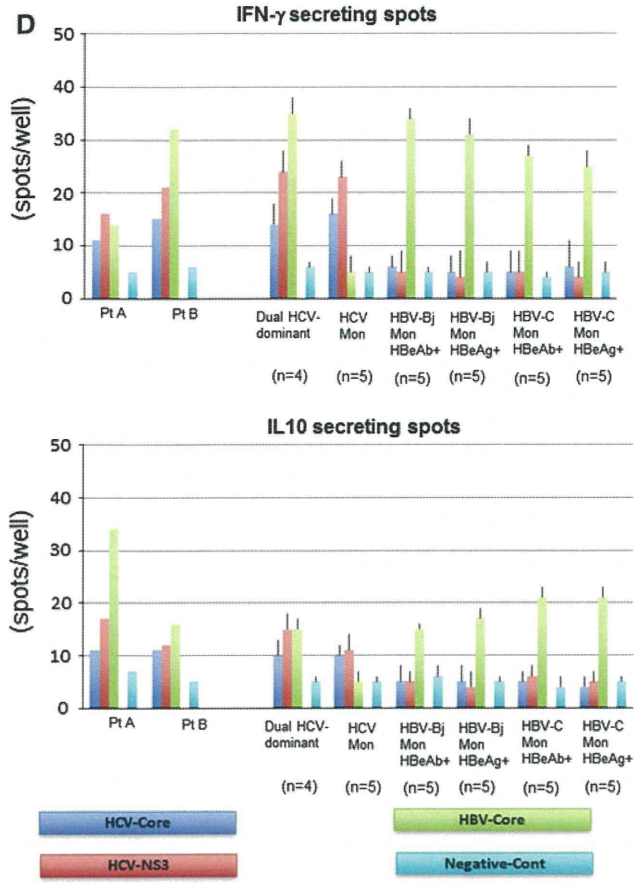

Fig. 4 continued

Discussion

The immunopathogenesis of dual hepatitis B and C infection is not clear, given the complexity of viral and host factors [19, 21, 48–50]. However, detailed understanding of specific patients with dual hepatitis B and C infection could contribute to improving the treatment and follow up of these patients. Therefore, we focused on two representative patients with HBV/HCV dual infection who received Peg-IFN/RBV therapy.

Concerning the virological results, patient A had genotype 1b, HCV-Core 70 wild-type and low mutation of ISDR HCV and genotype C HBV. It has been reported that genotype 1b HCV is common in Japan and is usually difficult to treat in comparison to genotypes 2a and 2b [51]. Among genotype 1b HCV strains, HCV-Core 70 wild-type HCV is easily decreased by Peg-IFN/RBV therapy [51]. On the other hand, it has been reported that in genotype 1b HCV low mutation of ISDR is difficult to treat [52]. Patient B had almost the same background of HCV–genotype 1b, HCV-Core 70 wild-type, and low mutation of ISDR–as patient A. However, the background of host factors that could affect the responsiveness of IFN-based therapy was different between patients A and B. For example, patient A had a hetero allele of the IL-28B polymorphism, advanced fibrosis, and fatty changes of the liver. On the other hand, patient B had the major allele of the IL-28B polymorphism and mild fibrosis. Moreover, the background of HBV in patient B was completely different from that in patient A. It has been reported that HBV genotype Bj is usually more susceptible to IFN-based therapy than genotype C [45, 53]. Therefore, not only the HBV factors but also the combination of host factors and HBV factors might affect the responsiveness to IFN-based therapy. In patient A, the responsiveness of HCV during Peg-IFN/RBV therapy was relatively poor. However, the viral titers of HCV were lower than 1.2 log copies/ml at 7 months after the start of therapy. During the reduction of the HCV viral titers, the titers of HBV and HBc-Ag specific IL-10-secreting cells were gradually increased. Although patient A had received Peg-IFN/RBV therapy for up to 18 months, HCV-RNA increased again 12 months after the start of the therapy. The sustained Th1 immune suppression might have contributed to the relapse of HCV. Not only weak up-regulation of HCV-specific Th1 immune reaction but also strong up-regulation of HBV-specific IL-10-secreting activity was detected during Peg-IFN/RBV therapy in patient A [26, 35]. Moreover, increased HBeAg could be detected 9 months after the start of the therapy. Fluctuations of activated CD4 cells, CD8 cells, NK cells, and NK-T cells could be seen in patient A. On the other hand, in patient B, the responsiveness of HBV and HCV during Peg-IFN/RBV therapy was good. Moreover, the immune response of patient B was almost comparable to the responses in the patients with HCV monoinfection and those with HBV-genotype Bj monoinfection. Previously, it has been reported that Peg-IFN/RBV therapy could achieve almost the same SVR rates in patients with HCV/HBV dual infection and those with HCV monoinfection [54–56]. We assume that the results in these studies were obtained from patients similar to our patient B, because the number of patients with HCV-dominant infection is much higher than the number of those with HBV/HCV dual active infection such as our patient A. Patients with HBV/HCV dual active infection such as patient A are relatively rare in Japan. However, it is necessary to understand the immunopathogenesis of these patients, because Peg-IFN/RBV therapy might not be sufficient to eradicate or control HBV/HCV in these difficult-to-treat patients. One of the candidate therapies for such patients might be Entecavir (ETV)/Peg-IFN/RBV sequential therapy. The effect of HBV specific regulatory T cells might contribute to the immunosuppression of not only HBV but also HCV [35]. In some previous studies, including ours, it has been reported that HBV replication might contribute to immune suppression [19, 29].

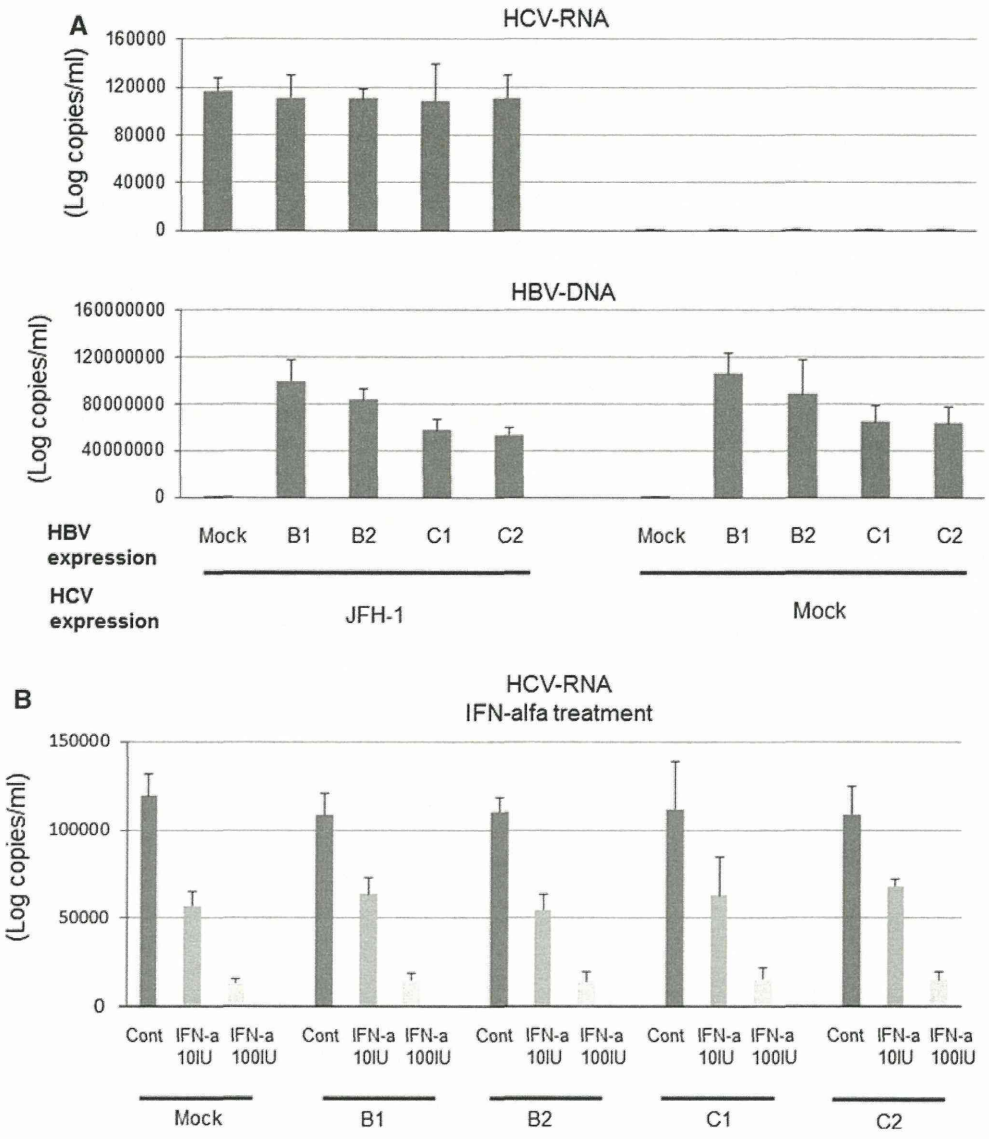

Fig. 5 In vitro analysis of HBV/HCV dual infection. The titers of HCV-RNA and HBV-DNA are shown. *B1* indicates genotype Bj35 clone. *B2* indicates genotype Bj56 clone. *C1* indicates genotype C-AT clone. *C2* indicates genotype C-22 clone (**a**). The titers of HCV-RNA after the IFN-α treatment are shown (**b**)

In the present study, we employed an in vitro coinfection system to analyze the direct interaction between HBV and HCV. In our system, we used several different HBV clones, because it is necessary to consider the effects of different genotypes. Although we could not detect the direct interaction of HBV/HCV in our system, we could not exclude the possibility of indirect interaction between cytokines and chemokines produced from virus-infected hepatocytes. We are now analyzing the chemokines produced from hepatoma cells with different HBV genotype clones (ongoing study).

In conclusion, we analyzed data from representative patients with HBV/HCV dual infection sequentially and precisely. Because many different kinds of backgrounds might affect immunoreactions, we focused on representative patients and analyzed the immunological responses extensively. There might be a group of patients with very difficult-to-treat dual infections. We need to understand the immunopathogenesis of such patients to develop the appropriate therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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Consumption of n-3 Fatty Acids and Fish Reduces Risk of Hepatocellular Carcinoma

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BACKGROUND & AIMS: Fish is a rich source of n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). Although consumption of fish and n-3 PUFA has been reported to protect against the development of some types of cancer, little is known about its association with hepatocellular carcinoma (HCC). **METHODS:** We investigated the association between fish and n-3 PUFA consumption and HCC incidence (n = 398) in a population-based prospective cohort study of 90,296 Japanese subjects (aged, 45–74 y). Hazard ratios and 95% confidence intervals (CIs) for the highest vs the lowest quintile were estimated from multivariable adjusted Cox proportional hazards regression models. We also conducted subanalyses of subjects with known hepatitis B virus (HBV) or hepatitis C virus (HCV) status, and of subjects who were anti-HCV and/or hepatitis B surface antigen positive. All tests of statistical significance were 2-sided. **RESULTS:** Among all subjects, consumption of n-3 PUFA-rich fish and individual n-3 PUFAs was associated inversely with HCC, in a dose-dependent manner. Hazard ratios for the highest vs lowest quintiles were 0.64 (95% CI, 0.42–0.96) for n-3 PUFA-rich fish, 0.56 (95% CI, 0.36–0.85) for EPA, 0.64 (95% CI, 0.41–0.98) for DPA, and 0.56 (95% CI, 0.35–0.87) for DHA. These inverse associations were similar irrespective of HCV or HBV status. **CONCLUSIONS: Consumption of n-3 PUFA-rich fish or n-3 PUFAs, particularly EPA, DPA, and DHA, appears to protect against the development of HCC, even among subjects with HBV and/or HCV infection.**

Keywords: Diet; Liver Cancer; Cancer Prevention; Omega-3 Fatty Acid.

The most important risk factor in the development of hepatocellular carcinoma (HCC) in human beings is chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[1] The markedly poor prognosis of HCC, with a 5-year survival rate in Japan of less than 20%,[2] emphasizes the need for effective preventive measures, particularly in hepatitis virus carriers. Although dietary factors also might be risk factors, the role of diet in the etiology of HCC remains unclear, except with regard to alcohol consumption and aflatoxin contamination.[3]

A recent prospective study showed an inverse association between white meat, including fish, and liver cancer.[4] Inverse associations with the consumption of white meat or fish were observed in some studies,[5–8] but were not confirmed in others.[9–11] Moreover, except for 2 case-control studies,[5,7] most previous epidemiologic studies of white meat or fish and HCC did not consider HCV or HBV infection status.

Fish is a rich source of n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), and several studies have documented a protective effect of dietary n-3 PUFA on the development of several cancers.[12,13] However, less is known about the influence of n-3 PUFA on HCC.

Here, we investigated the presence of an association between fish and n-3 PUFA consumption and HCC in a large-scale, population-based, cohort study in Japan, with consideration for HCV and HBV infection status.

Materials and Methods

Study Population

The Japan Public Health Center–based prospective study was launched in 1990. The study design has been described in detail previously.[14] The study population was defined as all residents of 11 public health center (PHC) areas across Japan who were aged 40–69 years at the start of the respective baseline survey (n = 140,420). In the present analysis, we excluded one PHC area (Tokyo) because data on cancer incidence were not available, as well as some subjects from a second PHC (Osaka) area for whom different definitions were used (n = 16,841). The study was approved by the Institutional Review Board of the National Cancer Center (Tokyo, Japan).

Abbreviations used in this paper: ALA, alpha-linolenic acid; ALT, alanine aminotransferase; CI, confidence interval; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; HBsAg, hepatitis B virus antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; PHC, public health center; PUFAs, polyunsaturated fatty acids.

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Baseline Survey

Cohort participants responded to a self-administered questionnaire at baseline in 1990 (cohort I) and 1993–1994 (cohort II). A 5-year follow-up survey was conducted in 1995 (cohort I) and 1998 (cohort II). The 5-year follow-up survey included more comprehensive information on food intake frequency than the baseline survey, and accordingly was used as baseline for the present study. We initially identified 113,378 participants as the study population at the baseline survey. The questionnaire also included information on personal medical history, smoking and drinking habits, diet, and other lifestyle factors. After exclusion of 205 participants who were found to be ineligible because of non-Japanese nationality (n = 44), late report of emigration that occurred before the start of the follow-up period (n = 155), incorrect birth date (n = 3), and duplicate registration (n = 3), the remaining 113,171 participants were considered eligible for the present study. Completed questionnaires were received from 94,999 subjects (response rate, 84%). Further, subjects who had been diagnosed with cancer before the starting point were excluded from analysis (n = 3022).

Food Frequency Questionnaire

The food frequency questionnaire (FFQ) asked subjects about their usual intake of 138 food items in standard portions/units during the previous year, including 19 fish questions. The questionnaire contained 9 frequency categories (never, 1–3 times/mo, 1–2 times/wk, 3–4 times/wk, 5–6 times/wk, once/d, 2–3 times/d, 4–6 times/d, and ≥7 times/d). Nineteen items inquired about fish and shellfish intake, including salted fish, dried fish, canned tuna, salmon or trout, bonito or tuna, cod or flat fish, seabream, horse mackerel or sardine, mackerel pike or mackerel, dried small fish, salted roe, eel, squid, octopus, prawn, short-necked clam or crab shell, vivipara, *chikuwa* (fish paste product), and *kamaboko* (fish paste product). Standard portion sizes were specified for each food item in the 3 amount choices of small (50% smaller), medium (same as standard), and large (50% larger). Fish consumption in g/day was calculated by multiplying frequency by standard portion size for each food item. In our FFQ, dishes in which food was just a constituent were not included. We calculated the daily intake of all n-3 PUFAs combined and of individual PUFAs, namely α-linolenic acid (ALA), EPA, DPA, and DHA, using a fatty acid composition table of Japanese foods.[15] Furthermore, based on the value of n-3 PUFA per 100 g edible portion of fish, we also calculated the consumption of n-3 PUFA-rich fish (salmon or trout, sea bream, horse mackerel or sardine, mackerel pike or mackerel, and eel).[15] Intake of food and nutrients was log-transformed and adjusted for total energy intake by the residual model.[16] We also used the nutrient density method and obtained similar results.

We documented the validity of the FFQ in the assessment of fish, ALA, EPA, DPA, and DHA consumption in subsamples using 14- or 28-day dietary records. Based on 102 men and 113 women in cohort I, the Spearman correlation coefficients between energy-adjusted intake of fish,[17] n-3 PUFA, ALA, EPA, DPA, and DHA[18] from the questionnaire and from dietary records were 0.37, 0.21, 0.27, 0.38, 0.32, and 0.34 for men, and 0.32, 0.34, 0.25, 0.45, 0.39, and 0.37 for women, respectively. The percentage differences between the dietary records and the FFQ for fish were −16% for men, and −1% for women.[19] Thus, validities for fish and n-3 PUFAs were considered moderate.

Among the 91,977 subjects who responded to the questionnaire and had no past history of cancer, subjects who reported extreme total energy intake (upper or lower 1.0%) were excluded, leaving 90,296 subjects for analysis.

Blood Collection and Laboratory Assays

Subjects were asked to voluntarily provided 10 mL of blood during health checkups in 1993–1995, at which time plasma alanine aminotransferase (ALT) level was determined. Samples were divided into the plasma and buffy layers, and preserved at −80°C until analysis. Among subjects who provided blood (n = 33,329), plasma samples from a portion of the subjects (n = 17,497) were screened for anti-HCV using a third-generation immunoassay (Lumipulse II Ortho HCV; Ortho-Clinical Diagnostics K.K., Tokyo, Japan)[20] and for hepatitis B virus antigen (HBsAg) by reversed passive hemagglutination with a commercial kit (Institute of Immunology Co, Ltd, Tokyo, Japan).

Follow-up and Identification of Hepatocellular Carcinoma

Subjects were followed up from the baseline survey until December 31, 2008. Changes in residence status, including survival, were identified annually through the residential registry in the respective public health center area. Among study subjects, 2775 (3.1%) moved out of their study area and 318 (0.4%) were lost to follow-up evaluation during the study period.

Incidence data on HCC were identified by active patient notification from major local hospitals in the study area and data linkage with population-based cancer registries, with permission from the local governments responsible for the registries. Death certificates were used as a supplementary information source, with 10.6% of cases in our cancer registry system based on death certificate only. Cases were coded using the International Classification of Diseases for Oncology, 3rd ed (code C22.0).[21] During an average follow-up period of 11.2 years (1,008,595 person-years), a total of 398 cases of HCC were newly diagnosed among 90,296 subjects who had returned the baseline questionnaire. In one subgroup, a total of 74 cases of HCC were newly diagnosed among 17,497 subjects who had data on anti-HCV and HBsAg status and ALT level.

Statistical Analysis

Person-years of follow-up evaluation were calculated for each subject from the date of completion of the baseline questionnaire to the date of HCC diagnosis, date of emigration from the study area, or date of death, whichever occurred first; or if none of these occurred, follow-up evaluation was through the end of the study period (December 31, 2008). Subjects who were lost to follow-up evaluation were censored at the last confirmed date of presence in the study area. Hazard ratios (HRs) of HCC were calculated by quintiles of consumption of the respective food items or nutrients, with the lowest consumption category as the reference. HRs and 95% confidence intervals (CIs) were calculated by the Cox proportional hazards model, and adjusted for age at baseline survey (continuous), sex, and study area (10 PHC areas) according to the SAS PHREG procedure (version 9.1; SAS Institute, Inc, Cary, NC). For further adjustment, additional possible confounders were incorporated into the model, namely smoking status (never, former, current); alcohol intake (almost never, 1–3 times/mo, ≥1 times/wk); body mass index (continuous); past history of diabetes mellitus (yes or

no); intake of coffee (almost never, 1–4 d/wk, ≥1 cups/d); and soy foods, vegetables, vegetable oil, protein, and iron (continuous). Because of a high correlation coefficient between vegetable oil and ALA, vegetable oil was not adjusted for in the analysis of the association between ALA and HCC. In the subgroup analysis among subjects who had data on hepatitis virus, further adjustment was added for HCV and HBV infection status (positive or negative) and ALT level (<30 IU/L, 30 to <70 IU/L, ≥70 IU/L). These variables are either known or suspected risk factors for cancer or were associated previously with the risk of HCC. Trends were assessed by assignment of the median value in each category. All *P* values were 2-sided, and statistical significance was determined at a *P* value of less than .05.

We also analyzed the association between fish and n-3 PUFA intake and HCC in the 17,497 subjects for whom HCV and HBV infection status and ALT level was known, as well as in the 1303 subjects who were either or both anti-HCV or HBsAg positive.

Results

During an average follow-up period of 11.2 years, a total of 398 HCC cases were identified in total subjects. Baseline characteristics of subjects according to total fish consumption are shown in Table 1. Subjects with higher fish consumption tended to be older, smoke less, and drink less alcohol and coffee. Body mass index and soybean intake was not substantially different according to consumption. Intake of vegetables, iron, and fatty acid increased as fish intake increased. The proportion of subjects positive for anti-HCV, HBsAg, or both among quintiles of fish consumption was similar. The pattern of characteristics was similar according to intake of n-3 PUFA-rich fish (data not shown).

Spearman correlation coefficients for the associations between total fish, n-3 PUFA-rich fish, n-3 PUFA, EPA, DPA, and DHA were analyzed. There were strong correlations between fish and n-3 PUFA (r = 0.73), EPA (r = 0.85), DPA (r = 0.83), and DHA (r = 0.87) and between n-3 PUFA-rich fish and n-3 PUFA (r = 0.73), EPA (r = 0.86), DPA (r = 0.87), and DHA (r = 0.84).

Table 2 presents hazard ratios in relation to fish and n-3 PUFA consumption for HCC cases. Total fish consumption had a weak inverse association with the risk of HCC, with a multivariable HR for the highest vs lowest quintile of 0.64 (95% CI, 0.41–1.02; P_{trend} = .07). n-3 PUFA-rich fish consumption was dose-dependently associated with a decreased risk of HCC, with a multivariable HR for the highest vs lowest quintile of 0.64 (95% CI, 0.42–0.96; P_{trend} = .04). In addition, inverse associations were seen between EPA, DPA, DHA, and HCC, with multivariable HRs for the highest vs lowest quintile of 0.56 (95% CI, 0.36–0.85; P_{trend} = .01) for EPA, 0.64 (95% CI, 0.41–0.98; P_{trend} = .05) for DPA, and 0.56 (95% CI, 0.35–0.87; P_{trend} = .03) for DHA. n-3 PUFA and ALA did not show statistically significant inverse associations with HCC, with respective multivariable HRs for the highest vs lowest quintile of 0.63 (95% CI, 0.36–1.10) and 0.78 (95% CI, 0.48–1.28). No substantial change in results was seen on additional analyses for HCC stratified by sex, smoking status, or body mass index (data not shown). Furthermore, our analyses did not change when restricted to cases that occurred after the first 3 years of follow-up evaluation (122 cases excluded) and when cases identified by death certificate only were excluded (42 cases excluded) (data not shown). Moreover, when subjects with self-reported pre-existing liver

Table 1. Subject Characteristics at Baseline According to Fish Consumption Among Japanese (n = 90,296) and Those Who Were Anti-HCV or HBsAg Positive (n = 1372) in the Japan Public Health Center–Based Prospective Study

	Total fish consumption				
	Lowest	Second	Third	Fourth	Highest
Median intake, *g*	35.0	60.6	82.8	109.9	160.6
Age ± SD, *y*	51.8 ± 8.2	51.4 ± 8.0	51.7 ± 7.8	52.2 ± 7.8	53.2 ± 7.7
Current smoker, %	27.1	25.4	23.6	20.6	18.6
Regular drinker (yes), %	22.3	22.7	21.4	20.5	18.1
Body mass index, mean ± SD, kg/m^2	23.7 ± 3.1	23.5 ± 3.1	23.5 ± 3.0	23.5 ± 3.0	23.6 ± 3.1
History of diabetes (yes), %	5.1	4.9	5.0	5.8	6.5
Coffee, daily, %	34.2	35.0	32.3	30.3	26.0
Soybean, mean ± SD, *g/d*	96.8 ± 119.8	91.2 ± 79.3	90.5 ± 72.9	89.6 ± 63.5	91.5 ± 65.9
Vegetables, mean ± SD, *g/d*	201.3 ± 165.5	217.1 ± 137.2	225.4 ± 132.5	232.9 ± 125.3	240.5 ± 131.4
Iron, mean ± SD, *mg/d*	8.7 ± 2.6	9.1 ± 2.2	9.4 ± 2.1	9.7 ± 2.0	10.8 ± 2.1
Vegetable oil, mean ± SD, *g/d*	9.0 ± 5.5	9.9 ± 4.4	10.4 ± 4.1	11.1 ± 3.8	12.5 ± 4.2
Fatty acid, mean ± SD, *g/d*	48.1 ± 19.4	50.1 ± 15.4	51.6 ± 14.2	53.2 ± 13.2	56.1 ± 13.6
n-3 PUFA, mean ± SD, *g/d*	2.3 ± 0.8	2.8 ± 0.7	3.2 ± 0.7	3.7 ± 0.1	4.7 ± 1.2
ALA, mean ± SD, *g/d*	1.92 ± 0.81	2.05 ± 0.66	2.15 ± 0.62	2.25 ± 0.58	2.39 ± 0.60
EPA, mean ± SD, *g/d*	0.16 ± 0.10	0.27 ± 0.09	0.37 ± 0.11	0.49 ± 0.16	0.78 ± 0.40
DPA, mean ± SD, *g/d*	0.04 ± 0.03	0.07 ± 0.02	0.10 ± 0.03	0.13 ± 0.04	0.20 ± 0.10
DHA, mean ± SD, *g/d*	0.30 ± 0.15	0.47 ± 0.13	0.62 ± 0.16	0.81 ± 0.22	1.25 ± 0.56
Infection status (n = 17,497)					
HCV(−)/HBV(−)	92.76	92.56	91.87	92.70	92.87
HCV(−)/HBV(+)	3.04	2.20	1.83	1.97	2.15
HCV(+)/HBV(−)	4.06	5.24	6.21	5.24	4.81
HCV(+)/HBV(+)	0.14	0	0.09	0.09	0.17

Table 2. Hazard Ratio and 95% Confidence Intervals for HCC According to Quintile of Intake of Fish and n-3 PUFA in the Japan Public Health Center–Based Prospective Study (n = 90,296)

	Lowest	Second	Third	Fourth	Highest	P_{trend}
Median fish intake, *g/d*	35.0	60.6	82.8	109.9	160.6	
Cases, n	92	79	78	74	75	
Person-years of follow-up evaluation	201,649	201,387	202,084	202,365	201,110	
Age, area, sex-adjusted HR (95% CI)	1	0.91 (0.68–1.24)	0.90 (0.66–1.22)	0.85 (0.62–1.16)	0.82 (0.60–1.13)	.19
Multivariate HR (95% CI)[a]	1	0.83 (0.59–1.17)	0.84 (0.59–1.20)	0.75 (0.51–1.11)	0.64 (0.41–1.02)	.07
n-3 PUFA-rich fish (median), g/d[b]	9.6	19.7	29.5	43.0	70.6	
Cases, n	89	83	79	71	76	
Person-years of follow-up evaluation	202,479	202,296	202,034	202,357	199,411	
Age, area, sex-adjusted HR (95% CI)	1	0.97 (0.72–1.31)	0.90 (0.67–1.23)	0.79 (0.57–1.08)	0.75 (0.55–1.02)	.03
Multivariate HR (95% CI)[a]	1	0.98 (0.70–1.37)	0.86 (0.61–1.23)	0.84 (0.58–1.21)	0.64 (0.42–0.96)	.04
n-3 PUFA (median), *g/d*[b]	1.95	2.65	3.18	3.77	4.80	
Cases, n	101	75	79	80	63	
Person-years of follow-up evaluation	200,491	200,103	201,864	203,023	203,115	
Age, area, sex-adjusted HR (95% CI)	1	0.84 (0.62–1.13)	0.97 (0.72–1.31)	1.01 (0.75–1.36)	0.73 (0.53–1.00)	.18
Multivariate HR (95% CI)[a]	1	0.77 (0.53–1.12)	0.99 (0.66–1.49)	1.02 (0.65–1.62)	0.63 (0.36–1.10)	.29
ALA (median), *g/d*[b]	1.25	1.68	1.98	2.31	2.84	
Cases, n	107	90	77	64	60	
Person-years of follow-up evaluation	199,727	199,879	201,557	203,044	204,388	
Age, area, sex-adjusted HR (95% CI)	1	0.97 (0.73–1.29)	0.97 (0.72–1.30)	0.90 (0.65–1.23)	0.95 (0.68–1.32)	.62
Multivariate HR (95% CI)[c]	1	0.84 (0.60–1.18)	0.78 (0.53–1.15)	0.75 (0.49–1.15)	0.78 (0.48–1.28)	.27
Median EPA, *g/d*[b]	0.14	0.26	0.36	0.48	0.74	
Cases, n	86	78	86	73	75	
Person-years of follow-up evaluation	201,446	200,959	200,759	202,205	203,226	
Age, area, sex-adjusted HR (95% CI)	1	0.88 (0.64–1.19)	0.94 (0.69–1.27)	0.76 (0.55–1.06)	0.70 (0.50–0.96)	.02
Multivariate HR (95% CI)[a]	1	0.76 (0.54–1.07)	0.85 (0.60–1.21)	0.73 (0.50–1.06)	0.56 (0.36–0.85)	.01
DPA (median), *g/d*[b]	0.04	0.07	0.09	0.12	0.19	
Cases, n	84	78	81	78	77	
Person-years of follow-up evaluation	204,239	201,463	200,839	200,190	201,864	
Age, area, sex-adjusted HR (95% CI)	1	0.93 (0.69–1.28)	0.95 (0.69–1.29)	0.88 (0.64–1.20)	0.76 (0.55–1.05)	.08
Multivariate HR (95% CI)[a]	1	0.84 (0.60–1.18)	0.91 (0.64–1.29)	0.85 (0.59–1.23)	0.64 (0.41–0.98)	.05
DHA (median), *g/d*[b]	0.28	0.46	0.61	0.8	1.19	
Cases, n	89	71	81	80	77	
Person-years of follow-up evaluation	202,203	200,834	200,568	202,231	202,759	
Age, area, sex-adjusted HR (95% CI)	1	0.79 (0.57–1.08)	0.87 (0.64–1.19)	0.82 (0.60–1.13)	0.71 (0.52–0.98)	.07
Multivariate HR (95% CI)[a]	1	0.73 (0.52–1.03)	0.77 (0.54–1.10)	0.77 (0.53–1.12)	0.56 (0.35–0.87)	.03

[a]Adjusted for age, area, sex, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, vegetable oil, protein, and iron.
[b]n-3 PUFA-rich fish included salmon or trout, sea bream, horse mackerel or sardine, mackerel pike or mackerel, and eel.
[c]Adjusted for age, area, sex, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, protein, and iron.

diseases were excluded (133 cases excluded), the results were attenuated but not substantially changed. The prevalence of fish oil supplement use was 0.06%; no change was seen when these users were excluded.

Fish consumption might reflect other lifestyle factors. In particular, subjects with higher fish consumption tended to drink less alcohol and coffee, and tended to have a past history of diabetes. Although we also assessed the effect of fish consumption according to alcohol, coffee drinking status, or history of diabetes, an inverse association between fish and n-3 PUFA-rich fish and HCC risk was shown in both regular (1–2 times/wk) and nonregular (<1 time/wk) alcohol drinkers, in both daily and nondaily coffee drinkers, and in both those with and without a history of diabetes (data not shown). Interaction between n-3 PUFA-rich fish and alcohol, coffee drinking status, or history of diabetes was not detected ($P_{interaction}$ =.25, .57, and .58, respectively).

To adjust for HCV and HBsAg status, we also analyzed the association between fish and n-3 PUFAs and HCC risk among subjects who had information on HCV and HBV infection status (Table 3). Although statistical significance was diminished because of a small sample size, similar results were seen, with multivariable HRs for the highest vs lowest tertile of 0.54 (95% CI, 0.23–1.24) for fish, 0.73 (95% CI, 0.35–1.53) for n-3 PUFA-rich fish, 0.51 (95% CI, 0.20–1.32) for n-3 PUFA, 0.70 (95% CI, 0.29–1.71) for ALA, 0.62 (95% CI, 0.28–1.39) for EPA, 0.80 (95% CI, 0.34–1.85) for DPA, and 0.63 (95% CI, 0.27–1.49) for DHA.

To clarify the association between fish and n-3 PUFAs and HCC risk among HBV- and/or HCV-infected subjects, we restricted analysis to subjects who were either or both anti-HCV or HBsAg positive (n = 1303) and anti-HCV positive (n = 911) (Table 4). Total fish consumption was not statistically significantly associated with the risk of HCC, with a multivariable HR for the highest vs lowest quintile of 0.52 (95% CI, 0.20–1.32; P_{trend} = .31), and the inverse association between total fish and HCC was strengthened when subjects were limited to those who were anti-HCV positive, with a multivariable HR for the highest vs lowest quintile of 0.30 (95% CI, 0.11–0.82; P_{trend} = .03). Higher n-3 PUFA-rich fish and n-3 PUFA consumption appeared to decrease the risk of HCC, but without statistical significance. Multivariable HRs for the highest vs lowest tertile among subjects who were either or both anti-HCV or HBsAg positive was 0.60 (95% CI, 0.25–1.40) for n-3 PUFA-rich fish and 0.41 (95% CI, 0.14–1.19) for n-3 PUFA, whereas the HR among subjects who were anti-HCV positive was 0.42 (95% CI, 0.16–1.12) for n-3

Table 3. Hazard Ratio and 95% Confidence Intervals for HCC According to Quintile of Intake of Fish and n-3 PUFA Among Japanese Men and Women Whose HCV and HBV Status Was Known in the Japan Public Health Center–Based Prospective Study (n = 17,497)

	Lowest	Middle	Highest	P_{trend}
Median fish intake, *g/d*	43.6	80.1	131.5	
Cases, n	24	30	20	
Person-years of follow-up evaluation	57,973	57,696	58,186	
Age, area, sex-adjusted HR (95% CI)	1	1.29 (0.75–2.22)	0.73 (0.40–1.34)	.36
Multivariate HR (95% CI)[a]	1	1.42 (0.73–2.76)	0.54 (0.23–1.24)	.24
Median n-3 PUFA-rich fish, *g/d*[b]	12.8	29.1	57.5	
Cases, n	25	23	26	
Person-years of follow-up evaluation	58,381	57,489	57,984	
Age, area, sex-adjusted HR (95% CI)	1	0.91 (0.51–1.61)	0.84 (0.48–1.46)	.53
Multivariate HR (95% CI)[a]	1	1.30 (0.66–2.57)	0.73 (0.35–1.53)	.42
Median n-3 PUFA, *g/d*[b]	2.27	3.05	4.12	
Cases, n	25	24	25	
Person-years of follow-up evaluation	57,776	57,683	58,115	
Age, area, sex-adjusted HR (95% CI)	1	1.02 (0.58–1.79)	0.84 (0.48–1.46)	.52
Multivariate HR (95% CI)[a]	1	0.67 (0.32–1.39)	0.51 (0.20–1.32)	.17
Median ALA, *g/d*[b]	1.49	2.02	2.63	
Cases, n	32	23	19	
Person-years of follow-up evaluation	57,211	57,962	58,401	
Age, area, sex-adjusted HR (95% CI)	1	0.96 (0.56–1.65)	0.97 (0.54–1.77)	.92
Multivariate HR (95% CI)[c]	1	0.75 (0.37–1.54)	0.70 (0.29–1.71)	.43
Median EPA, *g/d*[b]	0.17	0.33	0.58	
Cases, n	23	27	24	
Person-years of follow-up evaluation	57,994	57,447	58,133	
Age, area, sex-adjusted HR (95% CI)	1	0.95 (0.53–1.69)	0.71 (0.39–1.30)	.24
Multivariate HR (95% CI)[a]	1	1.39 (0.71–2.74)	0.62 (0.28–1.39)	.15
Median DPA, *g/d*[b]	0.05	0.09	0.15	
Cases, n	20	30	24	
Person-years of follow-up evaluation	58,066	57,585	57,923	
Age, area, sex-adjusted HR (95% CI)	1	1.30 (0.73–2.31)	0.89 (0.48–1.64)	.55
Multivariate HR (95% CI)[a]	1	1.72 (0.86–3.43)	0.80 (0.34–1.85)	.38
Median DHA, *g/d*[b]	0.32	0.57	0.96	
Cases, n	22	26	26	
Person-years of follow-up evaluation	57,926	57,460	58,187	
Age, area, sex-adjusted HR (95% CI)	1	1.03 (0.58–1.85)	0.88 (0.48–1.59)	.62
Multivariate HR (95% CI)[a]	1	1.15 (0.58–2.29)	0.63 (0.27–1.49)	.24

[a]Adjusted for age, area, sex, HCV, HBsAg, ALT level, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, vegetable oil, protein, and iron.
[b]n-3 PUFA-rich fish included salmon or trout, sea bream, horse mackerel or sardine, mackerel pike or mackerel, and eel.
[c]Adjusted for age, area, sex, HCV, HBsAg, ALT level, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, protein, and iron.

PUFA-rich fish and 0.44 (95% CI, 0.13–1.42) for n-3 PUFA. ALA, EPA, DPA, and DHA consumption also tended to be associated with a decreased risk of HCC among subjects who were either or both anti-HCV or HBsAg positive, albeit without statistical significance (highest vs lowest: multivariable HR, 0.69; 95% CI, 0.26–1.86; HR, 0.55; 95% CI, 0.22–1.39; HR, 0.55; 95% CI, 0.21–1.42, and HR, 0.59; 95% CI, 0.22–1.57, respectively). When subjects were restricted to those who were anti-HCV positive, a dose-dependent inverse association was seen, with multivariable HRs for the highest vs lowest tertile of 0.33 (95% CI, 0.12–0.92; P_{trend} = .03) for EPA, 0.30 (95% CI, 0.10–0.88; P_{trend} = .02) for DHA, and 0.37 (95% CI, 0.13–1.05; P_{trend} = .06) for DPA. ALA showed no association with HCC.

Discussion

Here, we investigated the relationship between fish and n-3 PUFA consumption and the risk of HCC in a population-based prospective study in Japan. Results showed a decreased risk in those with a higher consumption of n-3 PUFA-rich fish and n-3 PUFAs, particularly EPA, DPA, and DHA. Of particular note was the inverse association even when analysis was confined to subjects who were also either or both HCV and HBV positive.

A recent prospective study in the United States also reported that the consumption of white meat, including fish, was significantly inversely associated with the risk of HCC (HR for the highest vs lowest quintile of 0.52, $P_{trend} < .001$), but this study lacked information about HBV and HCV.[4] In a previous study of the association between fish intake and HCC, results from a prospective study in Japan showed a significantly decreased risk of HCC mortality in the second category (3–4 times/wk), albeit in univariate analysis.[6] In a case-control study in China, liver cancer mortality was associated with a curvilinear reduction of fish intake.[8] Another case-control study in China also

Table 4. Hazard Ratio and 95% Confidence Intervals for HCC According to Quintile of Intake of Fish and n-3 PUFA Among Japanese Men and Women Who Were anti-HCV or HBsAg Positive (n = 1303) and anti-HCV Positive (n = 911) in the Japan Public Health Center–Based Prospective Study

	Subjects who were anti-HCV or HBsAg positive (n = 1303)				Subjects who were anti-HCV positive (n = 911)			
	Lowest	Middle	Highest	P_{trend}	Lowest	Middle	Highest	P_{trend}
Median fish intake, *g/d*	41.0	76.7	126.4		48.1	80.6	131.1	
Cases, n	19	25	17		20	17	13	
Person-years of follow-up evaluation	4137	4073	4138		2809	2837	2831	
Age, area, sex-adjusted HR (95% CI)	1	1.44 (0.79–2.63)	0.80 (0.41–1.55)	.58	1	0.98 (0.51–1.88)	0.63 (0.31–1.29)	.22
Multivariate HR (95% CI)[a]	1	1.50 (0.71–3.15)	0.52 (0.20–1.32)	.31	1	1.15 (0.51–2.59)	0.30 (0.11–0.82)	.03
Median n-3 PUFA-rich fish, *g/d*[b]	12.8	29.5	58.0		15.1	31.7	59.3	
Cases, n	21	22	18		17	20	13	
Person-years of follow-up evaluation	4197	4048	4104		2872	2796	2809	
Age, area, sex-adjusted HR (95% CI)	1	1.11 (0.60–2.01)	0.70 (0.37–1.33)	.29	1	1.38 (0.71–2.67)	0.66 (0.32–1.37)	.31
Multivariate HR (95% CI)[a]	1	1.28 (0.61–2.69)	0.60 (0.25–1.40)	.27	1	1.40 (0.62–3.17)	0.42 (0.16–1.12)	.10
Median n-3 PUFA, *g/d*[b]	2.18	3.00	4.02		2.22	3.02	4.05	
Cases, n	20	21	20		19	15	16	
Person-years of follow-up evaluation	4137	4089	4123		2840	2820	2816	
Age, area, sex-adjusted HR (95% CI)	1	1.07 (0.58–2.00)	0.79 (0.42–1.49)	.46	1	0.82 (0.41–1.62)	0.68 (0.35–1.34)	.27
Multivariate HR (95% CI)[a]	1	0.59 (0.26–1.35)	0.41 (0.14–1.19)	.10	1	0.42 (0.17–1.07)	0.44 (0.13–1.42)	.16
Median ALA, *g/d*[b]	1.35	1.89	2.46		1.31	1.81	2.36	
Cases, n	24	20	17		22	12	16	
Person-years of follow-up evaluation	4003	4141	4205		2742	2858	2877	
Age, area, sex-adjusted HR (95% CI)	1	0.92 (0.51–1.68)	0.90 (0.47–1.73)	.75	1	0.60 (0.30–1.23)	0.88 (0.45–1.74)	.62
Multivariate HR (95% CI)[c]	1	0.85 (0.38–1.89)	0.69 (0.26–1.86)	.46	1	0.61 (0.24–1.53)	1.00 (0.35–2.83)	.97
Median EPA, *g/d*[b]	0.82	0.34	0.59		0.24	0.39	0.64	
Cases, n	18	25	18		18	19	13	
Person-years of follow-up evaluation	4221	4029	4098		2871	2780	2826	
Age, area, sex-adjusted HR (95% CI)	1	1.34 (0.71–2.51)	0.71 (0.36–1.41)	.22	1	1.15 (0.60–2.23)	0.60 (0.29–1.24)	.14
Multivariate HR (95% CI)[a]	1	1.35 (0.63–2.88)	0.55 (0.22–1.39)	.12	1	1.00 (0.45–2.21)	0.33 (0.12–0.92)	.03
Median DPA, *g/d*[b]	0.05	0.09	0.15		0.07	0.10	0.16	
Cases, n	18	24	19		15	22	13	
Person-years of follow-up evaluation	4211	4060	4078		2887	2781	2809	
Age, area, sex-adjusted HR (95% CI)	1	1.24 (0.66–2.31)	0.81 (0.41–1.57)	.43	1	1.49 (0.76–2.91)	0.77 (0.36–1.63)	.37
Multivariate HR (95% CI)[a]	1	1.45 (0.68–3.09)	0.55 (0.21–1.42)	.13	1	1.22 (0.56–2.69)	0.30 (0.10–0.88)	.02
Median DHA, *g/d*[b]	0.34	0.59	0.98		0.43	0.65	1.05	
Cases, n	16	25	20		16	18	16	
Person-years of follow-up evaluation	4223	4023	4102		2864	2803	2810	
Age, area, sex-adjusted HR (95% CI)	1	1.53 (0.80–2.92)	0.90 (0.45–1.77)	.56	1	1.22 (0.61–2.43)	0.86 (0.42–1.74)	.59
Multivariate HR (95% CI)[a]	1	1.33 (0.61–2.88)	0.59 (0.22–1.57)	.22	1	0.74 (0.32–1.71)	0.37 (0.13–1.05)	.06

[a]Adjusted for age, area, sex, ALT level, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, vegetable oil, protein, and iron.
[b]n-3 PUFA-rich fish included salmon or trout, sea bream, horse mackerel or sardine, mackerel pike or mackerel, and eel.
[c]Adjusted for age, area, sex, ALT level, smoking status, alcohol frequency, body mass index, past history of diabetes mellitus, and intake of coffee, soy foods, vegetables, protein, and iron.

showed that the frequent intake of fresh fish (≥3 times/week) decreased risk of HCC, with an odds ratio after adjustment for confounding factors, including HBV, of 0.32.[5] In contrast, several case-control studies showed no association between HCC and fermented fish in Thailand[10] or fish in Japan[11] or Italy.[9] Further, although adjusted by HBV and HCV, fish intake showed no association with HCC in a case-control study in Italy.[7] This inconsistency may be owing to errors in exposure measurement and limited variation in fish. Given that Japanese consume large quantities of fish, the inverse association between fish and HCC in our study might have been clarified by the comprehensive questionnaire and wide range of consumption.

Although fish are the principal source of n-3 PUFAs, we are unaware of any study of the association between n-3 PUFA intake and HCC. In the present study, we also observed that consumption of n-3 PUFAs, particularly EPA, DPA, and DHA, was associated inversely with HCC. In clinical trials, dietary supplementation with n-3 PUFAs for 1–3 months was associated with a decreased release of interleukin-1β and interleukin-6.[22–25] Given that HCC is an inflammation-related cancer that has a background of chronic inflammation, triggered by exposure to hepatitis virus infection or toxic compounds, such as ethanol,[26,27] the anti-inflammatory properties of n-3 PUFAs might decrease the risk of HCC. Of note, we showed that the risk of HCC was decreased with greater consumption of fish and n-3 PUFAs in subjects who were either or both anti-HCV or HBsAg positive. The intake of n-3 PUFA-rich fish might reduce the risk of HCC through the anti-inflammatory effects of n-3 PUFAs on chronic hepatitis.

Another possibility is that fish and n-3 PUFAs also might be associated with HCC through an improvement in insulin sensitivity. Given that recent epidemiologic data have suggested that diabetes and obesity are associated with an increased risk of HCC,[28–31] insulin resistance is now recognized as an independent risk factor for the development of HCC.[29] Animal experiments indicate that the intake of n-3 fatty acids from fish oils has a beneficial effect on insulin sensitivity in rats,[32] but not in human beings.[33–35] High concentration of n-3 PUFAs in human skeletal muscle cells have been associated with improved insulin sensitivity.[36] n-3 PUFAs from fish therefore might

improve insulin resistance. In addition, a clinical study has shown the induction of plasma adiponectin in response to a daily intake of EPA and DHA.[37] Thus, the induction of adiponectin also might contribute to the beneficial effect of n-3 PUFA on systemic insulin sensitivity. However, there was no difference in association between fish and n-3 PUFAs and HCC in participants with and without self-reported diabetes.

In contrast, ALA, which is another component of n-3 PUFAs, was weakly or not associated with HCC, although ALA might be converted to EPA and DHA. Other than fish, the other source of n-3 PUFA in this study population was vegetable oil, in which ALA is the only n-3 PUFA (EPA, DPA, and DHA are not included in vegetable oil). On adjustment for vegetable oil, results were not changed substantially. Therefore, EPA, DPA, or DHA among n-3 PUFA from fish might play particularly important roles as factors that lower the risk of HCC.

The strengths of the present study were its prospective design and negligible proportion of loss to follow-up evaluation (0.4%). Information on fish consumption was collected before the subsequent diagnosis of HCC, thereby diminishing the probability of the recall bias that is inherent to case-control studies. Another strength was that virus infection status was available at baseline, allowing us to clarify the association between n-3 PUFAs and HCC in a high-risk population, albeit the sample size was small. Further, dietary information was ascertained using a validated FFQ and the validity of fish and n-3 PUFAs intake was moderate.

Several limitations also warrant mention. First, because we estimated the consumption of fish and associated nutrients from self-reports and at one time point only, some measurement error in the assessment of consumption is inevitable. If present, however, this probably was nondifferential and likely would have lead to the underestimation of results. Second, we had no information on the clinical severity of hepatitis or on the treatment of subjects with hepatitis virus infection before or during the study period. If infected subjects had received treatment, the occurrence of HCC might have been decreased. However, this might have lead to the underestimation of HCC occurrence, which also would have biased the results toward the null. Finally, our study subjects were a middle-aged population, and caution accordingly is required in generalizing the present results to the young and elderly.

In conclusion, our large prospective study indicated that high consumption of n-3 PUFA-rich fish and n-3 PUFAs was associated with a reduced risk of HCC, even among a high-risk population. Given that the prognosis for HCC is extremely poor, our results would, if confirmed, have important implications for public health. Greater consumption of n-3 PUFA-rich fish and n-3 PUFAs may modify the development of HCC among HBV- and/or HCV-infected subjects.

Appendix

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Conflicts of interest

The authors disclose no conflicts.

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Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

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The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

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the world is divided conceptually into zones of high, intermediate, and low HBV endemic areas [Lavanchy, 2004]. In countries where the HBV infection is endemic, most infections result from the vertical transmission from the mother to the child in the peripartum period or from the infection in the early childhood. In the low HBV endemic regions, the neonatal or the childhood HBV infection is rare or even sporadic and the transmission of HBV occurs primarily among unvaccinated adults through the sexual transmission and injecting drug use [Custer et al., 2004].

Patients with chronic hepatitis B are considered to be the major reservoirs for the transmission of HBV. High incidence of infection with HBV is observed within the household contacts of chronic HBV carriers and it is not rare to have several members of the same household who have evidence of infection with HBV [Milas et al., 2000; Thakur et al., 2002]. However, the precise mechanisms of intra-familial spread have not been established clearly.

Different prophylactic strategies for controlling the HBV infection have been used by different countries depending on the prevalence of the HBV infection in each country [Poland and Jacobson, 2004]. The widespread immunization program against hepatitis B, which was implemented in more than 100 countries, was capable of dramatic reduction in the occurrence of chronic HBV infection and HCC [Zuckerman, 1997]. In Egypt, the HBV vaccine was included in 1992 in the Expanded Program of Immunization with injection at 2, 4, and 6 months of age [El Sherbini et al., 2006]. This program resulted in a significant reduction in the rate of acute symptomatic hepatitis B among the children in the age group eligible to receive the vaccine [Zakaria et al., 2007].

At least eight HBV genotypes have been identified based on the divergence of 8% or more of the entire nucleotide sequence and most of the HBV genotypes have a distinct geographical distribution [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Accumulated evidences indicated the difference in the virological characteristics among different HBV genotypes, which is reflected by the difference in the clinical outcome of infection with hepatitis B according to the infecting genotype [Miyakawa and Mizokami, 2003; Schaefer, 2005; Ozasa et al., 2006; Sugiyama et al., 2006]. However, data regarding the specificity of the transmission routes of each genotype is still scarce globally and need to be clarified.

The prevalence of HBV ranges between 2% and 6% in Egypt with the predominance of infection with HBV genotype D [Zekri et al., 2007]. It is widely known that Egypt is one of the countries with highest prevalence rate of infection with HCV in the world [el-Zayadi et al., 1992; Arthur et al., 1993; el Gohary et al., 1995]. However, the burden of HBV related progressive liver disease including liver cirrhosis and HCC in Egypt is observable either single or in a dual infection with HCV [Abdel-Wahab et al., 2000; el-Zayadi et al., 2005].

This study aimed to evaluate the prevalence of infection with HBV within the families of chronic HBV carriers in north Eastern Egypt. In addition, the intra-familial mode of transmission of HBV genotype D was also examined in the current cohort by the molecular evolutionary analyses. The impact of the HBV immunization programme in protecting this high-risk group was also investigated.

PATIENTS AND METHODS

Patients

The present study was conducted between January 2008 and June 2008 at the Communicable Disease Research and Training Centre, in Suez city. The study protocol was approved by the ethics committees of the participating institution and an informed consent was obtained from the included subjects.

Chronic HBV carriers were defined as individuals whose serum samples tested positive for HBsAg for at least 6-months period. Patients who fulfilled the criteria of chronic HBV carriers and were first detected within their families, were defined as the index cases ($n = 55$). The index cases included 40 (72.7%) men and 15 (27.3%) women. Their mean age ($\pm$ SD) was 41 ± 10.7 years and all the index cases were negative for HBeAg.

A total of 230 household contacts of the index cases were included in the study and defined as family members group. Data regarding their family relationship to the index cases, age, and the HBV vaccination history have been obtained.

According to the kinship of the family members to the index case group, the family members included 139 offspring, 4 parents, 46 spouses, 15 siblings, and 26 defined as other relatives who are living in the same house with the index cases.

Serological Methods

Serum samples were collected from the index cases and family members groups.

The Serum samples were examined for HBsAg, anti-HBc, anti-HBs, and HBeAg by the chemiluminescence enzyme immunoassay with the commercial assay kits (Fujirebio, Inc., Tokyo, Japan). The examination of the serum samples for anti-HCV and HIV was conducted using commercial kits (Abbott Laboratories, Abbott Park, IL).

Molecular Evolutionary Analysis

The HBV/DNA was extracted from 200 μl of serum samples positive for HBsAg using the QIAamp DNA MiniKit (QIGEN, Inc., Hilden, Germany), and re-suspended in 100 μl of a storage buffer (provided by the kit manufacturer).

The entire preS2 and S regions of the HBV genome (799 nucleotides; nucleotide positions 34–833) were amplified using the primers set and the conditions described previously [Sugauchi et al., 2001].

The amplified products were sequenced using Prism Big Dye (Pekrin–Elmer Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer according to the manufacturer's protocol. The sequences were aligned together with the CLUSAL X software programme [Thompson et al., 1994].

The phylogenetic tree was constructed using the neighbor joining method with Tamura-Nei's distance correction model using the Online Hepatitis Virus database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. The Bootstrap values were determined on 1000 database resampling tests. The sequences of other HBV isolates used for the construction of the phylogenetic tree were retrieved from the DDBJ/EMBL/GenBank sequence database and were indicated in their accession numbers. The new nucleotide sequences data that were reported in this manuscript will appear in the DDBJ/EMBL/GenBank sequence database with accession numbers AB561825-AB561856.

Statistical Analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent *t*-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL). *P*-values (two-tailed) <0.05 were considered to be significant statistically.

RESULTS

The family member included 96 (41.7%) males and 134 females (58.3%). Their mean age (±SD) was 20.6 ± 14.6. The rate of seropositivity for HBsAg and anti-HBc was 12.2% (28/230) and 23% (53/230) of the family members group with no statistical significant difference between the males and females members.

Age Group Distribution of HBV Infection Within the Family Members Group

Figure 1 illustrates the HBsAg and anti-HBc prevalences among different age groups of the family members. The highest prevalence of HBsAg seropositive cases was observed in the age group, 21–30 years old; (10/43; 23.3%) followed by the age group, 0–10 years old; (11/68; 16.2%). No statistical significant difference was found in the HBsAg seropositive rates between these two age groups. The prevalence of HBsAg was 7.7% (5/65), 3.4% (1/29), and 4% (1/25) in the age groups; 11–20, 31–40, and ≥41 years old, respectively. The prevalence of anti-HBc seropositive cases was significantly increasing with the age and the highest rate was observed in the age group ≥41 years old. The prevalence of anti-HBc was 8.8% (6/68), 20% (13/65), 25.6% (11/43), 37.9% (11/29), and 48% (12/25) in the age groups; 0–10, 11–20, 21–30, 31–40, and ≥41 years old, respectively.

The HBsAg and anti-HBc seropositive rates were analyzed in the family members with respect to their relationship to the index cases (Fig. 2A). As overall, the HBsAg was positive in 6.5% (3/46) spouse of index cases, 10.8% (15/139) of the offspring, 25% (1/4) of the parents, and 40% (6/15) of the siblings (Fig. 2A).

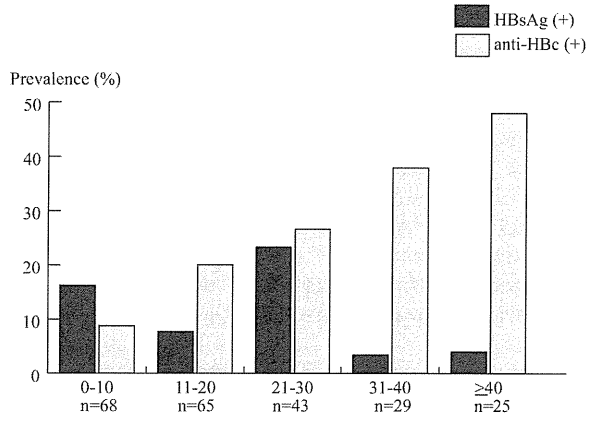

Fig. 1. Age distribution and HBV serological status among family members.

The prevalence of anti-HBc was 34.8% (16/46) in the spouse of index cases, 17.3% (24/139) in the offspring, 50% (2/4) in the parents, and 46.7% (7/15) in the siblings of the index cases (Fig. 2A).

Interestingly, the prevalence of HBsAg and anti-HBc was significantly higher in the family members of the females (19.2%, 15/78) than that of the males index cases (8.6%, 13/152; $P = 0.034$) and a trend of higher incidence of anti-HBc in the family members of the females than the males index cases (Fig. 2B). Among the offspring group, HBsAg and anti-HBc seropositive rates were significantly higher in the offspring of the females index cases (HBsAg; 23%, 11/47, anti-HBc; 29.8%, 14/47) cases than in the offspring of the males index cases (HBsAg; 4.3%, 4/92, antiHBc; 9.8%, 9/92), ($P = 0.001$, 0.003 respectively; Fig. 2C).

Further analysis was performed regarding the HBsAg seropositive rate in the offspring according to HBV infection of both one and two parents and the parent gender who is infected with HBV. Significantly higher rate of HBsAg positive (26.5%, 13/49) and anti-HBc positive (31.8%, 14/49) off spring were found in families where the mother was positive for HBsAg compared with families where the father was HBsAg positive (HBsAg; 4.7%, anti-HBc; 10.5%), ($P = 0.0006$, 0.009 respectively) (data not shown).

The seropositive rate of HBsAg was higher in the non-sexual contacts (13.6%, 25/184) of the index cases (parents, offspring, siblings, and cousins) than the sexual contacts (spouses; 6.5%, 3/46) with no statistical significant difference. Anti-HBc seropositive cases were observed more frequently in the sexual contacts (spouses) than in the non-sexual contacts (parents, offspring, siblings cousins) of the index cases. (Sexual vs. non-sexual contacts, 34.8% vs. 20.1%, $P = 0.049$) (data not shown).

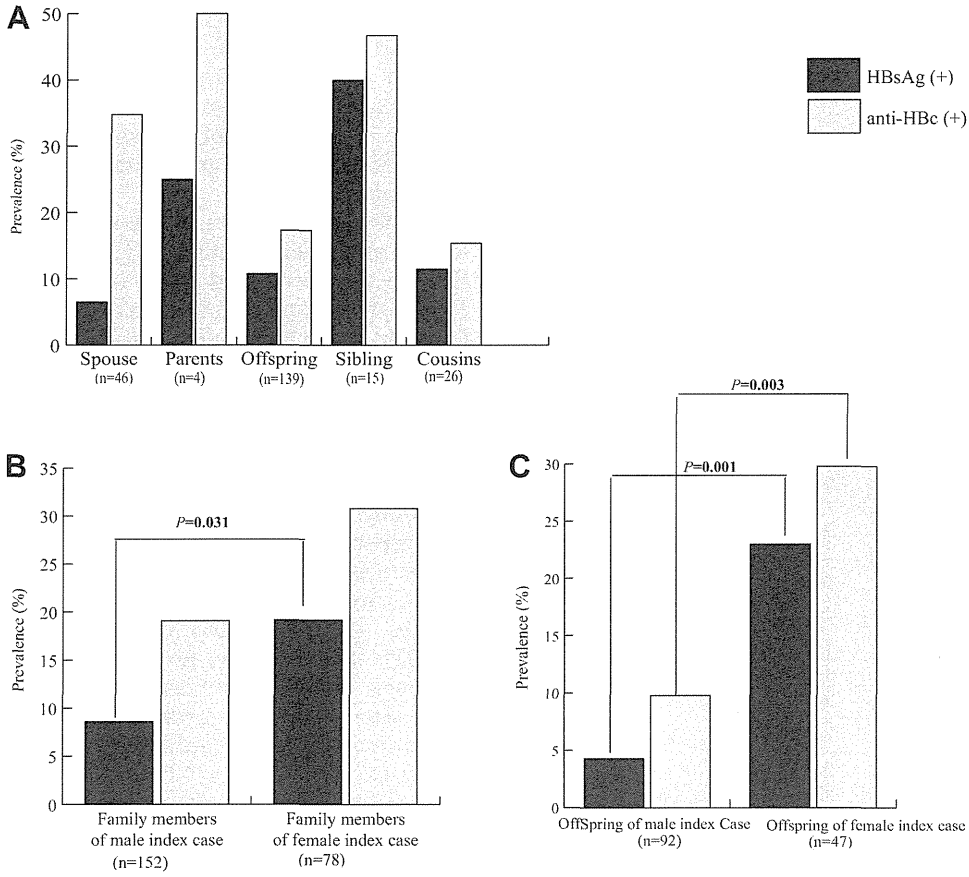

Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

Molecular Evolutionary Analysis and Transmission Pattern of Hepatitis B in the Family Members Group

Eighteen index cases out of 55 (32.7%) were found to have at least one family member positive for HBsAg. The age range of these index cases was 26–56 years and 50% (9/18) of them were male (Table I). Twenty-eight family members were found to be positive for HBsAg. The data regarding the degree of relativity of each family member infected with HBV to the index case, the age of the infected family member, and the vaccination status were indicated in Table I. The mean age (±SD) of the family members with active HBV infection was 17.8 ± 13.0 years old (Table I).

The HBV genomic region of 799-nt length and spanning PreS2 and S region was amplified in 44% (8/18) of the index cases and in 50% (14/28) of the family members infected with HBV. However, the target genomic region could be amplified and sequenced simultaneously in the index cases and their related family members in six subjects. These six subjects are defined in the present report, Table I and Figure 3 as F 3, F4, F19, F35, F37, and F 43 (Table I, Fig. 3).

To confirm the family clustering, a phylogentic tree was constructed by (1) the previous mentioned sequences (2) sequences isolated from the index cases whose family members were negative for HBsAg (3) HBV nucleotide sequences isolated from HBV chronic carriers residing in different districts in Egypt (North and South) either retrieved from the data base band or further included in the present study.

The phylogenetic analysis of the preS2 and S regions of the HBV genome revealed that the HBV isolates were of subgenotype D1 (Fig. 3). Using the phylogenetic analysis, in family 4 (F4), a high homology was detected between the HBV strains isolated from the grandmother together with her daughters and her grandchildren (Fig. 3). In the Family 35 and Family 43 (F35, and F43), the father and the child harbored very closely related HBV isolates and the phylogenetic analysis suggesting that the father may have been the source of infection for his child in Family 35 (F35) and Family 43 (F43). Similarly, very closely related HBV isolates were also detected in the

TABLE I. Descriptive Analysis of the Family Members Positive for the HbsAg

Subject	Relation (gender)	Age	HBV-vaccine[a]	PreS2 + S
F3	Index (F)	42		(+)
F3-1[b]	Daughter	13	Yes	(+)
F10	Index (F)	30		(−)
F10-1	Daughter	3	Yes	(+)
F11	Index (F)	33		(+)
F11-1	Daughter	8	Yes	(−)
F11-2	Cousin	10	Yes	(−)
F30	Index (F)	42		(−)
F30-1	Son	8	Yes	(−)
F34	Index (F)	30		(−)
F34-1	Son	7	Yes	(+)
F34-2	Son	9	Yes	(+)
F48	Index (F)	30		(−)
F48-1	Son	5	Yes	(−)
F35	Index (M)	29		(+)
F35-1[b]	Daughter	5	Yes	(+)
F39	Index (M)	33		(−)
F39-1	Daughter	5	Yes	(−)
F43	Index (M)	47		(+)
F43-1[b]	Daughter	12	Yes	(+)
F55	Index (M)	56		(+)
F55-1	Daughter	12	Yes	(−)
F37	Index (M)	45		(+)
F37-1[b]	Wife	26	Yes	(+)
F36	Index (M)	31		(−)
F36-1	Brother	26	No	(−)
F36-2	Brother	28	No	(−)
F36-3	Brother	22	No	(+)
F36-4	Mother	63	No	(+)
F4	Index (F)	54		(+)
F4-1	Daughter	35	No	(+)
F4-2	Daughter	20	No	(+)
F4-3	Grandchild	6	Yes	(+)
F4-4[b]	Grandchild	4	Yes	(+)
F19	Index (M)	29		(+)
F19-1[b]	Wife	27	No	(+)
F40	Index (M)	26		(−)
F40-1	Relative	24	No	(−)
F40-2	Relative	29	No	(−)
F41	Index (F)	53		(−)
F41-1	Daughter	23	No	(−)
F41-2	Daughter	17	No	(−)
F45	Index (M)	33		(+)
F45-1	Wife	27	No	(−)
F50	Index(F)	27		(−)
F50-1	Sister	25	No	(−)

[a]HBV vaccination history is provided for the family member.
[b]Index and family members who are positive simultaneously for the PreS2and S region.

man and his wife in Families 19 and 37 (F19 and F37) (Fig. 3). The molecular evolutionary analysis of the sequences isolated from the mother and her daughter in Family 3 (F3), yielded two separate but distinct groupings of the HBV isolates, suggesting that the presence of two different HBV viral isolates infecting the mother and her daughter (Fig. 3).

Serological Markers of HBV Infection in the Vaccinated and Unvaccinated Family Members

The family members group was subdivided into two subgroups according to the history of full regimen schedule of HBV vaccination as shown in Table II; (1) A group of vaccinated family members which includes a total of 142 subjects, who received the complete HBV vaccine regimen. (2) A group of unvaccinated family members, which included 88 subjects with no previous history or incomplete regimen of HBV vaccination.

The family members in the unvaccinated group were significantly older (mean ± SD; 32.5 ± 12.5 years old) than in the vaccinated group (mean ± SD; 13.3 ± 10.4, $P = 0.012$). No statistical significant difference was found in the male gender distribution between the two groups. The anti-HBs seropositive rate was significantly higher in the vaccinated group than the unvaccinated group [69.8% (99/142) vs. 33% (29/88), respectively, $P < 0.0001$] (Table II). The mean anti-HBs titre was significantly higher in the vaccinated than unvaccinated family members (70.1 ± 129.7 vs. 21.6 ± 51.7 mIU/ml, respectively $P < 0.0001$).

The prevalence of anti-HBc was significantly higher in the unvaccinated family members compared to vaccinated groups (37.5% vs. 14.1% respectively, $P < 0.0001$). Interestingly, no statistical significant difference was detected between the vaccinated and the unvaccinated groups regarding the prevalence of HBsAg [vaccinated vs. unvaccinated; 10.6% (15/142) vs. 14.8% (13/88), $P = 0.4$] (Table II). The HBV DNA was detected in 50% of family members positive for HBsAg with no statistical significant difference between the vaccinated (53%, 8/142) and unvaccinated groups (46.2%, 6/88) (Table II).

Mutations in the "a" determinant region. The available nucleotide sequences spanning the S gene of HBV isolated from the nine vaccinated and five unvaccinated members were translated into amino acid and aligned in correspondence to the reference sequences. The amino acid substitutions in the "a" determinant region that was reported to be associated with vaccine escape mutation were not detected. However, an amino acid substitution at the second loop of "a" determinant region (T143L) was clustered in the family subject F37 (F37 and F37-1) and found in one unvaccinated family member (F4-1). Another substitution was detected in the second loop of "a" determinant region (T140I) in an unvaccinated member (F36-1). P127A substitution in first loop of the "a" determinant region was clustered in the family 43(F43 and F43-1; Fig. 4).

DISCUSSION

The investigation of the intra-familial transmission in a particular region usually reveals valuable information about the routes of HBV spread in general and may help in exploring the HBV spread problem and local peculiarities. This study is the first one in Egypt done to explore the intra-familial spread of HBV infection and inclusively HBV genotype D transmission routes in Egypt. An evaluation of the impact of the universal HBV vaccination on the intra-familial transmission of HBV was also done.

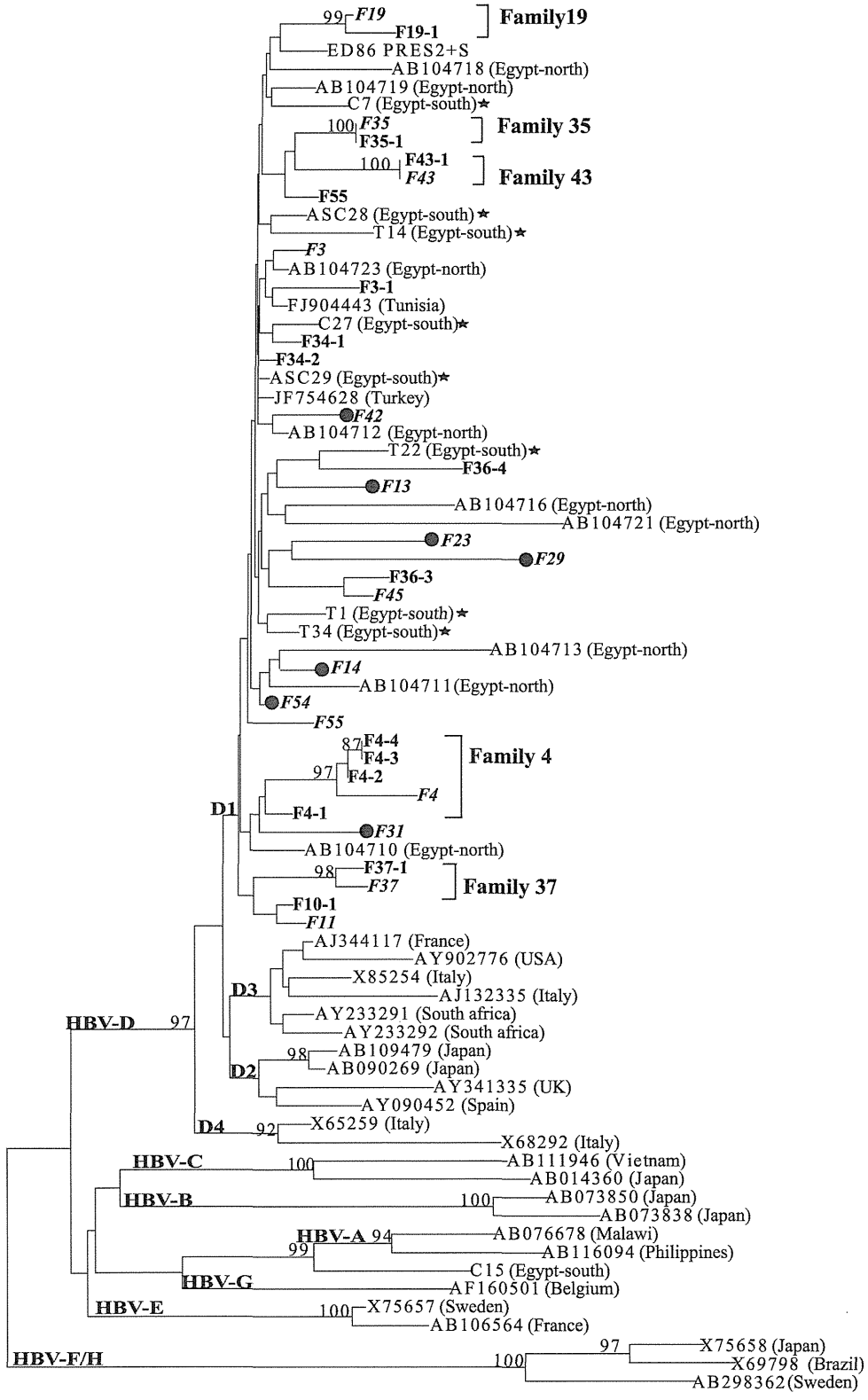

Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family members are indicated in italic bold and bold fonts respectively. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted in their accession numbers. Solid black rounds indicate sequences from index cases with family members negative for HBsAg. (★) Strains isolated from chronic hepatitis B carriers residing in Egypt south. The country origin of the reference sequences are indicated in brackets. HBV genotypes A–H are indicated in the cluster roots.

TABLE II. Comparison of Hepatitis B Serological Markers in Vaccinated Versus Unvaccinated Family Members Group

	Total (N = 230)	Vaccinated group (N = 142)	Unvaccinated group (N = 88)	*P*-value
Age[a]	20.6 ± 14.6	13.3 ± 10.4	32.5 ± 51.7	<0.0001
Gender (Male)[b]	96(41.7)	64 (45.1)	32 (36.4)	NS
Anti-HBc (+)[b]	53 (23)	20 (14.1)	33 (37.5)	<0.0001
HBsAg (+)[b]	28 (12.2)	15 (10.6)	13 (14.8)	NS
Anti-HBs (+)[b]	128 (55.7)	99 (69.8)	29(33)	<0.0001
HBV-DNA (+)[b]	14 (50)	8 (53.3)	6 (46.2)	NS

[a]Mean ± SD.
[b]N (%).

In the present study, 12.1% of the family members were infected with HBV. This incidence was much higher than that detected among the blood donors (1.4%) resident in the same area in Egypt (data not shown). Clustering of the HBV infection within the families has been described in nearby countries located within the same zone of the HBV endemicity but with different incidences; 30% in Turkey, 15.8% in Greece, and 11.9% in Iran [Alizadeh et al., 2005; Zervou et al., 2005; Ucmak et al., 2007]. An important risk factor was found to be implicated in acquiring the infection among the family was the presence of female infected with HBV. Furthermore, the higher incidence of HBsAg positive rate among the offspring of the females' index cases than that of males index cases illustrates clearly the role of the mother in the transmission of HBV. Similarly, Salkic et al. [2007] reported the same observation in his study from Bosnia [Salkic et al., 2007]. However, in Taiwan no significant difference was found in the HBsAg positivity among the offspring of the two groups, suggesting the importance of the paternal as well as the maternal transmission for the HBV intra-familial spread in Taiwan [Lin et al., 2005].

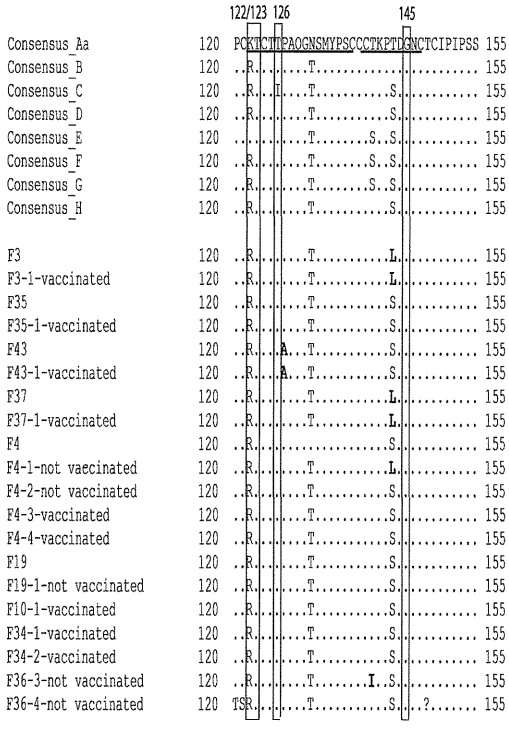

Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank database. Dots in alignment indicate identity of amino acids to the consensus sequence of genotype Aa/A1. First and second loop positions are underlined in the consensus sequence of the genotype Aa/A1 and positions of previously reported vaccine escape mutants are indicated in numbers and included in boxes.

Despite being a tedious and labor-intensive method, sequencing of the viral genomes isolated from different individuals, with the subsequent homology comparison and the phylogenetic analysis remains the golden approach for demonstrating the HBV transmission in a given population [Dumpis et al., 2001; Zampino et al., 2002; Tajiri et al., 2007].

The full length HBV sequence analysis is the gold standard for this purpose but remains a cost approach [Datta et al., 2007]. Highly variable HBV genomic region is recommended by some investigators to study the transmission event. Variability of the genomic region is affected by several factors one of which is the clinical characteristics of the studied cohort [Wu et al., 2005]. PreC/C region exhibit high variability in the cases of acute or fulminant hepatitis and thus analysis of this region is preferable for investigating the chain of recent/nosocomial fulminant cases [Bracho et al., 2006; Ozasa et al., 2006]. However, a high S gene variability is documented among the chronic hepatitis B carriers and their families, thus investigating the genotype, subgenotype, subtypes, and mutations by the sequence analysis of the S gene with further analysis by testing the constructed phylogenetic tree with the bootstrap resampling maximum-likelihood test, may provide enough confidence to prove the transmission event in the case of chronic HBV carriers [Thakur et al., 2003]. Hence, in the present study, the phylogenetic analysis of the HBV nucleotide sequences spanning the entire preS2 and S HBV genomic regions and isolated from chronic hepatitis B carriers which include index cases and their family members revealed the infection with HBV genotype D which coincides with the previous