) were identified. Although a significant time-dependent decrease in the rate of HBV-positive donors was observed before the start of the programme, a significant difference in the rate of HBV-positive donors was observed around the start of the programme by regression analysis for 16–19-year-old first-time blood donors. The selective vaccination programme has been effective to prevent the vertical transmission of HBV from the analysis of first-time blood donors. On the other hand, vaccination of blood donors should be considered to reduce the risk of post-transfusion HBV infection, because the horizontal transmission increases in HBV-positive blood donors.

Key words: first-time blood donors, HBV selective vaccination, principal component analysis, regression analysis.

South and East Asia including Japan was an epidemic area of hepatitis B virus (HBV). From the report of the Japanese Ministry of Health, Labour and Welfare in 2002, the number of HBV-infected patients was 97 000, and asymptomatic carriers were estimated to be 1·1–1·4 million. It is reported that the estimated number of HBV carriers was 0·63% and that of hepatitis C virus (HCV) carriers was 0·49% among Japanese first-time blood donors in 1995–2000 (Tanaka *et al.*, 2004). However, recently, HBV infection rate among Japanese first-time blood donors has been decreasing markedly. The recent rate of positive first-time blood donors for the HBV surface antigen (HBsAg) was < 0·22%.

Infection routes of HBV were divided into two main routes, the vertical (mother-to-infant) and horizontal routes. Most of the vertical infections become chronic and most of the horizontal infections end transiently.

Since January 1986, the government of Japan started a nationwide programme to prevent mother-to-infant infection by HBV (Shiraki, 1994; Shiraki *et al.*, 1996; Inui *et al.*, 2007). Every pregnant woman has been screened for serum HBsAg and the HBV e antigen

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(HBeAg). Newborn infants whose mothers were positive for HBeAg were received an immunoprophylaxis treatment by administrating a hepatitis B vaccine and hepatitis B immunoglobulin (HBIG)

The Ministry of Health and Welfare issued a notification on the use of disposable syringes in addition to disposable needles in 1988. This notification might contribute to reducing the risk of iatrogenic HBV infections when babies were administered several mandatory vaccines. With the implementation of this prevention programme, transmission of HBV decreased markedly yearly. On the other hand, horizontal infection with HBV genotype A, which seldom appeared in Japan several years ago, has increased recently in both patients and donors (Orito *et al.*, 2001; Murokawa *et al.*, 2005; Sugauchi *et al.*, 2006; Takeda *et al.*, 2006; Hayashi *et al.*, 2007).

To investigate the recent epidemiology of HBV infection and the effectiveness of the Japanese vaccination programme for the prevention of mother-to-infant transmission of HBV, the data of HBsAg-positive Japanese donors aged 16–25 years were used for principal component analysis (PCA) and regression analysis.

MATERIALS AND METHODS

The Japanese government started a nationwide hepatitis B vaccination programme in January 1986 for infants born to HBV-carrier mothers to prevent perinatal infection of HBV (Shiraki, 1994; Shiraki *et al.*, 1996; Inui *et al.*, 2007). Initially, the Japanese vaccination programme covered only neonates born to mothers who were positive for both HBsAg and the HBeAg. In 1995, the vaccination programme was extended to all neonates born to mothers who were HBsAg carriers regardless of the mother's HBeAg/antibody status. More than 92% of all the pregnant women in Japan were enrolled in the programme (Inui *et al.*, 2007).

The number of first-time blood donors and HBsAg-positive donors aged 16–25 years was extracted from JRC database from 1996 to 2007. To investigate the present state of HBV infection, the presence of the immunoglobulin-M antibody against the HBV core antigen (IgM-HBcAb) was determined among all HBsAg-positive donors from October 2006 to September 2007. The Japanese screening system was reported previously (Iizuka *et al.*, 1992; Yugi *et al.*, 2006). The nucleic acid amplification technology (NAT) system has been reported elsewhere (Mine *et al.*, 2003). IgM-HBcAb was tested by enzyme immunoassay (Abbott Laboratories, IL, USA).

Statistical analysis

The effect of the Japanese vaccination programme on the rate of HBsAg-positive donors was examined by principal component analysis (PCA) and regression analysis.

The rate of HBsAg-positive 16–18-year-old donors for every birth of year from 1980 to 1989 was visually grouped by PCA (Appendix) using the free software R (http://www.r-project.org/).

The difference in the rate of HBsAg-positive donors between before and after the implementation of the vaccination programme was analysed. We assumed the different slope around 1986 and intended to verify the assumption by regression analysis using the following equation

$$y_n = \alpha_n + \beta_n x_1 + \gamma_n x_2 + \delta_n D + \varepsilon_n, \qquad (1)$$

where α is a constant, β the coefficient of slope after 1980, γ the additional coefficient of slope after 1986, δ the coefficient (D) that shows the gap of HBsAg-positive rate around 1986, ε the error term that meets the standard assumption, n the age from 16 to 25 years old and x_1, x_2 the rate of positive donors of years born from 1980 to 1991. Regression analysis was carried out using the Microsoft Office Excel software.

RESULTS

Time-dependent changes in the rate of HBsAg-positive first-time blood donors of each generation from 1996 to 2007 are shown in Fig. 1. The rate of HBsAg-positive younger generations was lower than those of older generations. The rate of HBsAg-positive donors of all generations decreased yearly from 0·83% in 1996 to 0·22% in 2007 (data not shown).

Present state of numbers of blood donors and numbers of HBV-infected blood donors in Japan is shown in Table 1. From October 2006 to September 2007, the total number of donors was 4 974 911: the number of HBsAg-positive donors was 2043 (0·041%), the number of first-time blood donors was 594 096 and the number of HBsAg-positive first-time blood donors was 1362 (0·229%). Among 61 IgM-HBcAb-positive donors, 35 were repeat donors who have been infected after the last donation. Among 90 HBsAg-negative and NAT-positive donors, 22 were considered to have occult HBV infection on the basis of their being HBsAg-negative, HBV-DNA-positive and IgG-HBcAb-positive. Then serological window period donors were 68 among NAT-positive donors.

The rate of HBsAg-positive Japanese donors from 16 to 25 years old was extracted from JRC database from

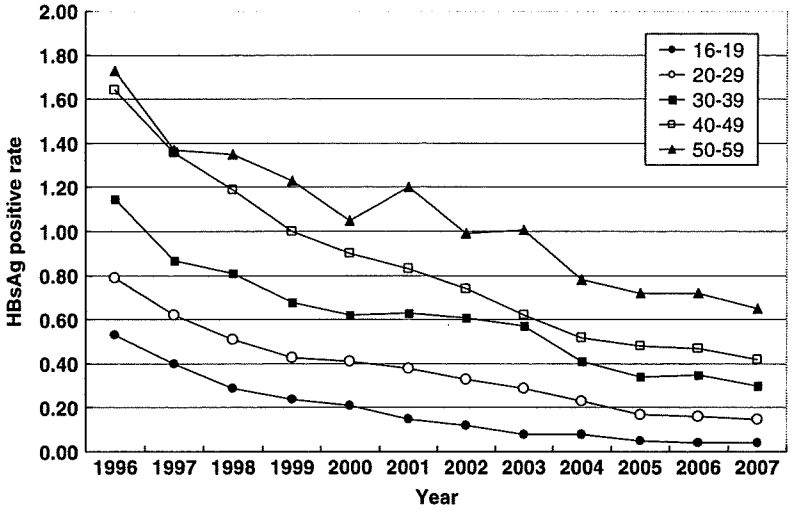

Fig. 1. Age–time-dependent rate of HBsAg-positive first-time blood donors from 1996 to 2007. Data of donors in their 60s are omitted because the number of first-time blood donors in their 60s is so small that the amplitude of the rate of HBsAg-positive donors becomes large unexpectedly.

Table 1. Present state of numbers of blood donors and numbers of HBV-infected blood donors from October 2006 to September 2007 in Japan

	Total blood donors			First-time blood donors			Horizontal infection	
Age	Number of donors	Number of HBsAg-positive donors	Rate (%) of HBsAg-positive donors	Number of donors	Number of HBsAg-positive donors	Rate (%) of HBsAg-positive donors	IgM-HBcAb-positive donors	NAT-positive donors
16	37 717	3	0·008	30 436	3	0·010	0	0
17	53 388	10	0·019	29 277	10	0·034	0	0
18	118 711	31	0·026	66 617	29	0·044	1	0
19	130 391	25	0·019	51 080	20	0·039	2(1)*	2
20	124 224	31	0·025	33 847	27	0·080	4(3)	2
21	120 609	28	0·023	25 583	22	0·086	3(2)	4
22	118 215	38	0·032	22 806	32	0·140	2(1)	1
23	118 974	38	0·032	20 640	30	0·145	3(2)	4
24	115 434	37	0·032	17 873	32	0·179	2(1)	3
25	110 247	38	0·034	15 574	30	0·193	2	4
26–29	452 645	172	0·038	50 433	130	0·258	6(3)	9
30–39	1 375 372	499	0·036	112 620	333	0·296	24(19)	25
40–49	1 077 348	487	0·045	64 232	286	0·445	9(2)	10
50–59	773 571	484	0·063	44 004	296	0·673	3(1)	15(12)†
60–69	248 065	122	0·049	9 074	82	0·904	0	11(10)
Total	4 974 911	2043	0·041	594 096	1362	0·229	61(35)	90(22)

*Number of repeated donors are shown in parenthesis.
†Number of IgG-HBcAb-positive donors (occult donors) are shown in parenthesis.

1996 to 2007 to investigate the effectiveness of the Japanese vaccination programme. The rates of HBsAg-positive first-time blood donors from 16 to 25 years old who are born from 1980 to 1991 are shown in Table 2.

The bold line between data in 1985 and those in 1986 shows the boundary before and after the implementation of the Japanese vaccination programme. The lowest column in Table 2 shows that the rate of HBsAg-positive 16-year-old donors who were born in 1991, and became acceptable as blood donors for the first time in 2007, was 0·018%.

To visualize the difference in the rate of HBsAg-positive donors around the start of the vaccination programme, PCA was carried out using the data within the frame of the dotted line in Table 2. From the result of the PCA of HBsAg-positive 16- to 18-year-old donors born from 1980 to 1989, the donors can be divided into four groups (Fig. 2).

Table 2. Rate of HBsAg-positive first-time blood donors born from 1980 to 1991

Year of birth	Age									
	16	17	18	19	20	21	22	23	24	25
1980	0·399	0·390	0·312	0·303	0·245	0·330	0·274	0·243	0·288	0·208
1981	0·313	0·279	0·281	0·285	0·289	0·232	0·326	0·240	0·219	0·205
1982	0·223	0·210	0·209	0·203	0·186	0·238	0·215	0·190	0·163	0·185
1983	0·142	0·179	0·164	0·144	0·157	0·157	0·096	0·170	0·154	
1984	0·129	0·105	0·117	0·130	0·106	0·076	0·139	0·134		
1985	0·105	0·110	0·086	0·126	0·078	0·070	0·126			
1986	0·055	0·035	0·056	0·067	0·061	0·098				
1987	0·040	0·044	0·049	0·058	0·071					
1988	0·044	0·020	0·038	0·041						
1989	0·017	0·024	0·044							
1990	0·041	0·036								
1991	0·018									

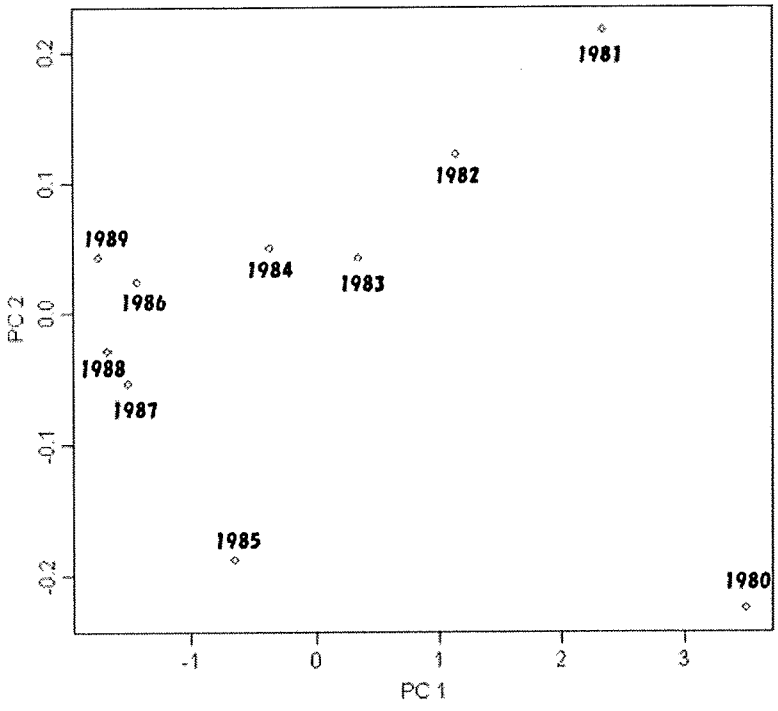

Fig. 2. Two-dimensional display of results of PCA of rate of HBsAg-positive first-time blood donors. Calculation was carried out with the free software 'R' (http://www.r-project.org/) using data within the frame of the dotted line in Table 2 (16-, 17- and 18-year-old donors born from 1980 to 1989). PCA is a statistical method to compress the multi-dimensional space of data into small-number- dimensions-space data (Appendix). The relations of data compressed into two dimensions are easy to understand by making a glance of this figure. The horizontal and vertical axes of PC 1 and PC 2 are composed of many types of variables. Therefore, it is difficult to show them as definite indexes. However, if we would be forced to consider the meaning of these axes, we had better regard them as the difficulty of determining infection by age. The group in 1986–1989 is obviously different from the other three groups in 1980, 1985 and 1981–1984.

Donors born after the implementation of the vaccination programme from 1986 to 1989 comprised one group. Donors born before the implementation of the vaccination programme can be divided into three groups; donors born in the transitional period in 1985 comprised one group, those born during the period of decreasing rate of HBsAg-positive donors from 1981 to 1984 comprised another group and those born during

the period of decreasing but rather high rate of HBsAg-positive donors in 1980 comprised an other single group.

The statistical significance of changes in decreasing curve and rate of HBsAg-positive donors in 1986 were investigated by regression analysis using Equation (1) described in Materials and Methods section

When 'n' is 16 years old, Equation (1) can be written as

$$y_{16} = \alpha_{16} + \beta_{16}x_1 + \gamma_{16}x_2 + \delta_{16}D + \varepsilon_{16} \quad (2)$$

When the birth of year is between 1980 and 1985, Equation (2) can be rewritten as follows, because x_1 = (1980–1991 : 0·399, 0·313, 0·223, 0·142, 0·129, 0·105, 0·055,0·040, 0·044, 0·017, 0·041, 0·018), x_2 = (1980–1991:0, 0, 0, 0, 0, 0, 0·055, 0·040, 0·044, 0·017, 0·041, 0·018, because γ is the additional coefficient of slope after 1986), D = (1980–1991: 0, 0, 0, 0, 0, 0, 1, 1, 1, 1, 1, 1, because δ is the coefficient (D) that shows the gap of HBsAg-positive rate around 1986).

$$y_{16} = \alpha_{16} + \beta_{16}x_1 + \varepsilon_{16} \quad (3)$$

When the birth of year is after 1986, Equation (2) can be rewritten as follows, because after 1986, x_1 is equal to x_2.

$$y_{16} = (\alpha_{16} + \delta_{16}) + (\beta_{16} + \gamma_{16})x_2 + \varepsilon_{16} \quad (4)$$

P-values of β_{16-19}, γ_{16-19}, δ_{16-19} are shown in Table 3. All the terms between 16 and 18 are significant ($P < 0·05$). That is, the rate of HBsAg-positive donors decreased significantly yearly after 1980 (β_{16-19}), and the trend of decreasing slope before 1985 was significantly different from that after 1986 (γ_{16-18}). The difference in infectious rate was significant at the border line between 1985 and 1986 (δ_{16-18}). These data show that HBsAg-positive rate has decreased significantly in donors born from 1980 to 1991 (β_{16-19} are negative) regardless of the hepatitis B vaccination programme. There was a significant decrease in HBsAg-positive rate in donors born before 1985 and after 1986 (δ_{16-18} are negative). This drop would result from the effect of the hepatitis B vaccination programme. The additional coefficient γ_{16-18} is positive means that after the significant drop, the decreasing curve of HBsAg-positive rate became flat, because there might be no room to decrease by vertical transmission except a few cases as intrauterine transmissions and inappropriate vaccinations. The remaining HBsAg-positive rate might be caused by horizontal transmission. A significant change was not observed for 19 years old [asterisk symbol (*) in Table 3], because the data of this age group were considered to be too small to obtain a significant difference statistically.

DISCUSSION

HBsAg-positive first-time blood donors consist of both horizontally and vertically infected donors. The minimum number of HBV-positive first-time blood donors with consistently horizontal infection from October 2006 to September 2007 was anticipated to be 94, on the basis of the sum of the numbers of HBsAg-negative and NAT-positive (68:90–22), and IgM-HBcAb-positive (26:61–35) donors, which was 4·4% (94/2133) of the total number of HBsAg-positive donors (2043) plus 90 HBsAg-negative and NAT-positive donors. The exact number of horizontally infected donors was obscure, making it difficult to determine the effectiveness of the Japanese vaccination programme for the prevention of mother-to-infant transmission of HBV. Moreover, the total number of HBsAg-positive donors was decreasing yearly before the start of the prevention programme. The number of HBsAg-positive first-time blood donors in several prefectures was actually too small to treat statistically in the investigation of the effectiveness of the Japanese

Table 3. Data obtained using Equation (1)

	Coefficient	Standard error	t-Value	P-value		Coefficient	Standard error	t-Value	P-value
α_{16}	0·429	0·025	17·375	1·2E–07	α_{17}	0·407	0·025	16·419	7·6E–07
β_{16}	–0·060	0·006	–9·482	1·3E–05	β_{17}	–0·056	0·006	–8·756	5·1E–05
γ_{16}	0·054	0·009	6·038	**3·1E–04**	γ_{17}	0·054	0·011	5·110	**1·4E–03**
δ_{16}	–0·336	0·066	–5·098	**0·001**	δ_{17}	–0·359	0·081	–4·454	**0·003**
α_{18}	0·362	0·009	39·749	1·7E–08	α_{19}	0·339	0·022	14·906	2·5E–05
β_{18}	–0·048	0·002	–20·394	9·0E–07	β_{19}	–0·040	0·006	–6·890	9·9E–04
γ_{18}	0·043	0·005	8·665	**1·3E–04**	γ_{19}	0·027	0·018	1·493	**0·196***
δ_{18}	–0·275	0·004	–7·130	**0·0004**	δ_{19}	–0·180	0·141	–1·278	**0·257***

*γ_{19} and δ_{19} are not significant ($P < 0·05$)

prevention programme (Chiyoda *et al.*, 2006; Uchida and Tadokoro, 2008). The situation was similar to the case of HCV. The rate of HCV-positive first-time blood donors in Japan has declined (data not shown). O'Brien *et al.* (2008) reported that they could not determine why the infection rates of HCV have decreased in Canada.

If the Japanese prevention programme succeeded completely, HBV infection would only be caused by horizontal transmission after 1986, and the trend of declining slope after 1986 would be different before 1985. However, in spite of the prevention programme, vertical transmission remained because of intrauterine transmissions and inappropriate vaccinations or problems of escape mutants. It was reported that 1·3% of infants became carriers immediately after birth before vaccination and another 2·1% became carriers during or immediately after the third vaccination because these infants were considered to be poor responders (Shiraki, 1994). Inui *et al.* (2007) reported that out of 27 patients who became infected, despite the immunoprophylaxis trials (selective vaccination and HBIG administration), 14 were infected by receiving an inappropriate Japanese vaccination programme, 11 were suspected to be infected by the intrauterine route and only 1 was infected by routes other than the mother-to-infant route. Therefore, a constant rate of vertically transmitted HBsAg-positive donors would remain in spite of universal or selective vaccination during infants. However, those infants who became infected despite the vaccination would be notified of the fact by a health centre or a hospital and should not donate blood or would be rejected to donate on the basis of their responses to a questionnaire. The problem might be the existence of donors infected by paternal or iatrogenic transmission routes, who were unaware of their being HBV carriers themselves or their parents. Some donors who were engaged in risk behaviour might visit a blood centre to donate and determine whether they were infected with viruses causing sexually transmitted diseases. In consideration of these factors, significant differences in the rate of HBV-positive donors were observed between before and after 1986.

The progressive decrease in the rate of HBV-positive donors from 1996, as shown in Fig. 1, might be due to the policy of the Ministry of Health and Welfare. To prevent an iatrogenic HBV infection, a vaccination enforcement regulation 'to use a disposable needle per person' was issued in September 1958 and the use of disposable syringes was permitted from September 1976. Afterwards, disposable needles and syringes were disseminated yearly and a memorandum 'to use a disposable syringe per person' was issued from the Ministry of Health and Welfare in January 1988.

According to the recommendation of the World Health Organization (WHO), many countries have implemented universal vaccination (World Health Organization, 1992) except UK (Hanè *et al.*, 2004) and Japan (Shiraki, 1994; Shiraki *et al.*, 1996; Inui *et al.*, 2007). Although there are many reports about the effectiveness of universal vaccination (Ni *et al.*, 2007; Gervais *et al.*, 2008; Mele *et al.*, 2008), reports about the comparison between the effectiveness of universal vaccination and that of selective vaccination are few. In Bulgaria, the period of selective vaccination of newborns to HBsAg-positive mothers was 1988– 1991, and that of the universal infant vaccination was thereafter (Hens *et al.*, 2008). Although they estimated the impact of vaccination using age–time-dependent incidence rates of hepatitis B, they did not show the superiority of universal vaccination to selective vaccination.

From the view points of cost–benefit and side effects, it should be considered which would be effective to implement, universal vaccination or selective vaccination with co-administration of HBIG to use the healthcare budget effectively. Although the side effect of HBV vaccine was estimated to be very low (Mikaeloff *et al.*, 2007), we cannot exclude the risk completely. Therefore, to continue the current Japanese strategy (selective vaccination) to control HBV infection or to implement universal vaccination is still open to discussion in Japan.

Although vertical transmission of HBV would be prevented sufficiently by the current selective vaccination, it might be necessary to prevent horizontal infection. The increase in horizontal HBV infection, especially HBV genotype A originated from United States or Western Europe, is apparent (Murokawa *et al.*, 2005). This might be supported by the finding that the HBV genotype A, which has been rare in Japan, was predominant among HBV–HIV dually infected Japanese men who had sex with other men (MSM). The sequences of genotype A spread by MSM were highly homologous to those of the strains isolated in the United States (Koibuchi *et al.*, 2001). In addition to genotype A, we have recently found genotype H in a Japanese HBsAg-negative and NAT-positive blood donor. The sequence of genotype H, which is prevalent only in the United States and Central America, was highly homologous to those of the strains isolated in Los Angeles (Ohnuma *et al.*, 2005).

HBV vaccination is not mandatory but recommended to workers engaged in medical services and to travellers who go to HBV endemic areas to reduce the horizontal infection. The implementation of universal vaccination as discussed above seems to be a solution; however, there is a problem of a 'waning-off' effect (Su *et al.*, 2008). The other solution is to immunize blood donors,

which was proposed in place of the implementation of NAT (Ringwald *et al.*, 2005). The vaccination of blood donors would reduce the risk of post-transfusion HBV infection. Although we do not know the effect of vaccination on occult HBV infection that is an important problem in the field of transfusion, it might also reduce the risk of occult HBV infection. In addition to reducing the risk, the vaccination of blood donors might be useful to produce HBIG because of the lack of HBsAb-positive plasma.

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APPENDIX

Principal component analysis (PCA) involves a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called principal components.

Actually, it functions as eigenvector-based multivariate analyses. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible.

Accordingly, its operation can be thought of as revealing the internal structure of the data in a way which best explains the variance in the data. If a multivariate dataset is visualized as a set of coordinates in a high-dimensional data space (one axis per variable), PCA supplies the user with a lower-dimensional picture, a 'shadow' of this object when viewed from its (in some sense) most informative viewpoint.

Therefore, if we apply PCA to the obtained data, we will be able to observe several groups which will have the same or similar features. In addition, PCA can show the result visually, it is rather easy to understand the grouping intuitively.

Outline of calculation: The correlation matrix is calculated using the data within the frame of the dotted line in Table 2.

X_{16}	X_{17}	X_{18}
1	0·9866428	0·9855587
0·9866428	1	0·9843252
0·9855587	0·9843252	1

The maximum eigen value is $\lambda_1 = 2 \cdot 96978028$. Corresponding eigenvector is 0·5762051, 0·8111528 and 0·1000940, respectively. The first component is shown as follows:

$$Z = 0{\cdot}5762051(X_{16} - 0{\cdot}1880000) + 0{\cdot}8111528 \times (X_{17} - 0{\cdot}1698782) + 0{\cdot}1000940(X_{18} - 0{\cdot}1597273) = 0{\cdot}5762051X_{16} + 0{\cdot}8111528X_{17} + 0{\cdot}1000940X_{18} - 0{\cdot}2620628$$

The component loading of first principal component (correlation coefficient between original data and principal component score) is 0·9929772, 0·9963599 and 0·9955117, respectively.

Incidentally, correlation coefficient is shown as (eigenvector) × √(eigen value).

Then the change of Z is calculated according to 10 group of values (1980, 1981, . . ., 1988, 1989), principal component score (PC 1) is shown as follows:

3·71767980, 2·29772699, 1·42799966, 0·51159502, −0·65596423, −0·86558646, −1·47020213, −1·51833807, −1·65244844, −1·69703017.

This represents the total change as a change of one series.

Similarly, second eigen value is $\lambda_2 = 0 \cdot 02113356$. Corresponding eigenvector is 0·5781680, −0·3179823 and −0·7514047, respectively.

$$U = 0{\cdot}5781680(X_{16} - 0{\cdot}1880000) - 0{\cdot}3179823 \times (X_{17} - 0{\cdot}1698182) - 0{\cdot}7514047 \times (X_{18} - 0{\cdot}1597273) = 0{\cdot}5781680X_{16} - 0{\cdot}3179823X_{17} - 0{\cdot}751404X_{18} + 0{\cdot}0653234$$

Among the change of X_{16}, X_{17} and X_{18}, $(\lambda_2 + \lambda_2)/3$ is shown as 0·9969713 using the values of Z and U. This means that most information (99·7%) is converged in first and second principal component.

Such a method of analysis is called component analysis. Z and U are called first and second principal components, respectively. Fig. 2 is drawn by plotting the first principal component in X-axis and the second principal component in Y-axis.

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Human papillomavirus infections among Japanese women: age-related prevalence and type-specific risk for cervical cancer

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To obtain baseline data for human papillomavirus (HPV) screening and vaccination in Japan, we analyzed HPV DNA data from 2282 Japanese women (1517 normal cytology, 318 cervical intraepithelial neoplasia [CIN] grade 1, 307 CIN2–3, and 140 invasive cervical cancer [ICC]) that visited the University of Tsukuba Hospital or Ibaraki Seinan Medical Center Hospital for screening or treatment of cervical diseases between 1999 and 2007. An L1-based PCR method was used for individual HPV genotyping. The most common HPV types in ICC were, in order of decreasing prevalence, HPV16 (40.5%), HPV18 (24.4%), HPV52 (8.4%), HPV58 (3.1%), and HPV33 (3.1%). Based on the comparison of HPV type distributions between normal cytology and CIN2–3 and ICC, estimated risk of disease progression varied considerably by genotype: HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, and HPV58 (prevalence ratio, 1.92; 95% confidence interval 1.58–2.34); other oncogenic types (0.31, 95% confidence interval 0.19–0.50); and non-oncogenic types (0.09, 95% confidence interval 0.03–0.43). HPV16 and/or HPV18, including coinfections with other types, contributed to 67.1% of ICC and 36.2% of CIN2–3 among Japanese women. More importantly, the overall prevalence of HPV16 and/or HPV18 varied greatly according to the women's age: highest in women aged 20–29 years (ICC, 90.0%; CIN2–3, 53.9%), decreasing with age thereafter, and lowest in women aged 60 years or older (ICC, 56.3%; CIN2–3, 25.0%). In conclusion, type-specific HPV testing may help identify Japanese women at high risk of progression to CIN2–3 and cancer. In Japan, current HPV vaccines are estimated to provide approximately 70% protection against ICC and may be more useful in reducing the incidence of cervical cancer and precancer in young women of reproductive age. (*Cancer Sci* 2009)

Persistent infection with oncogenic human papillomaviruses (HPV), most commonly types 16 and 18, leads to cervical cancer, the second most common cancer in women worldwide.[1] Therefore, oncogenic HPV testing combined with cytology was approved for primary screening in the USA, because of sensitivity and cost-effectiveness.[2] In addition, HPV vaccines have been licensed in the USA, Australia, and European and other countries, because of their efficacy and safety. Clinical studies of HPV vaccines have demonstrated close to 100% protection against HPV16- and HPV18-related infections and diseases,[3–5] implying possible cross-protection against HPV45, HPV31, and HPV52.[4,5] Based on evidence from clinical trials,[3–7] these two tools targeting HPV (detection assay and vaccine) are becoming increasingly attractive for cervical cancer prevention worldwide. In Japan, however, HPV DNA testing is still unavailable in mass screening and no HPV vaccine has yet been licensed. Type-specific and age-related data of HPV prevalence, both for women with normal cytology and for women with cervical diseases, are prerequisites to make a well-judged decision about the future role of HPV screening and vaccination in cervical cancer prevention, but these data are missing in Japan. A meta-analysis of Japanese HPV studies provided representative data of HPV type distribution, but no information about age-specific prevalence.[8]

In the present study, we analyzed HPV DNA data from 2282 Japanese women to obtain the prevalence data of HPV among women across a broad age range. Our data may help provide models for further evaluating potential impact and cost effectiveness of HPV screening and vaccination in Japan.

Materials and Methods

Study subjects. Our study subjects consisted of 2282 Japanese women (1517 normal cytology, 318 cervical intraepithelial neoplasia [CIN] grade 1, 307 CIN2–3, and 140 invasive cervical cancer [ICC]) who visited the University of Tsukuba Hospital or Ibaraki Seinan Medical Center Hospital for cervical cancer screening, treatment of cervical diseases, or other reasons between 1999 and 2007. Foreign women were excluded from the present study, based on self-reported ethnicity. Both hospitals are located in the south-west area of Ibaraki prefecture, approximately 50 km north of Tokyo. Histological diagnosis was made using hematoxylin–eosin-stained sections according to the World Health Organization classification. Written informed consent was obtained from all patients. The institutional ethical and research review board of each hospital approved the study protocol.

HPV detection and genotyping. Exfoliated cells from the ectocervix and endocervix were collected into a tube containing 1 mL PBS and stored at –30°C until DNA extraction. We detected HPV DNA in cervical samples by PCR-based methodology described previously.[9] In brief, total cellular DNA was extracted from cervical samples by a standard sodium dodecyl sulfate–proteinase K procedure. HPV DNA was amplified by PCR using consensus primers (L1C1 and L1C2 + L1C2 M) for the HPV L1 region. Direct comparisons of HPV detection methodology have demonstrated that the sensitivity of our PCR assay is higher than that of PCR assays using MY09 and MY11 and GP17 and GP18 primers.[10,11] A reaction mixture without template DNA was included in every set of PCR runs as a negative control. Also, primers for a fragment of the β-actin gene were used as a control to rule out false-negative results for samples in which HPV DNA was not detected. To avoid contamination, we used disposable utensils and discarded them after a single use. We also used aliquoted reagents and maintained separate locations for different stages of the assay. HPV types were identified by restriction fragment length polymorphism, which

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has been shown to identify at least 26 types of genital HPV. HPV detection and genotyping were carried out blinded to the clinical data collected from the study subjects. In the present study, we considered HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68 as oncogenic types. These 13 genotypes are detected by Hybrid Capture 2 (HC2) test (Digene, Gaithersburg, MD, USA). All other HPV types were classified as non-oncogenic types.

Statistical analysis. To estimate HPV genotype-specific risks for progression from HPV infections to CIN 2–3 or worse, prevalence ratios and odds ratios were calculated using JMP 7.0 J statistics package (SAS Institute, Cary, NC, USA). In addition, χ^2-test for trend was used to analyze the age-related prevalence of HPV16 and HPV18 in women with CIN 2–3 and ICC. Two-sided *P*-values were calculated throughout and considered to be significant at less than 0.05.

Results

This analysis included 2282 Japanese women (1517 normal cytology, 318 CIN1, 307 CIN2–3, and 140 ICC). The mean age of the study subjects was 35.9 years (range, 15–84 years); 35.0 years (range, 15–78 years) for women with normal cytology; 34.6 years (range, 15–75 years) for CIN1; 35.5 years (range, 18–78 years) for CIN2–3; and 49.2 years (range, 25–84 years) for ICC. HPV prevalence was 22.5% in women with normal cytology, 88.4% in CIN1, 94.8% in CIN2–3, and 93.6% in ICC. A total of 20 types were detected in women with normal cytology and the number of detected genotypes decreased according to disease severity (19 types in CIN1, 14 types in CIN2–3, and 12 types in ICC). HPV45 was not detected among our study subjects.

Age-related prevalence in cytologically normal women. In women with normal cytology, HPV prevalence peaked (35.9%) in women aged 15–19 years (n = 167), followed by a gradual decline in prevalence through 54 years; 28.9% among women aged 20–29 years (n = 499); 22.3% among women aged 30–39 years (n = 337); and 11.4% among women aged 40–54 years (n = 367) (Fig. 1). Interestingly, a second peak of HPV prevalence was observed in women aged 55 years or older (n = 147). Similarly, the age-related prevalence of vaccine types (HPV16 and HPV18) and HC2 (13 oncogenic types) was highest in women aged 15–19 years, with a second peak observed in women aged 55 years or older. A similar trend of age-specific HPV prevalence was observed for each HPV genotype separately (data not shown).

When the age-specific prevalence in cytologically normal women was applied to the age structure of the Japanese female population aged 15 years or older (56 869 466 women) reported in the 2005 Population Census,[12] approximately 12 million women (20.6%) were estimated to be carriers of HPV DNA, of whom 21.6% were estimated to be infected with vaccine types, and 46.1% with the other oncogenic types.

HPV genotype-specific risk for cervical cancer in Japan. To assess the progressive potential of each genotype, HPV type distributions among HPV-positive women were shown according to disease severity in Table 1. Among women with normal cytology, HPV16 and HPV51 (11.7%) were most frequently detected, followed by HPV52 (9.4%), HPV58 (7.0%), HPV56 (5.8%), and HPV18 (5.6%). In CIN1, HPV51 was the most common genotype (12.5%), followed by HPV52 (11.4%), HPV16 (9.6%), HPV56 (8.9%), HPV18 (6.8%), and HPV58 (6.8%). In CIN2–3, HPV16 was most prevalent (24.1%), followed by HPV52 (17.5%), HPV58 (10.6%), HPV18 (6.9%), HPV51 (6.5%), and HPV31 (4.5%). In ICC, the most common HPV types were, in order of decreasing prevalence, HPV16 (40.5%), HPV18 (24.4%), HPV52 (8.4%), HPV33 (3.1%), HPV58 (3.1%), HPV31 (1.5%), HPV39 (1.5%), and HPV53 (1.5%). Based on the comparison of

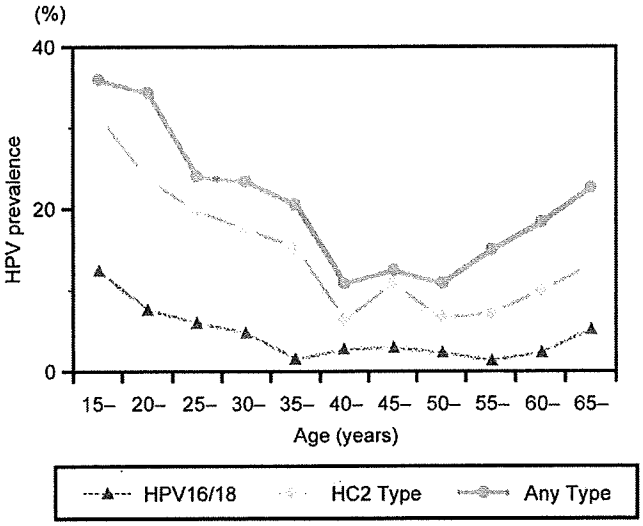

Fig. 1. Age-related human papillomavirus (HPV) prevalence among cytologically normal women in Japan. In women with normal cytology, HPV prevalence peaked in women aged 15–19 years, followed by a gradual decline in prevalence through 54 years. A second peak of HPV prevalence was observed in women aged 55 years or older. A similar trend of age-specific prevalence was observed for vaccine types (HPV16 and HPV18) and Hybrid Capture 2 (HC2) types (13 oncogenic types).

HPV type distributions between normal cytology and CIN2–3 and ICC, estimated risks for progression from viral infection to CIN 2–3 or ICC was highest in HPV31 (prevalence ratio, 3.04), followed by HPV16 (2.49), HPV18 (2.22), HPV35 (2.02), HPV52 (1.57), HPV33 (1.42), HPV58 (1.18), HPV82 (0.81), HPV53 (0.45), HPV51 (0.41), HPV56 (0.32), HPV39 (0.22), HPV59 (0.16), HPV70 (0.16), and HPV68 (0.12), suggesting that the seven genotypes of HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, and HPV58 (prevalence ratio >1.0) are 'higher-risk' types in Japan. The estimated risk was statistically significant for HPV16 and HPV18, but not for HPV31 because of limitations imposed by the low prevalence of this genotype. The prevalence of these seven types increased according to disease severity (normal cytology, 37.7%; CIN1, 41.6%; CIN2–3, 68.7%; ICC, 71.0%; prevalence ratio, 1.92). Conversely, the prevalence of the other oncogenic types decreased with disease severity (normal cytology, 27.2%; CIN1, 25.3%; CIN2–3, 10.7%; ICC, 3.1%; prevalence ratio, 0.31). A similar trend was observed for non-oncogenic types (normal cytology, 16.1%; CIN1, 8.9%; CIN2–3, 3.8%; ICC, 1.5%; prevalence ratio, 0.09).

The prevalence of multiple infections did not increase according to disease severity (prevalence ratio, 0.83), suggesting no association between multiple infections and disease progression.

We also calculated odds ratios to estimate type-specific risks of progression to CIN2–3 or ICC, although these analyses showed similar results (Table 1).

Estimating the impact of HPV16 and HPV18 vaccines in Japan. Clinical studies of HPV vaccines have demonstrated close to 100% protection against HPV16- and HPV18-related infection and diseases.[3–5] The prevalence of HPV16 and HPV18 was specifically analyzed to estimate the potential impact of current HPV vaccine against HPV16 and HPV18. When multiple infections were classified into each genotype, HPV16 and/or HPV18 were detected in 23.9% of CIN1, 36.2% of CIN2–3, and 67.1% of ICC. In women with CIN2–3 and ICC, the prevalence of HPV16 and HPV18 was highest among women aged 20–29 years, significantly decreasing with age thereafter (Table 2, χ^2 test for trend, $P < 0.05$). In ICC cases, HPV16 and/or HPV18

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Table 1. Human papillomavirus (HPV) type prevalence and risks for progression from viral infection to cervical intraepithelial neoplasia (CIN) 2–3 or invasive cervical cancer (ICC) in Japan

HPV type	Normal cytology (n = 342)		CIN1 (n = 281)		CIN2–3 (n = 291)		ICC (n = 131)		Prevalence ratios CIN2–3 + ICC: NL	Odds ratio[‡] CIN2–3 + ICC: NL
	n	%[†]	n	%	n	%	n	%	(95% CI)	(95% CI)
Oncogenic types (HC2 types)	222	64.9	188	66.9	231	79.4	110	84.0	1.24 (1.10–1.41)	72.2 (47.9–113.7)
HPV16/18/31/33/35/52/58	129	37.7	117	41.6	200	68.7	106	80.9	1.92 (1.58–2.34)	111.5 (72.7–178.1)
HPV39/45/51/56/59/68	93	27.2	71	25.3	31	10.7	4	3.1	0.31 (0.19–0.50)	17.7 (10.2–31.1)
HPV16	40	11.7	27	9.6	70	24.1	53	40.5	2.49 (1.61–3.87)	144.5 (86.3–251.3)
HPV18	19	5.6	19	6.8	20	6.9	32	24.4	2.22 (1.12–4.41)	128.6 (68.0–254.6)
HPV31	4	1.2	6	2.1	13	4.5	2	1.5	3.04 (0.73–12.7)	176.2 (59.3–653.3)
HPV33	8	2.3	7	2.5	10	3.4	4	3.1	1.42 (0.44–4.61)	82.2 (32.4–223.3)
HPV35	2	0.6	7	2.5	5	1.7	0	0	2.02 (0.24–17.1)	117.5 (24.1–848.2)
HPV39	11	3.2	2	0.7	1	0.3	2	1.5	0.22 (0.01–1.15)	12.8 (2.8–44.1)
HPV45	0	0	0	0	0	0	0	0	NA	NA
HPV51	40	11.7	35	12.5	19	6.5	1	0.8	0.41 (0.20–0.83)	23.5 (12.0–45.9)
HPV52	32	9.4	32	11.4	51	17.5	11	8.4	1.57 (0.90–2.74)	91.1 (51.7–166.0)
HPV56	20	5.8	25	8.9	8	3	0	0	0.32 (0.12–0.97)	18.8 (7.2–45.6)
HPV58	24	7.0	19	6.8	31	10.7	4	3.1	1.18 (0.59–2.38)	68.5 (36.1–133.9)
HPV59	15	4.4	4	1.4	3	1.0	0	0	0.16 (0.03–0.77)	9.4 (2.1–30.8)
HPV68	7	2.0	5	1.8	0	0	1	0.8	0.12 (0.01–1.34)	NA
Non-oncogenic (non-HC2 types)	55	16.1	25	8.9	11	3.8	2	1.5	0.09 (0.03–0.43)	11.1 (5.3–22.6)
HPV6/11	11	3.2	6	2.1	0	0	0	0	NA	NA
HPV53	16	4.7	13	4.6	7	2.4	2	1.5	0.45 (0.15–1.38)	26.4 (10.3–64.8)
HPV54	4	1.2	0	0	0	0	0	0	NA	NA
HPV61	11	3.2	3	1.1	0	0	0	0	NA	NA
HPV66	5	1.5	3	1.1	0	0	0	0	NA	NA
HPV70	5	1.5	0	0	1	0.3	0	0	0.16 (0.01–2.12)	9.4 (0.5–61.2)
HPV82	3	0.9	0	0	3	1.0	0	0	0.81 (0.10–9.00)	47.0 (8.4–265.1)
Undetermined[§]	24	7.0	16	5.7	16	5.5	6	4.6	0.65 (0.21–2.02)	43.1 (21.4–87.7)
Multiple	41	12.0	52	18.5	33	11.3	13	9.9	0.83 (0.37–1.81)	52.7 (29.9–95.4)

*Percentage among HPV-positive women.
[†]For estimation of odds ratios and 95% confidence intervals (CI), patients with cervical cancer who were negative for HPV (n = 9) and control individuals who were negative for HPV (n = 1175) were used as reference categories.
[§]Undetermined HPV types denote that HPV types were unclassified or not determined due to weak reactions. NA, not available; NL, nonual cytology.

Table 2. Age-related prevalence of human papillomavirus (HPV) 16 and HPV18 in women with cervical intraepithelial neoplasia (CIN) 2–3 and invasive cervical cancer (ICC)

(A) Women with CIN2–3

Detection of HPV16 and 18	Age (years)					
	20–29 (n = 78)	30–39 (n = 133)	40–49 (n = 55)	50–59 (n = 30)	60– (n = 4)	All (n = 307)
Single infection						
HPV16	26 (33.3%)	30 (22.6%)	9 (16.4%)	3 (10.0%)	1 (25.0%)	70 (22.8%)
HPV18	6 (7.7%)	10 (7.5%)	2 (3.6%)	2 (6.7%)	0 (0.0%)	20 (6.5%)
Multiple infection						
HPV16 and HPV18 only	1 (1.3%)	0 (0.0%)	0 (0.0%)	1 (3.3%)	0 (0.0%)	2 (0.7%)
HPV16, HPV18, and others	9 (11.5%)	5 (3.8%)	2 (3.6%)	1 (3.3%)	0 (0.0%)	19 (6.2%)
Total	42 (53.8%)	45 (33.8%)	13 (23.6%)	7 (23.3%)	1 (25.0%)	111 (36.2%)

(B) Women with ICC

Detection of HPV16 and 18	Age (years)					
	20–29 (n = 10)	30–39 (n = 29)	40–49 (n = 44)	50–59 (n = 25)	60– (n = 32)	All (n = 140)
Single infection						
HPV16	1 (10.0%)	9 (31.0%)	20 (45.5%)	11 (44.0%)	12 (37.5%)	53 (37.9%)
HPV18	6 (60.0%)	9 (31.0%)	8 (18.2%)	5 (20.0%)	4 (12.5%)	32 (22.9%)
Multiple infection						
HPV16 and HPV18 only	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
HPV16, HPV18, and others	2 (20.0%)	4 (13.8%)	1 (2.3%)	0 (0.0%)	2 (6.3%)	9 (6.4%)
Total	9 (90.0%)	22 (75.9%)	29 (65.9%)	16 (64.0%)	18 (56.3%)	94 (67.1%)

were associated with 90.0% of women aged 20–29 years and 75.9% of women aged 30–39 years. Also, HPV16 and/or HPV18 contributed to 64.9% of infections detected in squamous cell carcinoma and 84.7% of infections in adenocarcinoma.

To quantify the population-based impact of HPV vaccine in Japan, the age-specific prevalence of HPV16 and HPV18 in ICC cases was applied to the age distribution data of ICC case based on the Japanese cancer registration in 2002.[13] When excluding

HPV16- and HPV18-positive women from the data, the number of ICC cases was estimated to decrease from 8779 to 3074, suggesting that HPV16 and HPV18 vaccines may provide 65% protection against ICC in Japan.

Discussion

Our data showed that HPV infections are commonly detected among Japanese women, particularly of reproductive age, suggesting that approximately 12 million Japanese women may be carriers of HPV DNA. In the present study, the overall HPV prevalence in Japanese women with normal cytology (22.5%) was much higher than results from previous studies in Japan (range, 9.7–14.6%).[14–16] In a meta-analysis including these HPV studies, HPV prevalence in cytologically normal women was only 10.2%.[8] Because the meta-analysis data were from case-control studies, this discrepancy may be explained by the older age of control women that were selected to match the age of the case women with cancer or precursor lesions. For instance, Asato *et al.* reported that HPV DNA was detected in 10.2% of control women with normal cytology,[14] but they (average age, 52.4 years) were much older than our study subjects (average age, 35.0 years). In the present study, HPV prevalence in cytologically normal women aged 40–54 years was 11.4%. One may speculate that our data may be biased because the study subjects visited hospitals on their own initiative. However, our result was similar to results from recent large-scale population-based studies in the USA (26.8%), Denmark (22.9%), and Costa Rica (22.4%).[17–19] With regard to age-specific prevalence, HPV infection was most frequently detected in young women aged 15–25 years and a second peak was observed in women aged 55 years or older, which is also consistent with results from African, American, and European populations,[20] although the reason for the second peak is unknown.

In the present study, type-specific prevalence data in women with ICC and precursor lesions were very similar to those from the meta-analysis of previous Japanese studies:[8] for instance, HPV16 and HPV18 were less frequently identified in ICC cases in Japan compared with Southeast Asia, North America, and Europe,[21] with HPV31, HPV33, HPV52, and HPV58 accounting for approximately 20% of ICC; HPV45 was rarely detected in Japan. However, the prevalence of HPV18 in ICC cases was far higher in this study (24%) compared with previous studies conducted by us (14%)[22] and other groups (8–11%).[15,16] In a meta-analysis of Japanese HPV studies, HPV18 was identified in 12% of women with ICC.[8] This discrepancy may be explained by the difference in age of the study subjects. Our study subjects (mean age, 49 years) were much younger than the women participating in the previous studies: the mean age of ICC cases was 57 years in the data shown by Nakagawa *et al.* in 1996[22] and 53 years in the study reported by Asato *et al.* in 2004.[16] This was consistent with a recent report showing the increased incidence of young women with cervical cancer in Japan.[23] As shown in our previous[22] and present studies, HPV18 was more frequently identified among young ICC cases. Thus, the higher HPV18 prevalence in this study may be explained by the younger age of the study subjects. Although HPV18 is more commonly detected in adenocarcinoma than in squamous cell carcinoma, the incidence of adenocarcinoma did not differ between the present and previous studies.

Our data suggested that risks of progression to CIN2–3 and ICC vary greatly by HPV genotype, with seven types of HPV (HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, and HPV58) accounting for approximately 80% of ICC and 70% of CIN2–3. This was consistent with the results of a meta-analysis of Japanese HPV studies.[8] In a large-scale prospective cohort study of Japanese women with low-grade squamous intraepithelial lesion, the cumulative risk of CIN3 within 5 years was: 19.8% for HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, and HPV58; 6.7% for other oncogenic types; and 3.1% for non-oncogenic types ($P = 0.0001$; K. Matsumoto, unpublished data, 2009). These observations suggest that testing for a specific subset of oncogenic HPV types may be very useful for identifying populations at increased or decreased risk for disease progression in the follow up of women with cervical precursor lesions. Characterizing a woman's risk more precisely by partial or full genotyping may reduce the number of follow-up smears and colposcopy referrals, although further analyses of cost-utility are needed.

To date, clinical trials of HPV vaccines have demonstrated close to 100% efficacy in preventing infection and disease associated with types included in the vaccines.[3–5] Our preliminary calculations excluding HPV16- and HPV18-positive infections from the data suggested that the reduction in CIN2–3 and ICC that can be achieved by prophylactic HPV16 and HPV18 vaccination would be 36.2 and 67.1%, respectively, in Japan. Actually, the reduction may be smaller because some HPV16- and HPV18-positive infections include coinfections with other types, whereas cross-protection against HPV45, HPV31, and HPV52 may offer an additional prevention effect. A clinical trial of HPV16 and HPV18 vaccine demonstrated that vaccine efficacy against HPV45, HPV31, and HPV52 was 60, 36, and 32%, respectively.[5] Based on these data, the reduction in CIN2–3 and ICC that can be achieved by HPV16 and HPV18 vaccination would be 44.3 and 71.0%, respectively. These estimates suggested that current HPV vaccines would contribute substantially to reducing the incidence of cervical cancer and high-grade precursor lesions in Japan, but screening will remain necessary to detect approximately 60% of CIN2–3 and 30% of ICC cases that cannot be prevented by HPV16 and HPV18 vaccination. Equally important, the overall prevalence of HPV16 and/or HPV18 decreased significantly with the women's age: highest in women aged 20–29 years (CIN2–3, 53.9%; ICC, 90.0%) decreasing with age thereafter and lowest in women aged 60 years or older (CIN2–3, 25.0%; ICC, 56.3%). This may imply that HPV16 and HPV18 have an advantage of progressing rapidly to cervical cancer and pre-cancer compared with other oncogenic types, in accordance with recent reports.[24,25] Although data are limited with regard to the age-specific prevalence of HPV16 and HPV18 in cervical cancer and pre-cancer worldwide, a previous study also reported a high prevalence of HPV16 and HPV18 in young Japanese women with ICC.[22] These observations suggest that current HPV vaccines will be more useful in reducing the incidence of cervical cancer and pre-cancer in young women aged 20–39 years in Japan. Therefore, HPV vaccination could substantially reduce the number of young women receiving surgical treatment (cone biopsy or hysterectomy) that may result in negative consequences for pregnancy outcomes or inability to bear a child.[26]

In conclusion, our data suggest that risks of progression to CIN2–3 and ICC vary considerably by HPV genotype. Accordingly, testing for a specific subset of oncogenic HPV types may help identify Japanese women at particularly high risk of CIN2–3 and cancer. In Japan, current HPV vaccines are estimated to provide approximately 70% protection against ICC and may be more useful in reducing the incidence of cervical cancer and pre-cancer in young women of reproductive age.

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