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

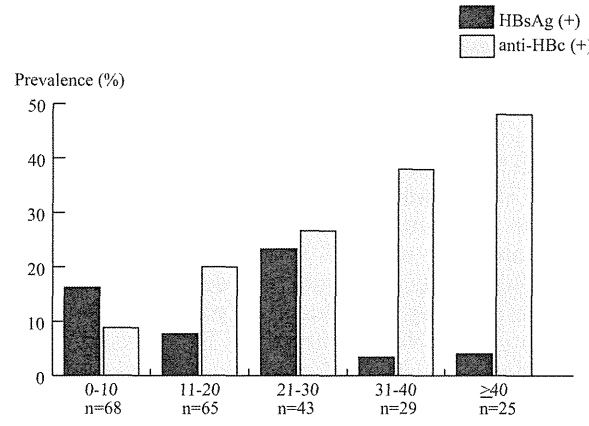

Fig. 1. Age distribution and HBV serological status among family members.

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).

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).

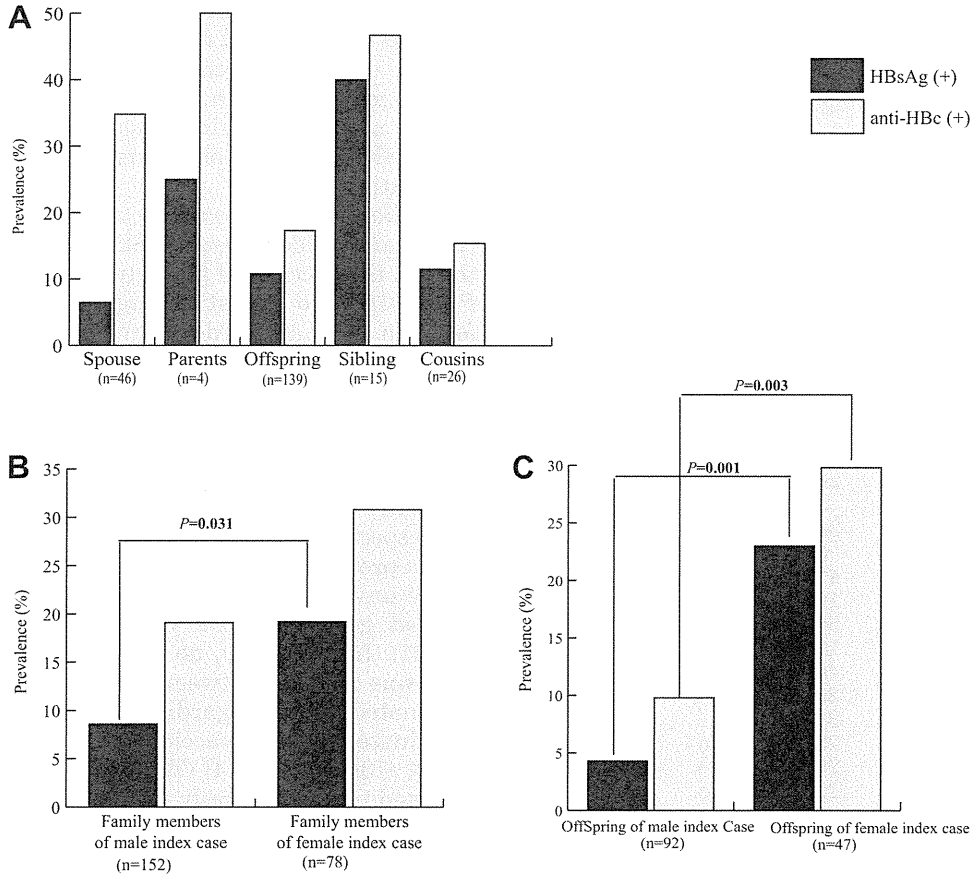

Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

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.

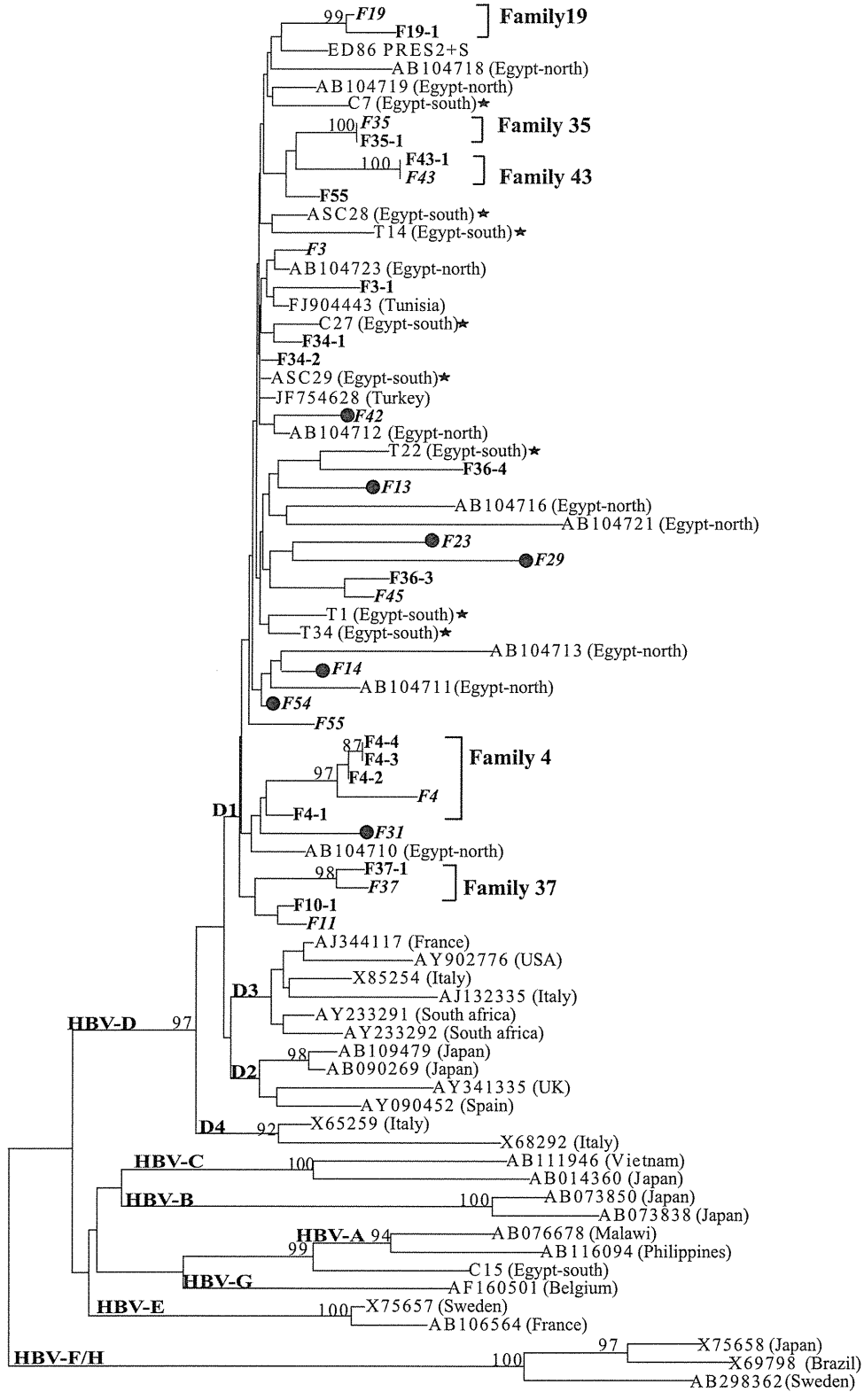

Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family 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].

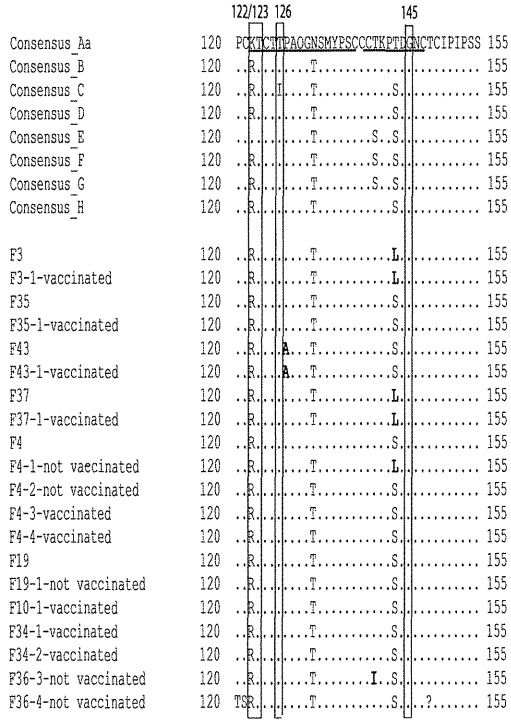

Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank 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

data regarding the predominance of infection with HBV genotype D in Egypt [Saudy et al., 2003]. In addition, the phylogenetic analysis documented the presence of three different patterns of HBV genotype D transmission within the families in Egypt; maternal transmission (from mother to child as in the family 4), paternal transmission (from father to child as in family 35 and family 43) and spousal transmission (between spouses as in family 19 and family 37). This was different from the transmission pattern characteristics of genotype D in Uzbekistan where the horizontal transmission was the predominant route of infection with HBV genotype D within a family [Avazova et al., 2008].

The Data regarding the difference of transmission routes of HBV infection between different genotypes are controversial and scarce. Based on the findings that the patients infected with HBV genotype C may exhibit delayed HBeAg seroconversion decades later than the patients infected with other genotypes, Livingston et al. [2007] speculated that genotype C is the most responsible for the perinatal transmission and that the other genotypes (A, B, D, and F) are mainly transmitted horizontally [Livingston et al., 2007]. A recent study has shown a different data through exploring that both genotypes B and C can be transmitted by maternal and horizontal routes [Wen et al., 2011]. Whether different HBV genotypes have different transmission routes remains a question, which needs further global studies to clarify this interesting and important issue.

In an attempt to evaluate the influence of the universal vaccination on the intra-familial HBV infection, it was surprising to find a high prevalence rate of HBsAg among the vaccinated members with no significant difference when compared to the unvaccinated group. In an agreement with the present data, El Sherbini et al. [2006] reported the unchangeable prevalence of HBsAg among the vaccinated school children across a decade despite the significant decrease of the anti-HBc rate [El Sherbini et al., 2006]. The possible explanation for this vaccine failure is the acquiring of the HBV infection in the lag period between the birth and the time of receiving the first HBV vaccine dose at the age of 2 months. Supporting our explanation is the recent data coming from Taiwan where a different HBV infection prophylactic strategy is applied by administrating the first dose of the HBV vaccine at birth with the administration of the hepatitis B immunoglobulin to the infants born to the HBeAg positive mother within 24 hr after birth. The recent study has clearly demonstrated that the current HBV prophylactic strategy in Taiwan was capable of reducing the intra-familial HBV transmission and reducing the overall HBsAg positive rate among the infants [Mu et al., 2011]. In Japan, the extension of the active and passive immunization to the babies born to HBeAg negative mother had greatly reduced the HBsAg prevalence to 0.2% of blood donors younger than 19 years old [Noto et al., 2003; Matsuura et al., 2009]. The present study recommends the changing of the current HBV prophylactic policy in Egypt. It would be needed to provide the first dose of the HBV vaccine at birth together with screening for HBV infection markers prenatally and administration of the HBIG to the infants born from HBeAg-positive mothers. The documented role of the HBV spousal transmission in the present study by the phylogenetic analysis (Family 19 and Family 37), coincides with the recent data conducted in Egypt that the first sexual contact with an infected spouse was a significant risk factor for infection with HBV among females and may further emphasize the importance of the premarital screening for HBV in Egypt [Paez Jimenez et al., 2009]. Investigating the " a" determinant region of viral isolates retrieved from the vaccinated members infected with HBV provides no evidence of breakthrough infection by previously reported vaccine escape mutant virus [Carman et al., 1990].

In conclusion, the present study has clearly explored the role of the HBV intra-familial transmission and spread in north Eastern Egypt. Three patterns of HBV transmission were determined in the current cohort infected with HBV genotype D; maternal, paternal, and spousal. The present study recommends the change of the current prophylactic policy against the HBV infection in Egypt by including the first dose of HBV vaccine at birth, screening of pregnant women for HBsAg and the administration of HBIG to the infants born from HBeAg positive mothers within 24 hr after birth. Further studies are needed globally to determine the transmission patterns of different HBV genotypes and locally in different districts in Egypt to explore the impact of familial transmission in HBV infection in Egypt.

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Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

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ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

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into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV precore/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of precore/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the *S* gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

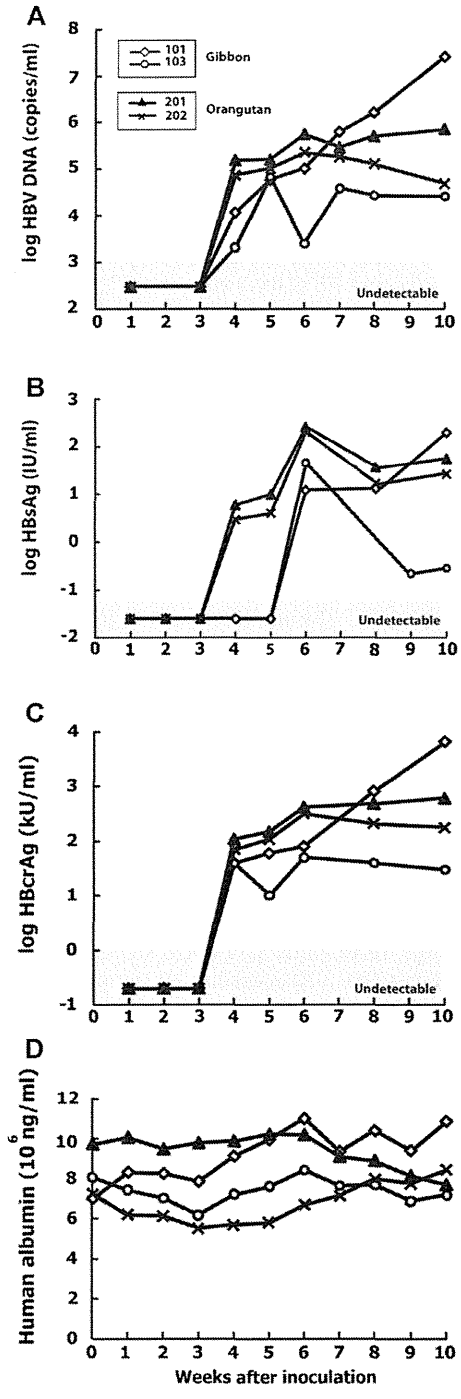

Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR (<10^3 copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

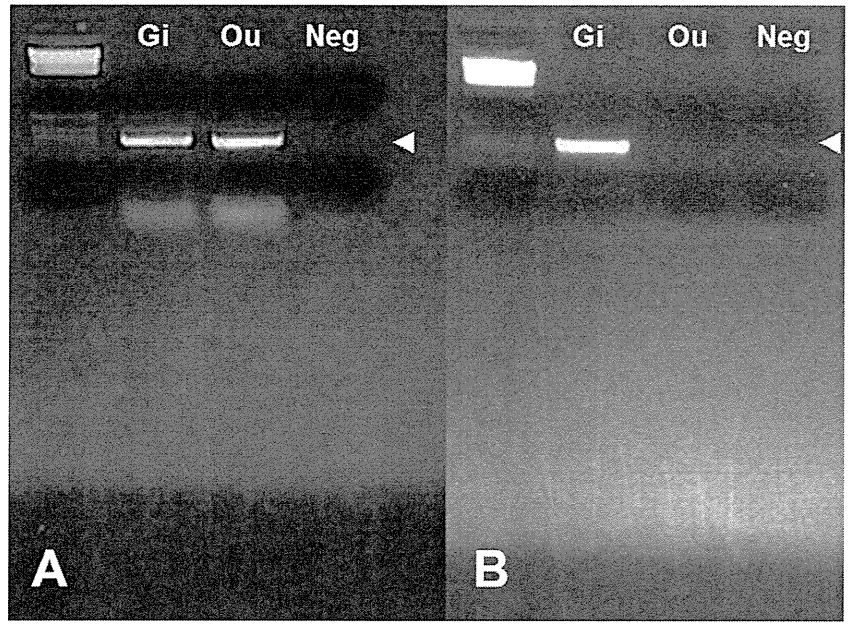

Fig. 2. CccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

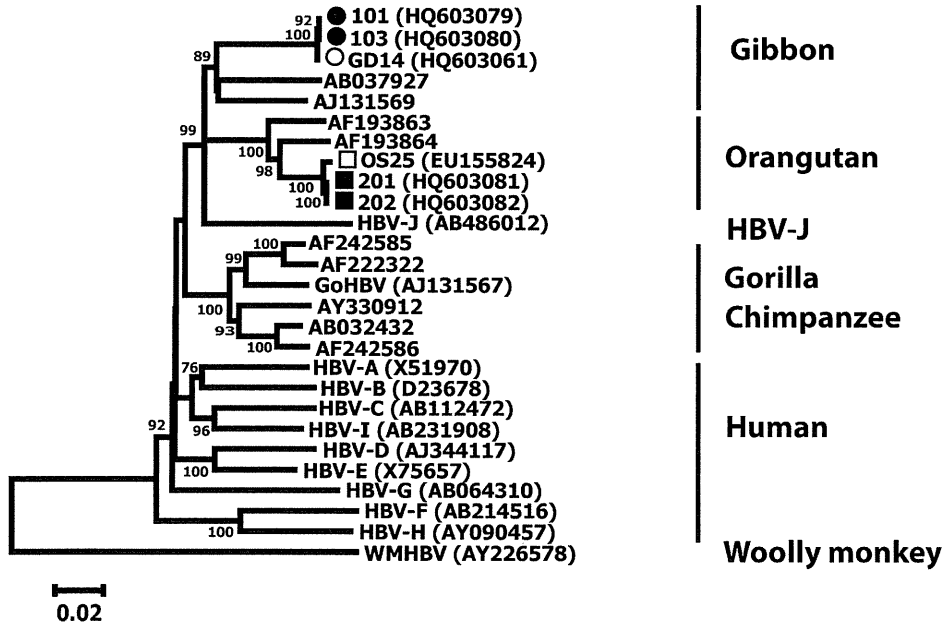

Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

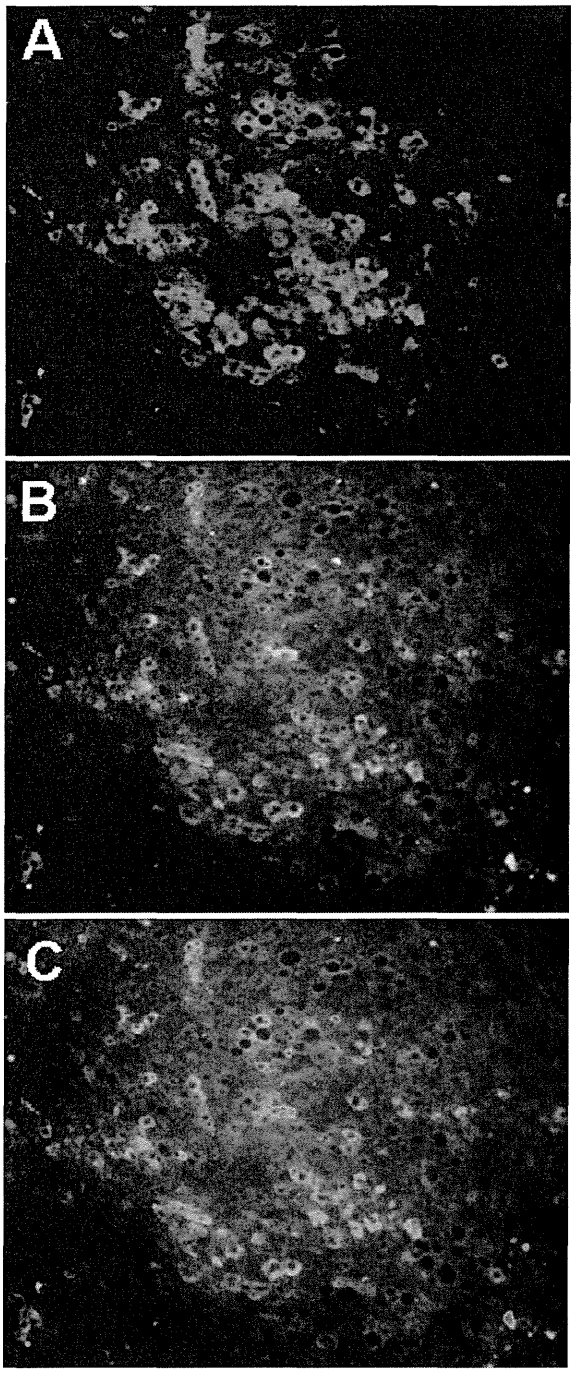

Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

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A computational procedure for identifying master regulator candidates: a case study on diabetes progression in Goto-Kakizaki rats

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Abstract

Background: We have recently identified a number of active regulatory networks involved in diabetes progression in Goto-Kakizaki (GK) rats by network screening. The networks were quite consistent with the previous knowledge of the regulatory relationships between transcription factors (TFs) and their regulated genes. To study the underlying molecular mechanisms directly related to phenotype changes, such as diseases, we also previously developed a computational procedure for identifying transcriptional master regulators (MRs) in conjunction with network screening and network inference, by effectively perturbing the phenotype states.

Results: In this work, we further improved our previous method for identifying MR candidates, by listing them in a more reliable manner, and applied the method to reveal the MR candidates for diabetes progression in GK rats from the active networks. Specifically, the active TF-gene pairs for different time periods in GK rats were first extracted from the networks by network screening. Another set of active TF-gene pairs was selected by network inference, by considering the gene expression signatures for those periods between GK and Wistar-Kyoto (WKY) rats. The TF-gene pairs extracted by the two methods were then further selected, from the viewpoints of the emergence specificity of TF in GK rats and the regulated-gene coverage of TF in the expression signature. Finally, we narrowed all of the genes down to only 5 TFs (Etv4, Fus, Nr2f1, Sp2, and Tcfap2b) as the candidates of MRs, with 54 regulated genes, by merging the selected TF-gene pairs.

Conclusions: The present method has successfully identified biologically plausible MR candidates, including the TFs related to diabetes in previous reports. Although the experimental verifications of the candidates and the present procedure are beyond the scope of this study, we narrowed down the candidates to 5 TFs, which can be used to perform the verification experiments relatively easily. The numerical results showed that our computational method is an efficient way to detect the key molecules responsible for biological phenomena.

Background

Recent developments in genome-wide computational analyses have successfully identified causal interactions [1], and showed promise in the identification of dysregulated genes in development and tumor progression pathways [2]. For example, a computational analysis procedure was applied to identify the MRs causally linked to the activation of a specific gene set, mesenchymal gene expression signature (MGES), in human malignant glioma [3]. Indeed, 53 TFs were obtained by the

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ARACNe algorithm and the MGES enrichment test, and among them, the top 6 TFs with the largest fraction of MGES genes were experimentally controlled, as the MR candidates. Finally, 2 of the top 6 TFs, STAT3 and CEBPB, were experimentally verified as MRs of mesenchymal transformation. Unfortunately, the computational method employed in the previous work was unsophisticated and required further improvement. For example, it is unclear why the method selected the top 6 TFs from 53 TFs, rather than 5 or 7 TFs. Although the coverage of the TFs for the MGES genes was carefully considered, there was no rational criterion for the final selection of the MR candidates. Furthermore, ARACNe considers the relationships between three genes for selecting MR candidates. However, there are some well known mathematical techniques that consider multiple relationships and have been applied to infer regulatory networks [4].

We previously reported 39 candidates of active networks for diabetes progression in the Goto-Kakizaki rat (GK), which were identified by network screening, in comparison with the Wistar-Kyoto (WKY) rat [5]. Network screening is a procedure to extract the regulatory networks activated under particular conditions, based on the known regulatory networks [5-7]. The candidates were characterized by the known biological pathways that were consistent with the previous knowledge about diabetes. Unfortunately, the plausibility of the active networks could not be verified experimentally. This was partly because the results were presented in a metaphysical form, and as the biological pathway, instead of the list of concrete target genes. Actually, the active networks were composed of many genes that were not amenable to experimental verification.

To overcome these problems, we recently developed a procedure for identifying MR candidates, by a combination of network screening and network inference [8]. The network screening strongly depends on the previous knowledge of the regulatory networks. To compensate for the limitations of network screening, we introduced a network inference method, which is a version of a path consistency algorithm (PC-A) [9] or a modified PC-A [10,11] that applies PC-A to biological data with high redundancy. The performance of our procedure was tested for MRs in human malignant glioma, using the same data set in ref. [3], and worked well [8]. In total, 22 TFs and 27 TFs were detected by the network screening and the network inference, respectively, and 3 TFs overlapped between them. Interestingly, 2 of the 3 TFs were STAT3 and CEBPB, which were verified experimentally as the master regulators in the previous report [3].

In this paper, we sought to identify the candidates of master regulators for diabetes progression, using the spontaneous diabetic GK rat model. Based on the networks specific to diabetes progression identified in our previous report [5] and the networks inferred by the modified PC-A, we intended to narrow down the candidate molecules responsible for diabetes further, by identifying the master regulators that play a central role in diabetes progression in GK rats. Furthermore, we improved our previous method [8] to narrow down the candidates in a more reliable manner, by considering the coverage of a TF for its regulated genes in a statistical manner, in addition to the specificity of the TF to the target biological phenomena. As expected from the previous case of the computational identification of MRs in a human brain tumor [8] and the present improvements, we identified a limited set of reliable MR candidates, and thus provided information for further experimental design for candidate verification.

Results

Overview of our computational procedure

In our computational procedure, we identified MR candidates by two approaches, which are schematically shown in Figure 1. One is a knowledge-based approach, which estimates the consistency of the network structures among the known networks with the measured data (named "network screening") [5-7]. The other is a data-driven inference approach, which estimates the conditional independency between the genes by calculating the partial correlation coefficients (named "modified path consistency algorithm") [10,11]. In both cases, we further selected the MR candidates by considering the enrichment of the gene expression signature in the networks. Finally, we refined the candidates by considering the TF specificity and the regulated-gene coverage. The details are described in the Methods.

MR candidates detected by network screening

In our previous study [5], we used network screening to identify 39 networks for GK and WKY rats in three growth periods: 4w, from 8w to 12w, and from 16w to 20w, among the 1,760 networks in the reference network set. Based on these results, we further selected the MR candidates. From the 39 networks, in total, we extracted 568 binary relationships of TFs and their regulated genes, which were specifically found in the three periods for GK and WKY rats, under the condition that the gene expression shows a difference with a false discovery rate (FDR) of less than 0.05, between the two strains of rats for each period (see the details in the Methods). The numbers of genes specifically found in each period in GK and WKY rats are as follows: 54 genes at the period of 4w in GK; 199 at 8w and 12w in GK; 56 at 16w and 20w in GK; 95 at the period of 4w in WKY; 125 at 8w and 12w in WKY; and 39 at 16w and 20w in WKY. Note that some TF-

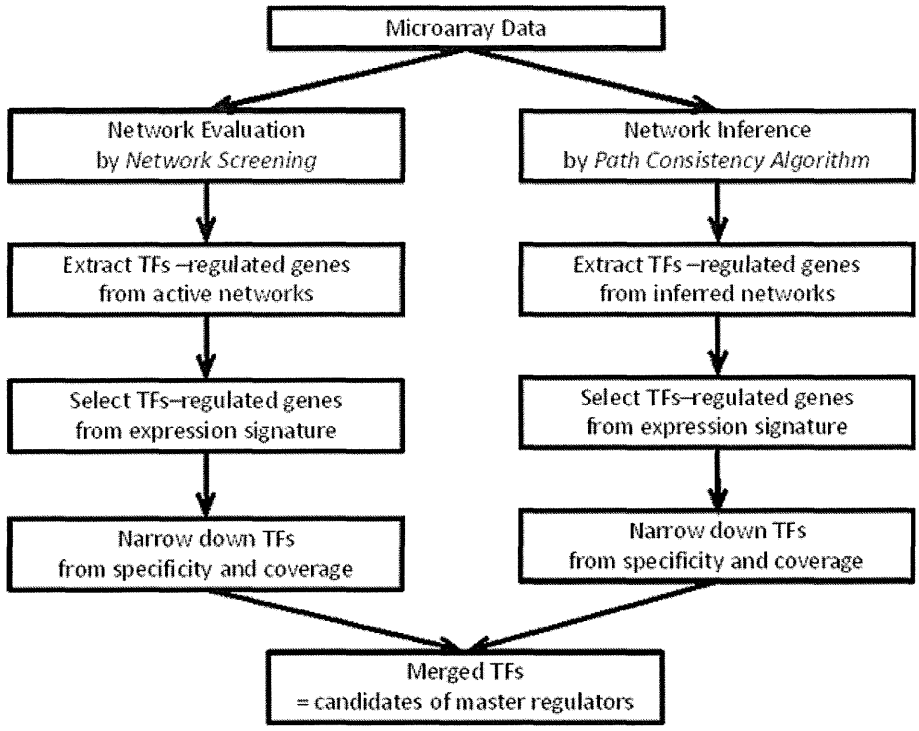

Figure 1 Workflow of the MR identification procedure.

gene relationships emerged iteratively for different periods in GK and WKY rats.

Among the TF-gene relationships selected above, the TFs were narrowed down in two ways. First, the TF-gene relationships were selected by the specificity, which means that the TFs emerge only in GK, but not in WKY. As a result, we found a total of 21 TFs, as shown in Table 1. Second, the TF-gene relationships were selected by the coverage, which means how many genes the TFs regulate, among the genes in the expression signature. The TFs thus selected were sorted according to the coverage, and then the MR candidates were further selected by a statistical test (see the Methods) for each period in GK and WKY listed in Table 2. As seen in the table, most of the TFs emerged in both GK and WKY, in terms of the coverage selection. We finally found 3 TFs (EGR1, NRF1, and TCFAP2A) among the genes by the initial selection in Table 2.

MR candidates inferred by the modified path consistency algorithm

We first inferred six networks of all genes on the microarray for each of the three periods in GK and WKY rats, by the modified path consistency algorithm [10,11], and then the TF-gene relationships were extracted from each network. After the extraction, only the relationships that included the genes with a significant difference between GK and WKY rats were further selected for the 6 sets of relationships.

Using the same procedure as that described in the preceding subsection, the TFs were narrowed down. First, we chose the relationships in terms of the gene-emergence specificity. As a result, 108 TFs were identified as the MR candidates in Table 3. The number of candidates seems to be large, even in comparison with the candidate number, 27 TFs, in the previous case of the brain tumor [3]. While one network was considered to identify the candidates in the previous paper, three networks for the three periods in GK rats were surveyed to select the candidates in the present study. Thus, the number of TFs extracted from one network, 36 TFs on average, is similar to that in the previous study. Second, the TF-gene relationships were selected by the coverage. We chose the TF-gene relationships by a statistical test (see the details in the Methods) for each period in GK and WKY, as shown in Table 4. In contrast to the

Table 1 TFs identified by network screening in terms of specificity.

Ar, Bcl6, Brca1, Etv4, Fus, Gli1, Hes1, Hnf1b, Hnrnpk, Klf10, Klf4, Lyl1, Mef2c, Nfia, Nr2f1, Nrl, Pax6, Sp2, Sp4, Tcfap2b, Wt1

All of the gene names are cited from the Rat Genome Database http://rgd.mcw.edu/ in all of the tables, the figures, and the text.

Table 2 TFs identified by network screening in terms of coverage.

4w				8w_12w				16w_20w			
GK		WKY		GK		WKY		GK		WKY	
TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes
SP1	10	**SP1**	19	**SP1**	39	**SP1**	18	**SP1**	12	**SP1**	5
		SP3	8	**SP3**	11	HNF4A	6	**SP3**	3	**FOXO3**	3
		TP53	4	**TP53**	11	**FOXO3**	4				
				EGR1	6						
				NRF1	6						
				TCFAP2A	5						

TFs found in both GK and WKY are indicated by bold letters.

coverage selection in network screening, only a few TFs emerged in both GK and WKY. Indeed, among the 44 TFs in Table 4, only two TFs (Tbpl1 and Cbfb) emerged in both GK and WKY. Finally, we found 42 TFs as MR candidates.

MR selection by comparison of the TF sets detected by the two methods

We obtained the final MR candidates by selecting the overlapped TFs detected by the two methods in terms of two criteria (Tables 1, 2, 3, 4), as shown in Table 5. Indeed, 21 TFs detected by network screening in terms of specificity overlapped with only 4 TFs (Etv4, Nr2f1, Sp2, and Tcfap2b) and 2 TFs (Fus and Sp2) by the modified path consistency algorithm by two criteria, respectively. In contrast, 3 TFs detected by network screening in terms of coverage showed no overlapped TFs by the path consistency algorithm by two criteria. This difference might reflect the restriction of the known TF-gene relationships in network screening.

As a result, we merged the MR candidates identified by the two methods, and 5 TFs were finally identified as the candidates of MRs for diabetes progression in GK rats. Note that Sp2 emerged in both the 4 TFs and 2 TFs. The 5 final MR candidates with their regulated genes, in total 54 genes, are listed in Table 6.

Discussion

In this study, we have identified the candidates of master regulators based on our previous study [5], by using an improved method for their identification [8]. The MR candidates were extracted from the active networks of many genes characterized by biological pathways, as the feasible gene candidates for experimental verification. From the methodological aspect, the method was improved by considering the coverage of TFs in a statistical manner, in addition to the specificity that was considered in the previous method. Although the experiments are beyond the scope of the present study, we consider experimental verification studies of the present candidates as our future research topic. Our study clearly illustrated a rational way to narrow down the genes of MR candidates, and is fundamentally different from metaphysical presentations, such as biological pathways or large network forms.

Our study intended to identify the MR candidates, which are those genes with large impacts on phenotype changes, in a biological sense [3]. Here, we logically identified MR candidates by the specificity of the TF appearance and the coverage of the regulated genes to the gene expression signature in the networks of GK and WKY rats. Apart from a biological sense, we further investigated the meaning of "master" from the viewpoint of the network structure. To do this, we revealed the hierarchical structures of the 8w-12w and 16w-20w networks by network screening, using a vertex sort algorithm [12], and allocated the present 5 TFs into the hierarchical structures (Figure 2A). As seen in the figures, all 5 TFs were allocated into the highest level. Indeed, Nr2f1 in the 8w-12w network and Tcfap2b in the 16w-20w network were definitely allocated into the highest level of the hierarchical structures. In addition, the remaining TFs were allocated into the levels including the highest and middle levels, but not into the lowest level. Furthermore, we investigated the hierarchical structure by another method, the BFS-level algorithm

Table 3 TFs identified by network inference in terms of specificity.

Alx1, Arnt, Cebpg, Ddit3, Dlx5, Dmrt2, Dnmt1, Dr1, Ebf1, Elf5, Elk3, Elk4, Erg, Etv4, Etv5, Fev, Fosl1, Foxe1, Foxg1, Foxo3, Foxp4, Gabpb1l, Gfi1, Gtf2a1, Gtf2b, Gtf2e1, Gzf1, Hcfc1, Hey1, Hhex, Hoxb3, Hoxb7, Ilf3, Irx2, Kcnip4, Klf1, Klf15, Klf3, Klf5, Klf7, Ldb2, LOC680117, Mafk, Meis2, Mnat1, Msx1, Msx2, Mybl2, Myc, Myocd, Myod1, Mzf1, Neurod2, Nfix, Nfx1, Nkx6-1, Notch1, Nr1h4, Nr2f1, Nr4a1, Nr5a1, Pax8, Pbx2, Phox2a, Pitx1, Pitx3, Pou2f3, Pou3f1, Ppard, Pparg, Ppargc1a, Rbl1, RGD1566107, Rreb1, Runx1, Shh, Six5, Six6, Skp2, Sox10, Sox11, Sp1, Sp2, Spdef, Srebf1, Ss18l1, Stat5a, Stat5b, Taf2, Tbx18, Tbx2, Tcf12, Tcfap2b, Tead1, Tfdp2, Tfec, Tmf1, Tp53bp1, Twist1, Vdr, Zbtb5, Zfhx3, Zfp191, Zfp238, Zfp423, Zfp444, Zhx1, Zic1

Table 4 TFs identified by network inference in terms of coverage.

4w				8w_12w				16w_20w			
GK		WKY		GK		WKY		GK		WKY	
TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes	TF	No. of regulated genes
Arntl	31	Max	10	Lhx5	24	Ywhae	18	Fus	10	Foxq1	32
Lhx2	22	Otx2	10	Etv1	23	Pfdn5	13	Smad5	10	Hoxa1	16
Sp2	18	Daxx	9	Ctnnb1	8	Atf1	11	Nfx1	9	Rbl2	16
Gabpa	13	Sim1	9	Rpa3	8	Cdk9	11	Hsf1	8	Zic2	12
Xpa	4	Tcf21	8	Zfp105	8	Hmgb2	11	Tlx3	8	Rorc	8
Foxs1	3	Gata5	7	Foxo3	7	Sfpq	9	Tp53	8	Tcfap4	6
		Tcfap2c	7	Hoxc5	6	Zfp281	9	Foxs1	7	Pttg1	5
		Meis3	5	Litaf	6	Cdk7	8	LOC679869	7	Ncoa3	4
		Rorc	5	Nr2f2	6	Ets2	8	**Cbfb**	6	Ccnh	3
		Snapc1	5	Foxo1	5	Hoxa1	8	Ctcf	6	Hif1a	3
		Zic2	5	Msx1	5	Nfe2l2	8	Glis2	6	Junb	3
		Meis1	4	Myocd	5	Nfil3	8	Irf7	6	Kcnip1	3
		Pou2af1	4	Pbx1	5	Six4	8	Nfkbib	6	Mtf1	3
		Srf	4	**Tbpl1**	5	Cux2	7	Nr1i2	6	Zfp148	3
		Stox2	4	Vdr	5	Mafg	7	Hdac1	5		
		Tcfcp2l1	4	Hltf	4	Nfkbia	7	Rfx5	5		
		Gtf2h2	3	Htt	4	Pgr	7	Tle1	5		
		Zfx	3	LOC680117	4	Ppp1r13b	7	Xpa	5		
				Mbd1	4	**Tbpl1**	7				
				Parp1	4	**Cbfb**	6				
				Rreb1	4	Ezh2	6				
				Smarcc1	4	Hbp1	6				
						Junb	6				
						Taf13	6				
						Tef	6				

TFs found in both GK and WKY are indicated by bold letters.

[13]. As shown in Figure 2B, the positions of the MR candidates are similar to those in Figure 2A. Indeed, previous hierarchical analyses of the regulatory networks by the BSF method in *Escherichia coli* and *Saccharomyces cerevisiae* suggested that the MRs were in the middle of the hierarchy [13]. In general, the vertex sort algorithm reports a linear ordering of nodes that contains all feasible solutions, while the BSF-level algorithm reports just a single solution, as shown in Figures 2A and 2B. Subsequently, unlike the BFS-level algorithm, the ordering in the vertex sort algorithm permits nodes to span an entire interval of possible positions with any feasible ordering. Despite this difference in the computational algorithms, the 5 TFs showed the common property as MRs. At any rate, although the verification experiments remain to be performed for the justification of the MRs in a biological sense, the 5 TFs may be regarded as the plausible MR candidates from the viewpoint of network structure.

Table 5 Summary of TFs identified by the two methods, in terms of specificity and coverage.

		path consistency algorithm	
		specificity (108)	coverage (42)
network screening	specificity (21)	4	2
	coverage (3)	0	0

A preliminary survey revealed that all 5 of the TFs have no reported causal relationship to diabetes. The 5 TFs are sequence-specific DNA-binding proteins, and they function as both transcriptional activators and repressors of large numbers of genes that are closely related to the cell cycle and tumorigenesis. Notably, the relationships of ETS translocation variant 4 (Etv4) and transcription factor AP-2 beta (Tcfap2b) to adipogenesis, which is strongly related to diabetes, have been reported, together with their association with the other pathways [14,15]. Nuclear Receptor subfamily 2, group F, member 1 (NR2F1) is a member of the steroid hormone receptor family, and has been shown to interact with estrogen receptor alpha (ESR1) [16]. There is a gender difference in the incidence of type 2 diabetes,

Table 6 Candidates of MRs and their regulated genes for diabetes progression in GK rat.

TF	Regulated genes						No. of genes	
Etv4	Mcm10	**Erbb2**	**Mmp7**	**Nid1**	Plau	Ptgs2	6	
Fus	Mcpt8l2	Mcpt9	**Paics**	**Ppat**	Ugt1a1	Ugt1a2	12	54
	Ugt1a3	Ugt1a5	Ugt1a6	Ugt1a7c	Ugt1a8	Ugt1a9		
Nr2f1	Alox5	**Cpt1b**	Cyp11b2	**Tf**	Ugt1a3	Ugt1a5	6	
Sp2	**Capns1**	**Irs2**	LOC685183	LOC685226	LOC685291	LOC685759	24	
	LOC688519	LOC688603	LOC689083	LOC689312	LOC689338	LOC689690		
	LOC689999	LOC690179	LOC690328	LOC690379	LOC690577	LOC691712		
	LOC691735	LOC691754	Papss2	Vom2r45	Vom2r46	Vom2r47		
Tcfap2b	Aqp1	Egfr	Krt14	Ptgds	Sod2	Tgm1	6	

The genes in bold characters are included in known TF-gene relationships detected by network screening.

which is largely due to the role of the sex hormone estrogen. The Sp family proteins, containing the conserved DNA-binding domain, are localized primarily within subnuclear foci associated with the nuclear matrix. Recent unpublished data from our lab have shown that another Sp family member, Sp1, has a major impact on the insulin signaling pathway. The Sp2 transcription factor interacts with E2F1, which mediates both cell proliferation and p53-dependent/independent apoptosis [17]. The recently discovered close relationships between diabetes and tumors in terms of these TFs are quite likely to play a crucial role in the control of diabetes. RNA-binding protein (FUS) is able to bind DNA, RNA and protein [18]. The interactions between the FUS recognition sites and Tcfap2, GCF, and Sp1 were identified recently. Thus, although direct evidence was not found in the previous knowledge, the 5 TFs are expected to be MR candidates, in consideration of the circumstantial evidence of their relationships to diseases, the hierarchical analysis of the 5 TFs, and the successful discovery of new MRs in brain tumor, by the previous version of the procedure. Actually, our current information in terms of important diabetes-related genes includes mostly functional proteins, located at the lowest level of our hierarchical structure, while the MR is deeply hidden and therefore must be revealed by systems biology methods. Thus, in addition to analyses of their regulated genes, some experimental verification of the MR candidates may be desirable to further examine

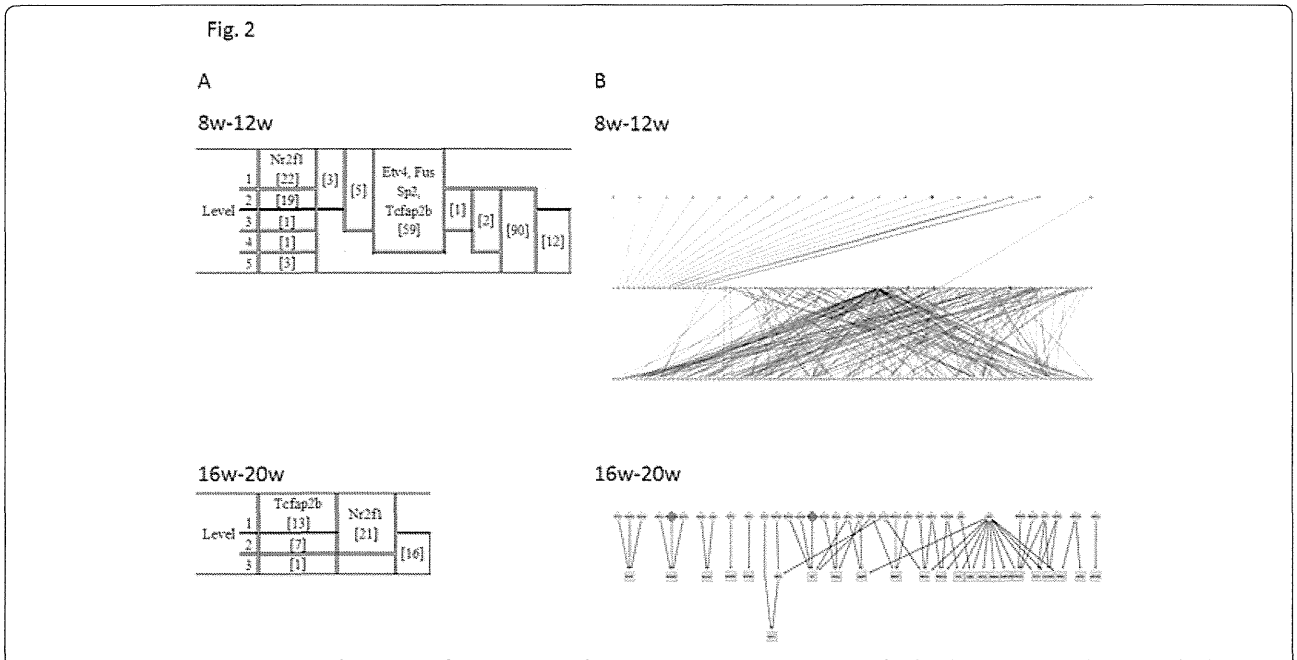

Figure 2 Hierarchical structures of networks for 8w-12w and 16w-20w by two previous methods. The 5 TFs are indicated at the levels in hierarchical structures obtained by the vertex-sort algorithm (A) [12] and those by the BFS method (B) [13], and the numbers of TFs in each level are indicated in parentheses in (A), and by red circles in (B). In (B), the TFs and the regulated genes are indicated by diamonds and rectangles, respectively.