Cohort Profile: Golestan Hepatitis B Cohort Study- A Prospective Long Term Study in Northern Iran

Hossein Poustchi1, Aezam Katoonizadeh1, Mohammad Reza Ostovaneh1,2, Shirin Moossavi1, Maryam Sharafkhah1, Saeed Emami1,2, Akram Poursam1, Ashra Mohamadkhani1, Sima Besharat1, Shahin Merat1, Mehdi Mohamadnejad1, Jacob George1, Reza Malekzadeh1*

ABSTRACT

Hepatitis B virus (HBV) infection is the most common cause of end stage liver disease in Iran and in Golestan province. Large-scale population-based prospective cohort studies with long term follow-up are the method of choice to accurately understand the natural course of HBV infection. To date, several studies of HBV epidemiology, natural history, progression to cirrhosis and association with HCC have been reported from other countries. However, few of these are prospective and fewer still are population-based. Moreover, the underlying molecular mechanisms and immunogenetic determinants of the outcome of HBV infection especially in low and middle income countries remains largely unknown. Therefore, the hepatitis B cohort study (HBCS), nested as part of the Golestan Cohort Study (GCS), Golestan, Iran was established in 2008 with the objective to prospectively investigate the natural course of chronic hepatitis B with reference to its epidemiology, viral/host genetic interactions, clinical features and outcome in the Middle East where genotype D HBV accounts for >90% of infections. In 2008, a baseline measurement of HBV surface antigen (HBsAg) was performed on stored serum samples of all GCS participants. A sub-cohort of 3,505 individuals were found to be HBsAg positive and were enrolled in the Golestan HBCS. In 2011, all first degree relatives of HBsAg positive subjects including their children and spouses were invited for HBV serology screening and those who were positive for HBsAg were also included in the Golestan HBCS.

Why was the cohort set up?

Hepatitis B virus (HBV) infection is a major chronic global health problem with an estimated prevalence of 400 million infected people at risk for the subsequent development of cirrhosis and hepatocellular carcinoma (HCC).1,2 HBV causes a spectrum of chronic liver diseases with a variable natural history.3 Upon initial infection with the virus, there is an immunotolerant phase in which high levels of viral DNA and HBeAg are detected. Nevertheless, due to the lack of an effective immune response to the infection, there is little histological liver injury and serum aminotransferases are usually within the normal range. Subsequently, the immune system mounts a robust response against HBV-infected hepatocytes resulting in hepatic necroinflammation. This stage is manifested by elevated serum aminotransferases and a decrease in the level of viral DNA. In most patients, this immune response is followed by a low- or non-replicative phase of the disease
marked by HBeAg seroconversion. The time to seroconversion from the first evidence of an immune response varies between individuals and hence different degrees of liver injury and fibrosis are observed. A proportion of patients will subsequently develop further immune activation against HBV despite seroconversion. These individuals as well as a minor proportion of patients with persistent serum HBeAg are at an increased risk of disease progression to cirrhosis and HCC.4-7

Large-scale population-based prospective cohort studies with long term follow-up are the method of choice to accurately define the natural history of chronic hepatitis B (CHB). To date, several such studies have been reported from other countries.8-16 However, few of these are prospective and fewer are population-based.8,15 The hepatitis B cohort study (HBCS), nested as part of the Golestan Cohort Study (GCS), Golestan, Iran17 was therefore established in 2008 with the principle objective of prospectively investigating the natural history of CHB in the Middle East, where genotype D HBV is the prevalent genotype.

In Iran, an estimated 2 million patients are positive for HBV surface antigen (HBsAg) and CHB is the most common cause of end stage liver disease and liver transplantation. Currently, the ideal strategy to treat genotype D HBV infected patients in Iran is largely unknown. Several national and international guidelines for the management of CHB are already in place including the American Association for the Study of Liver Disease (AASLD), European Association for the Study of Liver disease (EASL) and Asian Pacific Association for the Study of Liver (APASL); however, these are based on studies of non-D genotype HBV infection.16,18-21 Therefore a second objective of the Golestan HBCS was to investigate the best strategy to manage CHB in Iran and other neighbouring countries.

Who is in the cohort?

The GCS is a prospective study of 50,045 (58% female) adults (age 40-75 years) recruited between 2004 and 2008, designed to assess upper gastrointestinal cancers in Northern Iran. GCS subjects comprise 74% of Turkmen ethnicity and 80% live in rural areas. Eighty eight percent were married, 83% were non-smokers, and 70% had no formal education.17 Golestan province, shown in figure 1, is known to have a high prevalence of CHB (up to 9.7%).22,23 Therefore, for the purposes of Golestan HBCS, a baseline measurement for HBsAg was performed on stored serum samples of all GCS participants. Among the participants, 3,505 individuals were found to be HBsAg positive and were enrolled in the Golestan HBCS. All HBsAg positive individuals as well as their first degree family members were subsequently invited to consent to participate in the Golestan HBCS. At this stage we were able to obtain a new blood sample from 2,590 of the original 3,505 HBsAg positive subjects. The reasons for failure to obtain blood samples from the rest of the HBsAg positive subjects are discussed later in this report. We also collected blood samples from 5,644 family members including 5,488 first degree family members (spouses and children) of the 3,505 subjects who were HBsAg positive at the enrolment phase.

All subjects signed the consent before being interviewed and prior to blood sample collection. The study protocol and the text of the consent was reviewed and approved by the ethical committee of the Digestive Disease Research Institute, of the Tehran University of Medical Sciences.

How are they being followed?

Golestan HBCS is divided into three distinct phases (Figure 2). In phase I (enrolment in the GCS: 2004-2008), participants of the cohort were interviewed by well-trained and educated staff, examined by general practitioners and screened for CHB by measuring HBsAg. The participants were then contacted annually by telephone to ascertain major illnesses or mortality. In case of death, in addition to collection of all medical documents from the hospital or home including pathology and laboratory reports, a verbal autopsy was performed. The reliability of verbal autopsy in GCS has been previously established.24 In phase II (enrolment in Golestan HBCS and repeated measurements: 2008-2012), HBsAg positive individuals and their imme-
Immediate family members (spouses and children) underwent blood sampling for (re)assessment of HBV infection in a central cohort clinic where they were re-interviewed using structured questionnaires. The questionnaires addressed different aspects including socio-demographic factors, general health and family medical history, quality of life information and risk factors for HBV infection. Immediate family members who were found to be HBsAg positive were enrolled in Golestan HBCS. All participants in Phase II were contacted by telephone for annual follow-up. All family members who tested negative for HBsAg were offered HBV vaccination. It is the intention of Golestan HBCS to annually follow all participants during phase III (2012-2022). Subjects who need treatment were introduced to a special HBV follow-up clinic at Atrak centre in Gonbad that is staffed by a visiting gastroenterologist.

What has been measured?

Blood samples were collected from all GCS participants at the time of enrolment. Serum was separated and stored initially at -70°C. In 2008, all serum samples were tested for HBsAg using a commercially available (Enzygnost® HBsAg6.0) kit according to the manufacturer’s instructions (Siemens Healthcare Diagnostics Products, Marburg, Germany). The kit has a specificity of 99.89% and the cut-off to detect HBsAg is below 0.02 U/ml. Samples which were equivocal for HBsAg were re-evaluated. HBsAg positive individuals and their immediate relatives (spouses and children) were then invited to attend the central cohort clinic and their blood, nail, hair, and urine samples were collected and stored at -70°C. Serum was separated and examined for HBsAg, HBsAb (DIA.PRO, Milano, Italy: Sensitivity of 100% and specificity of 98.8%) and HBcAb (DIA.PRO, Milano, Italy: Sensitivity of 94.9% and specificity of 99.5% by enzyme-linked immunoassays). Serum samples were also tested for a complete blood count, liver function tests, renal function, electrolytes and lipid profiles.

What has the Golestan HBCS found?

Golestan HBCS is unique because CHB in this region is almost 90% of the D genotype. It is still too early to be able to provide prospective results, but we have analyzed and are going to present data on prevalence, baseline demographic characteristics, and outcomes including the incidence of end stage liver disease and hepatocellular carcinoma. This study also has the potential to explore several aspects of the natural history of D genotype CHB, such as the rate of spontaneous clearance of HBsAg and its determinants, the indications for therapy and defining the phenotypes that need to be treated. The high prevalence of nonalcoholic fatty liver disease (NAFLD) in GCS represents a good opportunity to study the impact of NAFLD on HBV infection and its management.

HBV prevalence

Of 50,045 GCS individuals, 3,505 (7%) were initially positive for HBsAg with a mean (SD) age of 52 (8.9) years at enrolment. The prevalence of HBsAg positivity was higher (9%) in men than in women (5.4%, p=0.0001) (figure3a). The prevalence of HBsAg positivity in different male age groups was similar (9.4%, 9%, and 8.5% in 50-59, 40-49 and ≥60 year-old age groups, respectively). The prevalence of HBsAg positivity in women was highest in the 50-59 year-old age group (6%). As expected, the prevalence of HBsAg positivity in women was lower in both the 40-49 and ≥60 year-old age groups (5.2%, p=0.009) (figure3b).

With respect to the first degree relatives (spouses and children) of the initially HBsAg positive sub-
In subjects, the overall HBsAg seropositivity rate was 6.6% with a male predominance (8.6% vs 5.6% in women, \( p=0.0001 \)). The higher prevalence of HBsAg positivity in men is in accordance with the gender difference observed in infections with HBV reported from elsewhere.\(^{8,25,26}\) The prevalence
of HBV in our cohort is similar to that of previous reports from Golestan province (5.1% and 9.7%) and higher than that of previous reports from other regions of Iran (2.3% and 2.1% in Tehran and Hormozgan respectively).23,27

The introduction of HBV vaccination in Iran’s expanded program on immunization (EPI) since 1993, as well as screening all pregnant women and implementation of a mass HBV vaccination campaign for adolescents who were born between 1989 to 1992 by the Ministry of Health (MOH), has resulted in a significant reduction in the inci-

Table 1: Characteristics of the 3,505 initially HBsAg positive individuals and the 2,590 HBsAg positive participants that took part in the second assessment

| Variable/characteristic                          | Initially HBsAg positive individuals (n=3,505) | Participants in the repeat assessment (n=2,590) |
|-------------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Mean (SD) age at enrolment /year                | 52 (8.9)                                      | 51.4 (8.8)                                    |
| Age distribution at sampling(year)              |                                               |                                               |
| <40 n                                            | 0                                             | 0                                             |
| 40-49 n (%)                                      | 1618 (46.1%)                                  | 638 (25%)                                     |
| 50-59 n (%)                                      | 1182 (33.7%)                                  | 1162 (45%)                                    |
| ≥60 n (%)                                        | 708 (20.2%)                                   | 791 (30%)                                     |
| Male gender n (%)                                | 1929 (55%)                                    | 1336 (51.6%)                                  |
| Marital status (married) n (%)                  | 3476 (99%)                                    | 2516 (97%)                                    |
| Ethnicity                                        |                                               |                                               |
| Turkmen n (%)                                    | 2808 (80%)                                    | 2086 (80.5%)                                  |
| Non-Turkmen n (%)                                | 700 (20%)                                     | 501 (19.5%)                                   |
| Place of residence                               |                                               |                                               |
| Urban n (%)                                      | 539 (15.4%)                                   | 440 (17%)                                     |
| Rural n (%)                                      | 2969 (84.6%)                                  | 2150 (83%)                                    |

Table 2: Demographic characteristics of 5,388 first degree family members of initially HBsAg positive individuals

| Variable                        | Spouses (n=1,454) | Children (n=3,934) |
|---------------------------------|-------------------|--------------------|
| Mean (SD) age years             | 53.1 (8.8)        | 26.5 (8.6)         |
| Male gender n (%)               | 451 (31%)         | 1452 (37%)         |
| Place of residence              | Urban n (%)       | 226 (15.6%)        | 496 (12.5%) |
|                                | Rural n (%)       | 1228 (84.4%)       | 3438 (87.5%) |
| HBsAg & HBcAb positive n (%)    | 32 (2.2%)         | 310 (7.9%)         |
| HBcAb positive (exposure rate) n (%) | 769 (52%)       | 914 (23%)          |

Fig. 4: Cohort Flow Diagram
idence of HBV infection nation-wide. Our main cohort population (3,505 initially HBsAg+ individuals) were all born before establishment of the vaccination program. Thus, the high prevalence of CHB in the GCS cohort can be explained in part by the specific age structure of the unvaccinated population. The aforementioned population consisted of older patients (76% >45 years) and most of them (85%) were living in rural areas with a lower socioeconomic level, which together with poorer social and health literacy in previous decades can all contribute to the higher prevalence of HBV infection in Golestan.

Characteristics of the cohort at phase II

During repeated measurement after an average 4.8 years, we re-assessed HBV infection using serological markers (HBsAg, HBcAb and HBsAb) in the 3,505 subjects initially positive for HBsAg. We were not able to obtain a second blood sample from 915 subjects because of death (303 subjects), inability to participate because of co-morbidity (75 subjects), relocation (72 subjects), and reluctance to give another blood sample (365). Other miscellaneous reasons accounted for failure of sampling in the remaining 100 individuals. Thus, we evaluated HBV serological markers in 2,590 individuals.

Selected baseline characteristics of this group of participants are shown in table 1. The mean (SD) age at enrolment (initial baseline sampling) was 51.4 (8.8) years. The mean (SD) age at second blood sampling was 56.1 (8.7) years and 1,336 (51.6%) subjects were male. More than 95% of HBsAg positive individuals were married, 83% of them living in rural areas, and ~70% of them were illiterate.

With respect to family members of the initially HBsAg positive subjects, blood samples were obtained in 5,644, including 5,488 children and spouses. A small number (156) of other relatives [grandchildren (n=81), sisters (n=31), brothers (n=29), and parents (n=15)] were also tested. This latter group was excluded from further analysis but was referred to the clinic for treatment and follow-up. Sera from 100 first degree family members were not suitable for further analysis and were also excluded from the study. The remaining 5,388 first degree family members included 3,934 children and 1,454 spouses with a mean (SD) age of 33.7 (8.8) years (range: 8-85 years) at the time of blood sampling. The number of first degree family members screened was 1-15 persons per family. Selected baseline characteristics of the 5,388 first degree family members are summarized in table 2. The mean (SD) age of spouses was 53.1 (8.8) years (range: 25-85 years) of whom 451 (31%) were husbands. The mean (SD) age of the children was 26.5 (8.6) years (rage: 8-60 years) and 37% (n=1,452) were male (Table 2).

Outcome of repeated measurement in 2,590 initially HBsAg positive individuals

Of the 2,590 individuals, 2,269 (87.6%) were still HBsAg positive and 321 (12.4%) were HBsAg negative of whom 135 were also negative for HBcAb and HBsAb (figure 4). Among the 321 HBsAg negative individuals, 175 (54.5%; annual rate: 1.4%) had positive HBcAb and 75 (23.4%; annual rate: 0.6%) developed HBsAb. Annual spontaneous HBsAg seroclearance thus occurred in 0.6%-1.4% of individuals during a follow-up period of 12,107 person-years.

Spontaneous HBsAg seroclearance is a favorable outcome of HBV infection and is reported as a rare event in the natural course of CHB. Annual HBsAg seroclearance rate in carriers have been reported as between 0.1% and 2.38% depending on the endemicity of the area for CHB, the mean age of participants at study entry, the study population type, and the years of follow-up. If we consider Iran as an area of intermediate endemicity for CHB in which Golestan province has a higher rate of HBsAg positivity, the reported rate of spontaneous seroclearance may be slightly high, but this is likely attributable to the higher average age of participants in this study.

Rates of end stage liver disease and liver cancer

All 3,505 HBsAg positive individuals were fol-
lowed up for an average of 7 years (25,785 person years). During this period, 32 CHB infected subjects (<1%) died of end stage liver disease (annual mortality rate: 1.2 per 1,000 persons). Despite the high prevalence of CHB in our cohort (7%), we observed a very low incidence of CHB (D genotype)-induced hepatocellular carcinoma (HCC). Only 13 (<0.5%) HBV infected individuals developed HCC with an overall incidence of 0.53 per 1000 person-years.

Ongoing and planned studies

A specialized HBV clinic was established in Atrak centre at Khatam Hospital in Gonbad city and all HBCS members are being invited to attend. In addition to a complete blood count, liver function tests, serum HBV DNA, abdominal sonography and liver elastography are being performed free of charge.

Our future plan is to offer therapy with Tenofovir in all subjects who have an indication for therapy based on available guidelines. We plan to follow all HBCS cohort members for the next 10 years.

The related data of Golestan HBCS participants has been compiled into a password protected computerized database. The true rate of HBsAg seroclearance and its determinants will be determined and a large subgroup of subjects who do not need therapy will be identified. The cost benefit of screening for HCC in this group will also be investigated.

Strengths and weaknesses

Strengths

This is the first prospective large scale cohort study of predominantly genotype D CHB and their immediate family members in the Middle East.

- We expect to undertake long term (2004-2022) follow-up (15 years) in all participants with a very low attrition rate.
- We have the unique opportunity to study the natural history and the true rate of HBsAg seroclearance in individuals who have acquired infection vertically (perinatally) or horizontally during early childhood.
- We will have the opportunity to undertake a nested interventionaltrial to treat subjects at high risk for end-stage liver disease and to determine the efficacy of therapy

- We will have a unique opportunity to study the interaction between NAFLD and HBV infection and their role in contributing to end stage liver disease.
- We will have access to liver stiffness assessments for a large subset of cohort participants.
- We have established infrastructure for (inter) national collaborative research on all aspects of HBV infection. International collaboration is already happening which will provide the opportunity to combine datasets and therefore to strengthen our findings.

Weaknesses

- The majority of Golestan HBCS participants are adults who were >40 years old at enrollment. This age structure of the participants is not representative of the general population of Golestan, or of Iran.
- Lack of liver function tests and serum HBV DNA measurement at the time of enrolment.

Can I get hold of the data? Where can I find out more?

Information about the study design, updated interim analyses, on-going new analysis, studies and interventions, and relevant publications are available at www.ddrc.ac.ir. Specific proposals for national and international collaborations are welcome.

Suggestions and new proposals, which should include at least a 2 page summary including the aims and methods of the study, the required data and samples, should be submitted to the principle investigator H.P (h.poustchi@gmail.com). The proposals will be discussed within the steering committee, which includes other principal investigators of the study and, if necessary, external experts.

Funding: This study was supported by the Digestive Disease Research Center of Tehran University of Medical Sciences (grant number 87/21) and Iranian Association of Gastroenterology and Hepatology.
ACKNOWLEDGMENTS

Many individuals have contributed to this study. We wish to thank the study participants for their cooperation over many years and the Behvarz working in the study areas for their help. We thank the Directors of the public health districts of Gonbad and Kalaleh for their collaboration. We express our special thanks to the general physicians, nurses and nutritionists in the enrolment teams for their collaboration and assistance. We received special support from the Social Security Organization of Iran, Golestan Branch. We have enjoyed the close collaboration of the Golestan health deputies and the Chief of the Gonbad health district. We would like to thank Keivan lab for technical support.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

REFERENCES

1. Sorrell MF, Belongia EA, Costa J, Gareen IF, Grem JL, Inadomi JM, et al. National Institutes of Health consensus development conference statement: management of hepatitis B. Hepatology 2009;49(Suppl):S4-S12.
2. EASL. International Consensus Conference on Hepatitis B. Consensus statement. J Hepatol 2003;38:533-40.
3. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol 2008;48:335-52.
4. Beasley R. Hepatitis B Virus. The Major Etiology of Hepatocellular Carcinoma. Cancer 1998;61:1942-56.
5. Liaw YF, Chu CM. Hepatitis B virus infection. Lancet 2009;373:582-92.
6. Kew MC. Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. Pathol Biol (Paris) 2010;58:273-7.
7. Neuvect C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. J Hepatology 2010;52:594-604.
8. Chen CJ, Yang HL. Natural history of chronic hepatitis B REVEALed. J Gastroenterol Hepatol 2011;26:628-38.
9. Lee MS, Kim DH, Kim H, Lee HS, Kim CY, Park TS, et al. Hepatitis B vaccination and reduced risk of primary liver cancer among male adults: a cohort study in Korea. Int J Epidemiol 1998;27:316-9.
10. Lai MS, Hsieh MS, Chiu YH, Chen TH. Type 2 diabetes and hepatocellular carcinoma: A cohort study in high prevalence area of hepatitis virus infection. Hepatology 2006;43:1295-302.
11. Wang LY, You SL, Lu SN, Ho HC, Wu MH, Sun CA, et al. Risk of hepatocellular carcinoma and habits of alcohol drinking, betel quid chewing and cigarette smoking: a cohort of 2416 HBsAg-seropositive and 9421 male residents in Taiwan. Cancer Causes Control 2003;14:241-50.
12. Raptopoulou M, Papatheodoridis G, Antoniou A, Ketikoglou J, Tzourmakliotis D, Vasiliadis T, et al. Epidemiology, course and disease burden of chronic hepatitis B virus infection. HEPNET study for chronic hepatitis B: a multicentre Greek study. J Viral Hepat 2009;16:195-202.
13. Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, et al. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. Hepatology 2010;51:1531-7.
14. Tai DI1, Tsay PK, Chen WT, Chu CM, Liaw YF. Relative roles of HBsAg seroclearance and mortality in the decline of HBsAg prevalence with increasing age. Am J Gastroenterol 2010;105:1102-9.
15. Kusakabe A, Tanaka Y, Inoue M, Kurbanov F, Tatematsu K, Nojiri S, et al. A population-based cohort study for the risk factors of HCC among hepatitis B virus mono-infected subjects in Japan. J Gastroenterol 2011;46:117-24.
16. Lok AS, McMahon BJ. Chronic Hepatitis B: Update 2009. Hepatology 2009;50:661-2.
17. Pourshams A, Khademi H, Malekshah AF, Islami F, Nouraei M, Sadjadi AR, et al. Cohort Profile: The Golestan Cohort Study--a prospective study of oesophageal cancer in northern Iran. Int J Epidemiol 2010;39:52-9.
18. Coffin CS, Fung SK, Ma MM, Canadian Association for the Study of the Liver. Management of chronic hepatitis B: Canadian Association for the study of liver Diseases consensus guidelines. Can J Gastroenterol 2012;26:917-38.
19. European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 2012;57:167-85.
20. Liaw YF, Kao JH, Piratvisuth T, Chan HL, Chien RN, Liu CJ, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2012 update. Hepatol Int 2012;6:531-61.
21. Buster EH, van Erpecum KJ, Schalm SW, Zbaar H, Brouwer JT, Gelderblom HC, et al. Treatment of chronic hepatitis B virus infection - Dutch national guidelines. Neth J Med 2008;66:292-306.
22. Alavian SM. Hepatitis B virus infection in Iran: Changing the epidemiology. Iran J Clin Infect Dis 2010;5:51-61.
23. Abdollahi N, Keshkar AA, Semnani S, Roshandel GR, Beirat S, Joshaghani HR, et al. HBV seroprevalence among Golestan adults. Iran J Epidemiol 2006;4:35-40. (In Persian).
24. Khademi H, Etemadi A, Kamangar F, Nouraie M, Shakeri R, Abaie B, et al. Verbal autopsy: reliability and validity
estimates for causes of death in the Golestan Cohort Study in Iran. *PloS One* 2010;5:e11183.

25. Baig S. Gender disparity in infections of Hepatitis B virus. *J Coll Physicians Surg Pak* 2009;19:598-600.

26. Oertelt-Prigione S, Regitz-Zagrosek V. Springer-Verlag; Sex and Gender Differences in Gastroenterology and Hepatology in Sex and Gender Aspects in Clinical Medicine edited London Limited 2012.

27. Merat S, Rezvan H, Nouraie M, Jamali A, Assari S, Abolghasemi H, et al. The prevalence of hepatitis B surface antigen and anti-hepatitis B core antibody in Iran: a population-based study. *Arch Iran Med* 2009;12:225-31.

28. Kabir A, Alavian SM, Ahanchi N, Malekzadeh R. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus in infants born to HBsAg positive mothers in comparison with vaccine alone. *Hepatol Res* 2006;36:265-71.

29. Alavian SM, Gooya MM, Hajarizadeh B, Esteghamati AR, Moeinzadeh AM, Haghazali M, et al. Mass Vaccination Campaign against Hepatitis B in Adolescents in Iran: Estimating Coverage using Administrative Data. *Hepat Mon* 2009;9:189-95.

30. Liaw YF, Sheen IS, Chen TJ, Chu CM, Pao CC. Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B virus infection: a prospective study. *Hepatology* 1991;13:627–31.

31. McMahon BJ, Holck P, Bulkow L, Snowball M. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. *Ann Intern Med* 2001;135:759–68.

32. Chu CM, Liaw YF. HBsAg seroclearance in asymptomatic carriers of high endemic areas: Appreciably high rates during a long-term follow-up. *Hepatology* 2007;45:1187-92.

33. Liu J, Yang HL, Lee MH, Lu SN, Jen CL, Wang LY, et al. Incidence and determinants of spontaneous hepatitis B surface antigen seroclearance: a community-based follow-up study. *Gastroenterology* 2010;139:474–82.