Advances in Research on Hepatitis B Virus DNA Integration

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Abstract: Since HBV DNA integration was discovered for the first time in 1980, various methods have been used to detect and study it, such as Southern Blot, in situ hybridization, polymerase chain reaction and so on. HBV DNA integration is thought to be random on the whole although some hot spots of integration were described by some researchers, one of which might be the repetitive sequences of the genomic DNA. Besides, DNA damage, especially double-strand breaks could promote HBV DNA integration into host genome. HBV DNA integration into cells may damage the stability of the genome, cause DNA rearrangement, promote DNA deletion and induce the formation of HCC.

Key words: Hepatitis B virus; Integration; Hepatocellular carcinoma

Hepatitis B virus (HBV) infection is a leading cause for chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (HCC). After decades of chronic HBV infection, about 30-40% of patients progress into liver cirrhosis, of which around 1-5% subsequently develop HCC annually. HBV DNA integration into the genome of liver cells is one important factor of hepatocarcinogenesis (14). Researches on the regularity and consequence of HBV DNA integration will elucidate the molecular mechanisms of HBV-induced HCC and pave the way for the discovery of new targets for treatments. This review will discuss the methodology of detection of HBV DNA integration, the integration modes and sites, DNA damage and its effects on the hosts.

THE DISCOVERY OF HBV DNA INTEGRATION

In 1980, Brechot detected integrated HBV DNA for the first time in human liver cancer tissues and PLC/PRF/5 cell line by Southern Blot (2), which directed a new way to study the mechanism by which HBV induced HCC at the molecular level. In 1982, Duck Hepatitis B Virus (DHBV), a virus similar to HBV, was found to replicate itself through reverse transcription (22). Therefore, researchers speculated that the integration of HBV DNA might be the direct cause for HBV-induced HCC since it had been recognized that the integration of HBV into host genome would lead to canceration.

METHODS TO DETECT INTEGRATED HBV DNA

Southern blot is the most popular method to detect integrated HBV DNA and can be used to determine whether HBV DNA is free or integrated in liver cells.

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At the beginning, a commonly used method was to construct a genomic library of HBV-induced HCC. Different fragments of HBV DNA were used as probes for hybridization with the genomic library and the positive clones were examined by DNA sequencing (11). Tsuei et al constructed a genomic library of the liver tissue of a nine-year-old HCC patient, using phage L47.1 as a vector (5). Then the positive clones were picked out through hybridization and sequencing. Results showed that there were deletion and rearrangement in the integrated HBV DNA. The integrated sequences included X gene and S gene, which had been rearranged. The integrated X gene still kept its function of trans-activating heterologous promoter.

A following method was in situ hybridization by which 3H-HBV DNA probe was used for in situ hybridization in paraffin sections of HCC and matched surrounding liver tissue. Results showed that HBV DNA was mainly distributed in cytoplasm with little in nucleus. Most of HBV DNA in matched surrounding tissue was free while that in cancer cells was integrated. Blum et al hold that the HBV DNA copies and replication activity could be estimated according to the quantity of the positive particles of HBV DNA in cells. Therefore, HBV DNA in situ hybridization provided a reliable method to investigate the relationship between HBV infection and HCC.

The invention and application of polymerase chain reaction (PCR) is also of significance for the studies on HBV DNA integration. The key issue of PCR is designing primers, which directly affect the efficiency of amplification (16, 29). Minami *et al* designed primers according to known sequences and human Alu repetitive sequences, and invented a PCR technique to clone integrated HBV DNA and consecutive sequences (8). In order to avoid amplification of the DNA fragments between Alu repetitive sequences, dUTPs were introduced into the primers. Then the products were treated with uracil DNA glycosylase after ten cycles of amplification. By using this technique, the consecutive sequences to integrated HBV DNA has been successfully cloned in three cases of HCC tissues, which proves that it is a comparatively ideal method to study the targets for HBV DNA integration.

Another important method is reverse PCR, which is based on PCR amplification (7). Tsuei et al found that HBV DNA could be detected in 80% of the HBV-induced HCC tissues, but the mechanism by which HBV DNA induce HCC was not clear (27). They cloned integrated HBV DNA and consecutive sequences by using reverse PCR, in which primers were designed according to direct repeat1 (DR1) and direct repeat2 (DR2) (26). Results showed that the amplified HBV DNA resided near DR1 and DR2 in nine out of ten cases of HCC, and all of them were Integron I. The results of Southern blot showed that in two out of five cases there were sequences specific to men, homologous to human long interspersed DNA element (LINE-1). In one integrant, it was found that HBV DNA was integrated into RNA-binding motif on Y chromosome (RBMY).

In addition, in situ PCR is also widely used in studies on HBV DNA integration. Matsui *et al* used fluorescence in situ polymerase chain reaction (FISPCR) to detect integrated HBV DNA in HCC tissues (15). Strong fluorescence was found in Alexander cells and could be analyzed quantitatively.

INTEGRATION AT DIFFERENT STAGES

Integration is not necessary for HBV replication, so it is not a routine in the natural history of HBV infection, which is quite different from that of retroviruses (18). Most researchers used to think that HBV DNA integration occurs at the chronic stage of HBV infection. With the advance of research, HBV DNA integration was found at various stages of infection. Murakami et al used Alu-PCR to detect the liver samples of 19 acute and 12 chronic hepatitis B patients. Results showed that HBV DNA integration was found in 3 acute and all the chronic hepatitis patients (17). In the liver sample of one acute patient, HBV DNA was found to be integrated into the intron sequences of TNF inducible protein.

THE MODES OF HBV DNA INTEGRATION

Both the DNA double-strand and DNA fragments of HBV can be integrated into liver cells in the form of monomer or oligomer. This kind of integration was also found in the liver cells of chimpanzees and woodchucks whose were infected with woodchuck hepatitis B virus. This indicates that the integration might be a direct nucleic acid-nucleic acid process. Integration may occur at different positions with more than one position in the same liver cell. It is known that HBV genome is complicated in construction and structural genes are overlapped with regulatory genes or other structural genes. Therefore, the integration of a fragment of DNA might involve several coding genes with different functions.

No complete HBV DNA integration has been found in the samples of spontaneous HBV infection in researches so far, and no integrated HBV DNA sequence has been found to be identical with another.

Therefore, most researchers believe that HBV DNA integration is random. However, in 30% of different cancer tissues, the integration was found to occur at the adhesive terminus of HBV DNA, and the region of single chain was also a frequent position.

HBV DNA integration usually occurs at DR1 and DR2, two direct repeats at the end of HBV DNA chain. Since X gene is near direct repeats, it is the most frequent integrated fragments, and the next is S gene, which is upstream X gene. In sum, integrated DNA fragments focus on the coding gene domains of two transactivating proteins, which are HBsAg and X protein (6, 10, 12).

THE SITES OF HBV DNA INTEGRATION

The sites of HBV DNA integration were analyzed to be random on the whole, although some hot spots had been discovered. In order to find preponderant integration targets of HBV DNA, Murakami *et al* used improved Alu-PCR to detect the targets in 60 samples of HCC (13). Results indicated that integration could occur in all chromosomes except chromosome 13, X and Y. Through this analysis, 68 sequences of HBV DNA integration targets were identified, including 7 repetitive sequences, 42 cell genes and 19 sequences encoding unknown proteins, of which 15 sequences were closely related to the occurrence of cancer. Some preponderant integration targets of HBV DNA were also found in this research, including hTERT (3/60) and some signal transduction factors.

In addition, Zhang *et al* found that the sites of HBV DNA integration were near oncogene erb B in HCC tissues (31). Analysis of the sequences revealed that the integrated HBV DNA was near epidermal growth factor (EGF) and other sites of tyrosine protein kinase.

Berger *et al* did not find rearrangement of cell DNA in AL-14 and AL-26, two integrated clones of HBV DNA in PLC/PRF/5 cells. They only found deletion of small DNA fragments. A fragment of 2kb was deleted in AL-14 and 17bp was deleted in AL-26. There was a sequence of 182bp on the right lateral of AL-26, which was thought to be a part of the cell gene. But further study proved that it was an unusual fragment of HBV DNA. Surprisingly, this fragment was highly homologous to WHBV DNA. There were minisatellite-like repetitive sequences in AL-14 and AL-26, so they were thought to be the possible hot spots of HBV DNA integration (5).

HBV DNA INTEGRATION AND DNA DOUBLE-STRAND BREAKS

DNA double-strand breaks (DSB) are potentially lethal lesions, which can be caused by ionizing radiation, chemicals, biological factors and so on. DSBs can be repaired by homologous recombination (HR) or non-homologous end jointing (NHEJ). In the past decade, there have been many studies on the relationship between HBV DNA integration and DNA damage and repair. All these studies indicated that DNA damage, especially DSBs could promote HBV DNA integration into host genome.

Petersen found that H_2O_2 could induce DHBV to integrate into LMH-D21-6, and the inhibitor of poly (ADP-ribose) polymerase (PARP) could increase DHBV integration (19). This indicated that the oxidative stress and DNA damage caused by H_2O_2 could promote HBV DNA integration. On the other hand, PARP was a ribozyme in eukaryotes, catalyzing poly ADP to be ribosylated. It played an important role in repairing DNA damage and maintaining

stability of genome. In sum, oxidative DNA damage and inhibition of DNA repair could increase HBV DNA integration, which provided new ideas to study its molecular mechanism. After that, Zhang Lunli made further research on the effects of oxygen-derived free radicals on DHBV DNA integration in infected cells and on the possibility of transmission of integrated DHBV DNA from parent cells to offspring ones (30). In this study, he treated hepatoma cell line LMH-D21-6 with 10 mol/L of H_2O_2. Results showed that the occurrence of integration was 50% at this concentration, while that of integration in the cell without treatment with H_2O_2 was only 8.3%. He also found that the integrated DHBV DNA could be passed on to the DNA of daughter cells. In 2002, Dandri proved again that DSB could promote HBV DNA integration in *in vitro* infectious models (3). Recently, studies by Bill indicated that DNA DSBs might be a potential target for HBV DNA integration and it was possible that this integration can be done through NHEJ (1). Although the effects of DSBs and DNA repair on HBV DNA integration need further verification, these results shed new light on the mechanism of HBV DNA integration undoubtedly.

EFFECTS OF HBV DNA INTEGRATION ON HOSTS

HBV DNA integration into cells may damage the genome stability, cause DNA rearrangement and deletion and even induce canceration. The abnormal translocation of chromosomes was firstly observed in studies on these effects. Simon et al found integrated HBV DNA in HCC tissues and Hep40 cells (20). There was an abnormal chromosome 1p36 in HCC tissues, which did not exist in normal liver cells.

Livezey *et al* found that 2.2.15 cells growed more slowly than Hep G2 cells (13). The former could grow into tumor in nude mice gradually while the latter could not. Besides, several chromosome rearrangements and loss of heterozygosity (LOH) at the site of p53 were detected in 2.2.15 cells. Su *et al* cloned complete HBV DNA from Hep3B cells (23). Analysis of its sequence indicated that the open reading frame (ORF) of HBsAg was kept intact, but pre-S1 gene and C gene were rearranged. X protein was cut short at the carboxyl terminal, but its transactivation for SV 40 enhancer/promoter was reserved. Analysis with S1 nuclease revealed that there were HBV RNAs of 4.0, 2.9 and 2.2 kb in Hep3B cells, and their transcription was carried out under the guidance of pre-S2/S promoter. Somatic-cell hybrid mapping demonstrated that the flanking sequences belonged to chromosome 13 and chromosome 4, respectively, which indicated that HBV DNA integration caused rearrangement of DNA and the transactivation of X protein also played an important role in the formation of HCC.

Tokino *et al* found deletion of large fragments of genomic DNA in liver tissue integrated by HBV DNA and the fragments were 25kb, 12kb and 11kb, respectively (25). Every integron contained small fragments of viral DNA. After analyzing sequence of integrated the random clones, Tokino *et al* found that chromosome 11 and chromosome 17 were the most frequent ones to be integrated, but no specific integration sites were discovered (24). Slagle *et al* hold that there were possibly many reasons for HCC occurrence, and one of them was that the integrated HBV DNA activated the expression of important genes (21). Henderson *et al* found that the sequence at chromosome 18q11.1-q11.2 was located on the left of

HBV DNA and the sequence at the end of chromosome 4 was located on its right, which indicated that HBV-induced HCC could be associated with the translocation of chromosomes (9).

HBV DNA might induce and promote the formation of HCC. Recently, Dejean found that in the cells of HCC, the ORF of chromosomes which contained HBV DNA had encoding constructions homologous to those of V-erb-A and steroid receptor genes (4). Further more, Zhang found fragments similar to oncogene erb-B and rearrangement of base sequence in the cells of liver cirrhosis and HCC (31). In sum, the integration of HBV DNA into liver cell causes chromosome aberration and DNA rearrangement, which may initiate the regulatory sequence of adjacent proto-oncogenes with the help of some carcinogenic agents. If the enhancer of HBV was inserted into a specific site, the transcription of oncogene may be enhanced and canceration induced. As Brechot speculated, the metabolism of liver cells was impaired by HBV infection to lead to the accumulation of carcinogene which induced canceration. Then the cancerous cells reproduced themselves quickly due to HBV DNA integration and gene rearrangement. This process was accelerated by the regeneration of liver cells and abnormality of immunity at the later stage of hepatitis.

In sum, since HBV DNA integration was discovered in 1980s, a great deal of research has been done and great progresses have been achieved. Through these studies, we obtained various methods to detect HBV DNA integration, the status of integration at different stages of infection, putative integration modes and the sequence of integrated HBV DNA as well as that of cellular DNA at integrated sites. And

the roles of HBV DNA integration in the formation of HCC were also confirmed. However, there still exist many problems. Firstly, the complexity and diversity of HBV DNA integration need to be explained systematically and theoretically. Secondly, the mechanism by which DSBs and their repair affect HBV DNA integration needs further study. Thirdly, HCC cells and Hep G2 cells are the most frequently used cell models in which HBV DNA has already been integrated into these cells stably, so the natural process of HBV infection, integration and canceration can not be observed. In a nutshell, there is a lot of work to be done in order to make clear the molecular mechanism of HBV DNA integration.

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