PREVALENCE OF INTEGRATED HBV DNA AMONG BLOOD DONORS IN EGYPT

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Blood transfusion service (BTS) is a necessary and key part of the medical services framework. The main objective of the BTS is to ensure safety, adequacy, availability, and effectiveness of blood supply at all levels (Islam, 2009). Transfusion of blood and blood derivatives, as a specific methodology of patient administration spares a large number of lives worldwide every year and decreases HBV transmission. It is outstanding that blood transfusion is associated with a substantial number of complexities, some are just trifling and others are conceivably life, undermining, requesting for fastidious pre-transfusion testing and screening. Unscreened blood transfusion expose the patient to infect with many transfusions transmitted infections (TTI) like hepatitis virus (HBV, HCV), human immune-insufficiency virus (HIV), syphilis, malaria, etc. Transfusion units have dependably been a noteworthy entry to monitor, screen, and control diseases transmitted by blood transfusion. (Khan et al., 2007).

Hepatitis B virus (HBV) infection is a critical worldwide health problem influencing two billion individuals worldwide and 400 million people suffer from chronic HBV infection (Dhawan et al., 2008). HBV transmitted sexually, perinatally, and percutaneously, manipulate 350 to 400 million people around the world. HBV disease caused every year for 1 million deaths worldwide from liver failure, cirrhosis, and hepatocellular carcinoma. (Lee, 1997; Hoofnagle et al., 2007).

HBV is a member of Orthohepadnavirus genus of the Hepadnaviridae family, which is classified to Retroid viruses (Kann and Gerlich, 1998). HBV genome around 3.2 KB, its smaller, incompletely double-stranded DNA genome, HBV has four highly overlapping open reading frames (ORFs) (Nassal, 1999; Seeger and Mason, 2000). HBV has a higher rate of mutation than other DNA viruses, because of using RNA polymerase without proofreading function. Thus, generally it is assumed that reverse transcription step is a source for the majority of point mutations and deletions or insertions that can be observed in the HBV genome (Petzold et al., 1999).
HBV DNA integration has been detected in HBsAg-negative patients (Brechoht et al., 1987). It has been recorded that HBV DNA could integrate into the host hepatocytes as well as the spermatozoa. (Hino et al., 1991; Peng et al., 1988).

HBV DNA usually integrates in cellular genes involving cell growth or signaling control. When HBV DNA integrates into the host chromosome chromosomal stability is also decreased (e.g., large inverted deletions, duplications and chromosomal translocation) (Chamberlain, 2016).

**MATERIAL AND METHODS**

Ninety thousand and one hundred five volunteer blood donors were collected from blood donation campaigns for the company blood transfusion services (VACSERA) during 2014. Blood samples are usually collected in EDTA tubes. Then, samples were centrifuged and the plasma was transferred to a screw-cap cryo tube within 1 h of collection.

**Nucleic Acid Test (NAT)**

For the qualitative detection of HBsAg, Chemiluminescent immunoassay (ChLIA) was used. NAT test was used as a confirmatory test to screen samples with negative ChLIA of HBsAg in collected plasma. Samples, only which diagnosed as positive NAT for HBV with no co-infection with other hepatitis viruses such as HCV and HDV or HIV, had been aliquot and stored at -20°C until use.

**Extraction of HBV DNA and PCR amplification**

DNA was extracted from two plasma samples; Total DNA was extracted from 200 μL plasma by using QIAamp DNA Blood Mini Kit (Qiagen, Cat. No. 5114) (Goodarzi et al., 2007).

PCRs designed to amplify the complete surface antigen of HBV. A simple and efficient way for virus primer design was used based on the alignment of HBV genotype sequences published at gene banks database (Stuyver et al., 2000). Taq PCR Master Mix kit (Qiagen, Cat. No. 201443) was used to amplify HBsAg. Final volume of PCR was 100 μl, according to the instruction of the manufacturer. Thermal profile of PCR reaction consist of: preheating at 95°C for 5 min, 35 cycles of 95°C for 45 sec, 60°C for 40 sec, and 72°C for 60 sec; finally at 72°C for 10 min as a final extension step. 2% agarose gel with ethidium bromide stain (0.5 µg/ml) was used to separate the PCR products. PCR products were purified by using gel extraction Kit. (Qiagen, Cat. No. 28704).

**Cloning and sequencing**

When direct sequencing for amplified fragments were failed according to the protocol provided by the manufacturer of DNA sequence kit. The PCR products ≈1200 bp were cloned into pPCR - Script™ Amp SK+ vector (Stratagene, La Jolla, sCA, USA) according to manufacture protocol, sequencing had been carried out at Genetic Engineering Research De-
department (VACSERA). BigDye Terminator v3.1 Kit (Life Technologies) was used as a ready reaction to perfume cycle sequencing then read and analyzed on a 310 DNA automated sequencer (Life Technologies Inc.). Primers Used to read the sequence were T3 (5’ - AATTAACCCCTCACTAAAGGG- 3’) and T7 (5’ -GTAATACGAC-TCACTATAGGGC-3’) in the tow direction forward and reverse sequences were assembly and analyzed by using the NCBI Blast tool and to identify viral genome sequences, and to locate the integration sites in the human genome. (Takahashi et al., 1998).

RESULTS AND DISCUSSION

Insurance blood transfusion service and safety of blood products is one of the major issues in the area of transfusion medicine. Blood screening is playing a major role to decrease the risk of transfusion of infected units. Firstly, testing of antigen/or antibody markers of blood borne disease was established. Wherefore, limitation of these serological techniques including the window period between infection and detection, and antigenic variability enhance implementation of the second testing for nucleic acid technique (NAT). (Hollinger and Liang, 2001).

From ninety thousand and five hundred volunteer blood donors only two samples had a negative result for HBsAg by using Chemiluminescent immunoassay and diagnosed positive HBV for NAT test with no co-infection with other hepatitis viruses such as HCV, HDV or HIV. Perchance, chromosomal Human DNA was amplified by using primers designed for viral DNA. In this study, two human chromosomal DNA were amplified by using primers designed to amplify surface antigen of HBV. These two samples carried integrated virus DNA. Both of them had both "left” and "right” intersection of the integrated virus DNA. The structure of integrated virus genomes were determined by constructing the sequence of cloned PCR product that amplified with specific primer for HBV.

The expected PCR Product size is 1.3 kb, the subgenomic PCR designed to amplify the surface antigen of HBV gave rise to smaller amplicons ranging in size from 300 to ≈1200 bp. Only amplicon with size ≈1200 bp were successfully cloned into a pPCR-ScriptTM Amp SK+ vector after direct sequence failed to give a clear and significant reading (Fig. 1) and the two cloned samples were sequenced (Table 1).

DNA sequences were processed in the pPCR SK(+) plasmid containing amplicons soon after sequences were checked against a gene bank. The identities of the sequenced samples were 98% with human chromosome 5. Integration point in the 1st and 2nd samples were located and determined in Table (1).

Integration sites of HBV DNA in human HCCs have been found on almost all chromosomes, a random distribution throughout the host DNA (Tokino et al., 1991) this confirmed by finding of Slagle et al. (1992) that HBV DNA insertions are
found at random sites in human cellular DNA.

Albeit a few chromosomes are affected more often than others in the present study four sites were found to be identical as integrated HBV DNA into Human chromosome 5 and fifth site locate in the 1st sample only. The length of integrated fragment ranging from 10 to 19 nucleotides which supported by the finding of Nagaya et al. (2015) who found that the inserted fragments HBV DNA were less than 24 bp. Sites of chromosomal fragile are large regions of DNA, which replicate late during S phase of the cell cycle and are predisposed to deletions or breaks. So hot spots for foreign DNA integration multiply. Unlike retroviruses, in which viral DNA inserted into host DNA is a step of replication cycle, integration does not carry out during normal Hepadnaviruses replication. Integration of Hepadnaviruses DNA may take place as a result of an illegitimate recombination mechanism mediated by cellular enzymes. (Yang and Summers, 1999; Wang and Rogler, 1991).

In the current study, the integration sites mapped to show direction of integrated fragment into chromosomal DNA (Fig. 2). Three intersections were integrated in reverse direction (Plus/Minus) of chromosome 5 and only one intersection was integrated in forward direction (Plus/Plus). Fifth intersections site in 1st sample was integrated in forward direction (Plus/Plus).

Genome of human HBV does not contain known oncogenes. One of the possible mechanisms whereby integration of viral DNA, a random phenomenon, activates carcinogenesis is its integration upstream of an enhancer sequence which is located near an oncogene in the chromosomal DNA of human hepatocyte. Many gastrointestinal centers have been notifying increasing numbers of patients with HCC who have a history of blood transfusion. The time between blood transfusion and detection of liver cancer was ranging from 20 to 35 years (Kunio, 1989).

Early studies confirmed that integration of HBV occurs in patients with occult HBV infection and with HCC (Lai et al., 1990). In most of cases, HBV integration was common in tumorous than in nontumorous tissue. (Matsuzaki et al., 1997; Matsuzaki et al., 1999)

A higher rate of chromosomal abnormalities is found in HBV-related to HCC than can be clarified alone by the accumulation of chromosomal mutations. HBV is an oncogenic virus and its DNA can be integrating into the genome of hepatocytes. Integration of HBV DNA sequences is present in about 80% of human HBV related HCCs. Integration of HBV DNA may confer an eclectic growth advantage on infected hepatocytes and leads to the onset of tumor progression. (Chamberlain, 2016).
SUMMARY

This study presented important data about the gap between serology screening (surface antigen) and nucleic acid testing (NAT) testing of blood donors. Effectiveness of NAT for blood donors screening is a debating area in blood transfusion. This study highlight on the riskiness of transfuse blood contain integrated HBV DNA owing to the carcinogenic effect of integrated HBV in human liver. Wide-national study is required to ensure blood transfusion safety and effectiveness by using traditional (surface antigen, core antigen as a serological test) confirmed by NAT testing to screen blood donors and introduce new techniques for the detection of viral integrated with the human genome.

REFERENCES

Brechoht, C. (1987). Hepatitis B virus (HBV) and hepatocellular carcinoma: HBV-DNA status and its implications. J. Hepatol., 4: 269-279.

Dhawan, H. K., N. Marwaha, R. R. Sharma, Y. Chawla, B. Thakral, K. Saluja, S. K. Sharma, M. K. Thakur and A. Jain (2008). Anti-HBc screening in Indian blood donors: Still an unresolved issue. World J. Gastroenterol. 14: 5327-5330.

Goodarzi, Z., R. Malekzadeh, G. Montazeri, S. M. Alavian and M. Qurbanalizadgan (2007). Phylogenetic analysis of HBV based on PreS region in iranian hepatocellular carcinoma patients. Hepatitis, 7: 201-205.

Hino, O., S. Tabata and Y. Hotta (1991). Evidence for increased in vitro recombination with insertion of human hepatitis B virus DNA. Proc. Natl. Acad. Sci., (USA), 88: 9248-9252.

Hollinger, F. B. and T. J. Liang (2001). Hepatitis B Virus. In: Knipe DM et al. eds. Fields Virology, 4th ed. Philadelphia, Lippincott Williams & Wilkins, p. 2971-3036.

Hoofnagle, J. H., E. Doo, T. J. Liang, R. Fleischer and A. S. Lok (2007). Management of hepatitis B: summary of a clinical research workshop. Hepatology, 45: 1056-1075.

Islam, M. B. (2009). Blood transfusion services in Bangladesh Asian J. Transf. Sci., 3: 108-110.

Kann, M. and W. H. Gerlich (1998). Hepatitis B, in: Virology (Mahy, B. W. 3., Collier, I., Eds.), p. 745-773. London, Sydney, Auckland: Arnold.

Khan, Z. T., S. Asim, Z. Tariz, I. A. Ehsan, R. A. Malik and Ashfaq (2007). Prevalence of Transfusion transmitted infections in healthy blood donors in Rawalpindi Dis-
strict, Pakistan-a five year study. Int. J. Pathol., 5:21-25.

Kunio, O. (1989). Primary Liver Cancer Quadrennial Review Lecture. Digestive Diseases and Sciences, 31: 133S-146S.

Lai, M. Y., P. J. Chen, P. M. Yang, J. C. Sheu, J. L. Sung and D. S Chen (1990). Identification and characterization of intrahepatic hepatitis B virus DNA in HBsAg-seronegative patients with chronic liver disease and hepatocellular carcinoma in Taiwan. Hepatology, 12: 575-581.

Lee, W. M. (1997). Hepatitis B virus infection. N. Engl. J. Med., 337: 1733-1745.

Matsuzaki, Y., T. Chiba and T. Hadama (1997). HBV genome integration and genetic instability in HBsAg-negative and anti-HCV-positive hepatocellular carcinoma in Japan. Cancer Lett., 119: 53-61.

Matsuzaki, Y., M. Sato and Y. Saito (1999). The role of previous infection of hepatitis B virus in HBs antigen negative and anti-HCV negative Japanese patients with hepatocellular carcinoma: etiological and molecular biological study. J. Exp. Clin. Cancer Res., 18: 379-389.

Nagaya T., Nakamura, T. Tokino, T. Tsurimoto, T. Imai, M. T. Mayumi and K. Kamino (2015). The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. Genes & Development, 1: 773-782.

Nassal, M. (1999). Hepatitis B virus replication: novel roles for virus-host interaction. Inter. Nirology, 42: 100-116.

Chamberlain, N. R. (2016). Blood-borne hepatitis. https://www.atsu.edu/faculty/chamberlain/website/lectures/lecture/hepatit2.htm.

Peng, H. W., T. S. Su, S. H. Han, C. K. Ho, C. H. Ho and K. N. Ching (1988). Assessment of HBV persistent infection in an adult population in Taiwan. J. Med. Virol., 24: 405-412.

Petzold, D. R., B. Tautz, F. Wolf and J. Drescher (1999). Infection chains and evolution rates of hepatitis B virus in cardiac transplant recipients infected nosocomially. J. Med. Virol., 58: 1-10.

Seeger, C. and W. S. Mason (2000). Hepatitis B virus biology. Microbiol Mol. Biol. Rev., 64: 51-68.

Slagle, B. L., T. H. Lee and J. S. Butel (1992). Hepatitis B virus and hepatocellular carcinoma. Prog. Med. Virol., 39: 167-203.

Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi
and R. Rossau (2000). A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J. Gen. Virol., 81: 67-74.

Takahashi, K., Y. Akahane, K. Hino, Y. Ohta and S. Mishiro (1998). Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. Arch. Virol., 143: 2313-2326.

Tokino, T., H. Tamura, N. Hori and K. Matsubara (1991). Chromosome deletions associated with hepatitis B virus integration. Virology, 185: 879-882.

Wang, H. P. and C. E. Rogler (1991). Topoisomerase I-mediated integration of hepadnavirus DNA in vitro. J. Virol., 65: 2381-2392.

Yang, W. and J. Summers (1999). Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. J. Virol., 73: 9710-9717.

Table (1): Summary of integration site (Length, Identities, Gaps and Strand direction) on chromosome 5(CH5) compared with HBV DNA.

| Sample No. | Seq. Source | Sequence alignment | Length | Identities | Gaps | Strand direction |
|------------|-------------|--------------------|--------|-------------|------|------------------|
| 1st        | CH5         | ACTCTAAAATCTCAGTGAT 159 | 17/19  | 89.4%       | 0/19 (0%) | Plus/Plus |
|            | HBV         | ACTCTAAAATCTCAGTGAT 856 |        |             |      |                  |
|            | CH5         | GCAGGGTTTGGACAG 785 | 15/15  | 100%        | 0/15 (0%) | Plus/Minus |
|            | HBV         | GCAGGGTTTGGACAG 514 |        |             |      |                  |
|            | CH5         | AAATIGCAACTA---AAATT 910 | 16/18  | 88.9%       | 2/18 (11%) | Plus/Minus |
|            | HBV         | AAATIGCAACTA---AAATT 674 |        |             |      |                  |
|            | CH5         | CCAGTCTGATAGCTTGGT 1109 | 19/19  | 100%        | 0/19 (0%) | Plus/Minus |
|            | HBV         | CCAGTCTGATAGCTTGGT 2834 |        |             |      |                  |
|            | CH5         | CGAATCAGACGAC 294 | 12/12  | 100%        | 0/12 (0%) | Plus/Plus |
|            | HBV         | CGAATCAGACGAC 2733 |        |             |      |                  |
| 2nd        | CH5         | ACTCTAAAAACTCAGTGAT 159 | 19/19  | 89.4%       | 0/19 (0%) | Plus/Plus |
|            | HBV         | ACTCTAAAAACTCAGTGAT 856 |        |             |      |                  |
|            | CH5         | GGAGGTGGTTGGACAG 785 | 15/15  | 100%        | 0/15 (0%) | Plus/Minus |
|            | HBV         | GGAGGTGGTTGGACAG 514 |        |             |      |                  |
|            | CH5         | AATTGCACTA---AATT 910 | 16/18  | 88.9%       | 2/18 (11%) | Plus/Minus |
|            | HBV         | AATTGCACTA---AATT 674 |        |             |      |                  |
|            | CH5         | CCAGTCTGATAGCTTGGT 1109 | 19/19  | 100%        | 0/19 (0%) | Plus/Minus |
|            | HBV         | CCAGTCTGATAGCTTGGT 2834 |        |             |      |                  |
Fig. (1): Panel (A): 2% agarose pPCR-Script Amp SK (+) plasmid containing amplicons gel stained with ethidium bromide showing. Panel (B): The cloned fragments were amplified with primers T3 and T7.
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Fig. (2): Panel (A): 1st sample with five integration site and its direction. Panel (B): 2nd sample with four integration site and its direction.
