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Molecular mechanisms that Hepatis B virus use to induce Hepatocellular Carcinoma.

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Course: BIO680

December 2020

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Hepatitis B virus infection

Hepatitis B is an infectious liver disease caused by the Hepatitis B virus (HBV). HBV is a small DNA virus that mainly affects hepatocytes, which are the main liver cells, and it has the ability to replicate by reverse transcription. It can cause both acute infection, which last less than 6 months or chronic infection which last more than 6 months. Most of the times newly infected patients do not develop any symptoms, depends on the ability of the host's immune system and their general health. However, some of the most common symptoms are jaundice, dark urine, abnormal abdominal pain, vomiting and fatigue. Chronic HBV infection can cause severe complications such as liver cirrhosis or even liver cancer, called hepatocellular carcinoma (HCC). Hepatitis B virus can be transmitted through blood or any other body fluids and from mother to child during birth. Since 1982 the HBV infection has been preventable by vaccination. Other prevention measures are the use of condoms during any type of sexual activity, all the bloods to be tested for HBV before transfusion. In patients with chronic infection there are antiviral medication for example tenofovir interferon and even liver transplantation is also used to patients with severe liver damage (cirrhosis). The molecular mechanisms of are still not well understood. However, many studies showed that one of the most important causative factors for carcinogenesis is the viral protein HBx. HBx protein is a multifunctional protein and it plays a vital role in the viral replication. HBx regulates the expression of many important cellular and viral genes which are involved in the cell survival (apoptosis), cell replication, DNA repair and protein degradation. It also modulates several signalling pathways including Ras/Raf/MAPK, PI3K/Akt, NF-κB, and JNK. This review will summarize the important mechanisms of HBx-induced hepatocellular carcinoma (HCC), as well its potential use in new therapeutic strategies against the HBV infection.

Hepatitis B is a life-threating liver disease caused by the Hepatitis B virus (HBV). As the name suggests, the virus specifically affects hepatocytes leading to both acute and chronic infection and ultimately liver cirrhosis and cancer. HBV is estimated to affect almost 2 billion people worldwide, according to the World Health Organization (WHO). In 2015, WHO estimated that 257 milion people were infected and living with chronic HBV infection while 887 000 people died from HBV, mainly because of liver cirrhosis and hepatocellular carcinoma (HCC) (Watashi, 2008). More recent studies showed that HBV infection appears to be reduced in Europe, the US and South-East Asia, where people have better access to vaccination and treatment as opposed to third world countries (Africa and Western Pacific) where this is not possible (Liang, 2009). The percentage in these countries ranges between 6,1 and 6,2% comparatively to the percentage of the other countries which ranges

between 3.3% to even 0,7% (Watashi, 2008), (Liang, 2009). Hepatocellular carcinoma (HCC) is the most common type of primary cancer caused by Hepatitis B infection. It is considered to be a highly prevalent and lethal neoplasia worldwide. HCC is the 5th most common cancer in men with 500 000 new cases per year and the 9th most common in women with 200 000 new cases per year. According to the World Health Organization (WHO), HCC is more common in Asia regions where the cases are estimated to be 76% in contrast with European Countries where the cases are 2.1% and North America where the cases are 4.2% of the whole population. The world-wide distribution of the new cases every year as well as the etiological factors for HCC differ for each country. For example, in Eastern Asia, in some African regions and generally third world countries where the living conditions and the medical treatment are extremely poor the main causative factor of HCC is the Hepatitis B infection. Compare with North America, Europe, some regions in Africa and Japan where the main causative factor of HCC is mainly from the combination of excessive alcohol intake, obesity together with Hepatitis C infection, which is caused by Hepatitis C virus (HCV) (Liang, 2009). Hepatis B is commonly transmitted from mother to infant during birth or delivery, there is not enough evidence if the virus can also be transmitted from mother to baby though breastfeeding. Another common way that the virus can be spread is through blood or other body fluids, during any kind of sexual contact with an infected person, between drug users that share the same needles, syringes or any other drug preparation equipment such as needles sticks (Ivanov et al., 2016). It can be rarely transmitted through blood transfusion given the fact that the blood is checked for sexually transmitted disease including Hepatitis A, B and C, before it is transfused to the patient. Even though the virus can be found in saliva it is not proved to be transmitted through kissing, sharing food or utensils, sneezing, coughing and generally by casual contact (Liang, 2009), (Rajbhandari and Chung, 2016). Most of the HBV patients, who are recently infected do not develop any symptoms especially or their symptoms are not specific, this includes loss of appetite, weakness and abdominal pain. However, patients with acute HBV infection or with severe liver damage can develop symptoms that range from mild to serious and even life-threating. These symptoms include jaundice where the skin and the white of the eyes become yellow and this indicates liver disfunction, dark urine, abdominal pain and high fever. Other common symptoms include nausea with or without vomiting, joint pain and fatigue. Some HBV patients with severe liver dysfunction can also develop brain damage called hepatic encephalopathy, because the liver is not able to remove the toxins, such as ammonia which is increased from the intestine, as a result these toxins remain in the body and reach the brain causing confusion, memory loss, problems with concentration, changes in the patient's behaviour and drowsiness. In some cases, they can even lead to coma (Rajbhandari and Chung, 2016). In adults the symptoms usually appear between 1 to 4 months after the infection, however, some signs can be developed two weeks after the infection. in contrast with the younger patients where most of the times

do not develop any symptoms (Liang, 2009). There are various diagnostic method depending on the patient's condition. The main diagnostic method is through blood test which is used to determine the type of the Hepatitis virus, whether is Hepatitis A, B or C. Blood tests are also used to distinguish the acute from chronic HBV infection. The diagnosis focuses on the antigen detection called HBsAG which is locate in the surface of the Hepatitis B virus. This is the main test used in blood donation in order to prevent the HBV transmission to people who received blood products. In order to differentiate acute and chronic infection there are some specific markers, for example acute HBV infection is characterized by the presence of HBsAg and IgM (immunoglobulin M), patients with acute HBV infection, especially in early stages are also positive for HBeAg. While chronic HBV infection the presence of HBsAg can last for at least 6 months with or without the presence of HBeAg. Persistence of HBsAg indicates high risk of liver damage or even the development of liver cancer called Hepatocellular carcinoma (Liang, 2009), (Rajbhandari and Chung, 2016). There is no specific treatment for acute Hepatitis B infection, however treatment targets to control the symptoms and replace the nutritional balance by replacing the fluids that the patient lost from the severe vomiting or/and diarrhoea. Unnecessary medication should be avoided in order to protect the liver from more damage. However, chronic Hepatitis B infection can be treated with antiviral medicines, which have the ability reduce the progression of the infection and by reducing the incidence of liver cirrhosis or/and the development of Hepatocellular carcinoma. Most of the time, patients with chronic HBV infection have to take the antiviral medicines for the rest of their life once they start the treatment (Rajbhandari and Chung, 2016). Nonetheless, in some countries there is limited access to diagnosis and treatment for HBV infection and this results to serious complications and most of the times life-threating conditions for the patients, such as liver damage (cirrhosis) and the development of Hepatocellular carcinoma repetitive. Since the treatment options are limited in some cases and the progression of HCC is rapidly the success for complete treatment is poor. In Third-World countries patients with HBV infection that developed HCC die within months after their diagnosis, whereas in High-income countries, like European countries, North America there are more treatment options including, chemotherapy, surgery and liver transplantations mostly used for patients with liver cirrhosis due to HBV rather than HCC the life expectancy is much higher (Liang, 2009), (Rajbhandari and Chung, 2016)

HBV structure and its components function

Hepatitis B virus is a small double-stranded DNA virus with unusual characteristics similar to retroviruses. The virus belongs to the Orthohepadnavirus species and it is a member of the Hepadnaviridae family, and it has been classified into eight different genotypes from A to H and each genotype has a different geographic distribution. It is considered one of the smallest animal viruses

and its virion has diameter of 42nm. HBV consist of a virus particle, called Dane particle (virion), it also contains an outer lipid envelope and an icosahedral nucleocapsid core where the proteins of the virus are located (Zhang et al., 2019). The nucleocapsid core also contains the Viral DNA and a DNA polymerase which has the ability of reverse transcriptase which is similar to the retrovirus family such as HIV (Yang and Roberts, 2010). The HBV contains pleomorphic forms, such as filamentous and spherical bodies lacking a core which are particle that are not infectious, and they are composed of lipid and protein which form part of the surface of the Dane particle (virion). This is called the surface antigen HBsAg and it is produced in high levels during the viral life cycle. HBsAG was the first protein that discovered, and it is consisted of three regions called the small (S), medium (M) and the large (L) protein. HBV also contains HBcAg which is one of the most important structural proteins of the icosahedral nucleocapsid and it plays a vital role in the viral replication and it is the key factor for infection of the cell. Another important component of Hepatitis B virus is the Hepatitis B protein HBx and it has a viral in the viral replication as well in the development of sever liver damage or/and the development of Hepatocellular carcinoma. The Hbx will be further analyse in a separate section of the article (Liang, 2009), (Zhang et al., 2019).

Viral life cycle

Hepatitis B virus is a non-retroviral virus which uses reverse transcription in order to replicate in the host cell. The virus enters the cells by binds to its receptors located in the surface of the cell, then either through direct fusion or endocytosis the membrane of the virus fuses with the host cell's membrane and releases its nucleocapsid into the cytoplasm of the host (Watashi, 2008). Then, the virus follows the uncoating procedure and it replicates through RNA made using a host enzyme in order the viral DNA to be transferred into the host's nucleus, from the capsid to the microtubules to the nuclear pore. Once the viral DNA enter the nucleus the core proteins dissociate from the partially double-stranded DNA of the virus and then using the DNA polymerase of the host it forms into fully double-stranded and changed into circular DNA (cccDNA) which acts as a template for transcription of the four viral mRNAs. Then, the largest mRNA is used int order to create new copies of the genome as well to make the capsid core protein which leads to the viral RNA-dependent-DNA -polymerase. Further processes occur in order to make progeny virions using the four viral mRNAs (transcripts), which later are released form the cell or returned to the nucleus for produce more new copies with the viral DNA (re-cycled). Finally, the long mRNA is released to the cytoplasm where the synthesis of the DNA takes place by reverse transcriptase which is used by the virion P protein (Watashi, 2008).

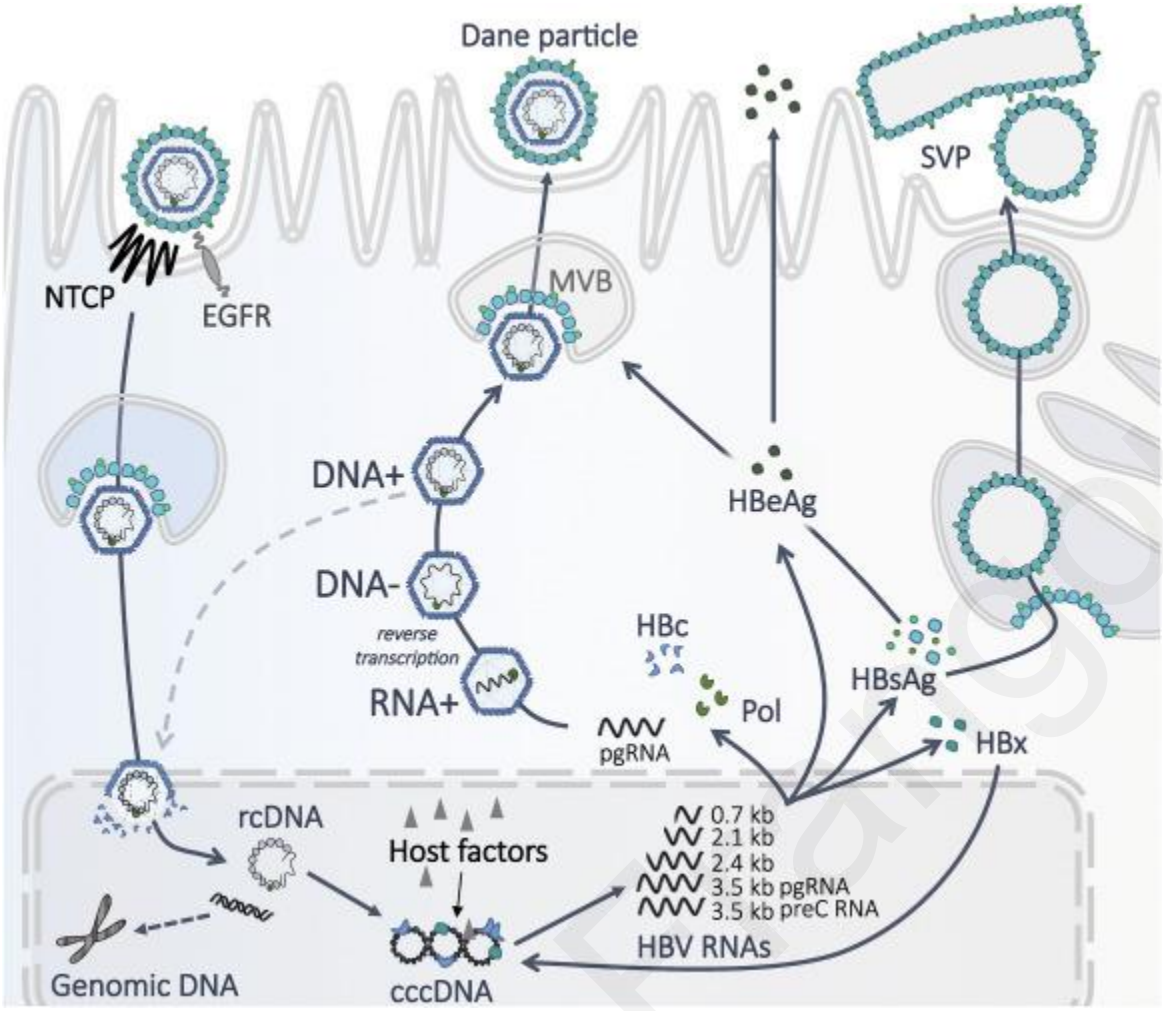

Figure 1: this schematic shows the HBV life cycle from the entry to the release in the cytoplasm of the host's cell and the main components that take part of the viral replication (Watashi, 2008).

Molecular mechanisms of HBV induced liver cancer

Hepatitis B virus has various molecular mechanisms to induce liver carcinogenesis, including DNA damage due to persistent inflammation, the induction of oxidative stress, alteration of endogenous genes as wells as chromosomal instability. Alternatively, HBV can induce hepatocellular carcinogenesis using the viral Hepatitis b proteins HBx and HBs. Additionally, HBV has the ability to regulate the microRNA expression (Ali, 2014). This review will describe some of the above-mentioned molecular mechanisms and focus on the role of HBx protein in the development and the progression of hepatocellular carcinoma (Watashi, 2008).

Immune and inflammatory factors

Inflammation considered to be the key factor in carcinogenesis in different types of tissues including liver tissues. More specific studies showed that chronic inflammation in liver contribute to apoptosis

and hepatocyte regeneration. Hepatocytes are important cells in the liver that have metabolic, secretory and endocrine functions. Both apoptosis and hepatocyte regeneration increase the risk of liver cancer development. HBV infection according to many studies induces NF-kB activation. NF-kB is a vital factor for the regulation of DNA transcription as well as genes which are responsible for innate and adaptive immune response usually through the activation of T- or B- cells. It is also responsible for the production of cytokines and for the cell life cycle. HBV infection causes persistence liver inflammation which lead to the activation and interaction of NF-kB and STAT3 pathway (Ali, 2014). These pathways play a vital role in the development of HCC as they control the communication between inflammatory and carcinogenic cells. NF-kB activation promotes immune escapes, which cause the dysfunction of the immune response cells such as T- and B-cells leading to the development of HCC. STAT3 activation caused by interleukin (IL)-6, IL-6 cytokine family, and IL-22can also cause the formation of HCC (Ali, 2014), (Watashi, 2008).

Apart from NF-kB and STAT3 activation, HBV infection is also responsible for the upregulation immunomodulatory activity of Treg through the overexpression of FoxP3 (forehead box P3 transcriptional regulator), CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) and tumor necrosis factor (TNF). According to Li et al Treg cells were found in liver tissues from HBV-related Hepatocellular cacrcinoma (Ali, 2014), (Zhang et al., 2019).

As a conclusion, HBV affects the immune response cells and it has the ability to cause persistence inflammation in the liver which later leads to the development of HCC (Ali, 2014).

HBV and oxidative stress

Furthermore, studies showed that Hepatitis B virus has the ability to elevate oxidative stress levels, as lipid perocidation and sulfhydryl were observed in chronic HBV patients. Increase level of products of reactive oxygen species (ROS) were also observed in HBV patients due to oxidative (Ali, 2014). ROS can cause DNA, RNA, protein and lipid damage, which lead to cell aging and eventually in the formation of hepatocellular carcinoma. Reactive oxygen species also contribute to the activation of different cellular signalling pathways including, mitogen-activated protein kinase (MAPK), NF-kB, p53 and β-catenin/Wnt and others are related with angiogenesis. These signalling pathways play an important role in mutagenesis, tumor promotion and development reactive oxygen species are considered potential carcinogens. Recent studies in vivo and in vitro have shown that HBV infection can cause oxidative stress. Scientists also found increase levels of peroxide and alanine aminotransferase (ALT) in chronic HBV patients in contrast with asymptomatic carries, as result

oxidative stress plays a main role in hepatic damage and eventually in the formation of HCC (Ali, 2014).

HBV- DNA Integration

Another important mechanism of Hepatitis B virus is that it has the ability to integrate inot the host's DNA. The integration of the HBV into the host genome is achieved in the early stages of both acute and chronic infections. According to studies 80 to 90% of the HBV sequences have been observed in HBV-related Hepatocellular carcinomas. These HBV-DNA insertions have been linked with fundamental genetic alterations in the host's cell genome (Ali, 2014). These alterations include genomic instability such as chromosomal deletions, translocations, changes in miRNA expressions, as well mutations in oncogenes and tumour suppression genes which result in the development of HCC. According to some early studies HBV-DNA insertions occurred randomly into the host genome, however more recent studies have been shown that HBV integrate its DNA close to or inside specific genes for example telomerase reverse transcriptase (TERT), Fibronectin 1 (FN1), cyclin E1 (CCNE1) and much more. These findings suggest that the viral integration in specific genes which control either cellular proliferation or apoptosis or differentiation play a vital role in the development of hepatocarcinogenesis (Ali, 2014).

HBx protein

Hepatis B virus genome encodes 4 viral gene products including Hepatis B X proteins also called HBx. HBx is a 154 amino acid long protein (17k-Da) and it is one of the main viral protein of Hepatitis B. Many studies have shown that it plays a vital role not only in the efficient replication of the virus in the host cell by interacting with the host's protein, but it is also a key factor in hepatocellular carcinogenesis (Ivanov et al., 2016). According to recent studies HBx is overexpressed in the liver and in the hepatocellular carcinoma of the majority of the HBV patients. It is considered a multifactorial regulatory protein and it is responsible of the dysregulation of many vital cellular pathways that affect the normal life cycle of the cell. These pathways include transcription, signal transduction, cell cycle progress, apoptosis, DNA repair, inflammation and it is also responsible for chromosomal instability (Wang et al., 2014). Furthermore, HBx contributes to alterations in the expression of miRNAs and this affects the histone methyltransferase (Zhang et al., 2019). The HBx protein does not have the ability to bind to the DNA of the host however, it acts on cellular promoters by protein-protein interactions and it controls signalling pathways in the cytoplasm. HBx protein has the ability to maintain and transcribe into the host HCC tumor cells even if HBV replication is absent and it achieves that through DNA integration into the host genome. It has different functions in the

cytoplasm and in the nucleus, in the cytoplasm it activates mitogenic signalling cascades and in the nucleus, it regulates gene expression through interaction with various transcription factors. Furthermore, some studies in transgenic mice that express HBx protein had increase level of reactive oxygen species (ROS), as result HBV has various mechanisms to induce oxidative stress (Ali, 2014). ROS are mostly located in mitochondria inside the cells (Wang et al., 2014). The main target of the HBx protein is to bind to mitochondria and more specifically it binds to voltage-dependent anion-selective channel protein 3 (VDAC3) and changes the mitochondrial membrane potential, as a result it elevates the level of ROS inside the mitochondria. Another way that HBx induces oxidative stress is via the cytosolic calcium signalling which leads to changes in the levels of Ca++ into mitochondria which results in high levels of reactive oxygen species as well as the activation of cellular kinases called PYK2 and SRC. Thus, HBx can cause the activation of NF-kB and STAT3 which play an important role HBV replication and also in the early development of HCC. The oxidative stress caused by HBV infection it is an important causative factor of DNA damage, which according to many studies has been observed in mice with HBV-related HCC (Zhang et al., 2019), (Liu, Koh and Lee, 2016), (Ali, 2014).

One of the most important mechanisms of HBx protein is that it targets to the p53 and p53 family inhibition. It achieves that by directly binding negatively affects its function. This interaxction leads to the alterations in apoptosis (cell death), DNA repair and tumor suppressor genes and generally the regulation of the cell cycle, which leads to the development of the HCC (Zhang et al., 2019), (Ali, 2014).

Additionally, more studies showed the HBx protein ability to upregulate the vascular endothelial growth factor (VEGF) and proangiogenic growth factor called angiopoietin 2 (ANG2) which are the main causative factors for angiogenesis in HBV-related HCC (Liang, 2009). HBx has the ability to bind and stabilize HIF1a (hypoxia inducible factor HIF-1 cellular level) transcription which again leads to angiogenesis. process in the hepatocellular carcinoma. As a result, HBx considered to be a regulatory factor of the angiogenic process in the HBV-related HCC (Zhang et al., 2019), (Ali, 2014).

Last but not least, HBx protein has two mechanisms in order to activate the signalling pathway of Wnt/beta-catenin. The first is to upregulate the cytoplasmic beta-catenin and the second is through hypermethylation of the E – cadherin promoter. Consequently, these mechanisms lead to transcription repression (Liu, Koh and Lee, 2016).

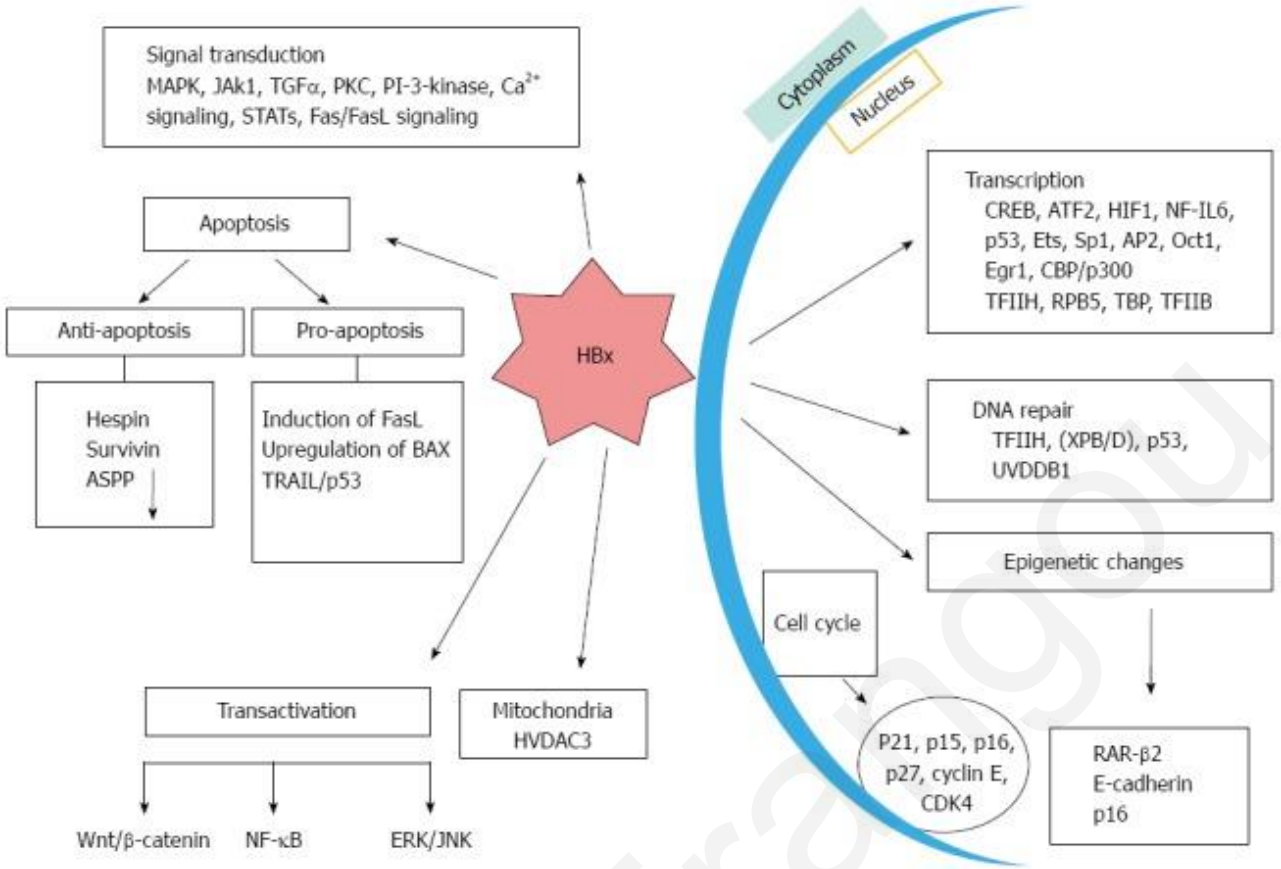

Figure 2: This figure shows the effects of HBx in both cytoplasm and in the nucleus of the host's cell. HBx protein plays important role in many vital pathways for the cell life cycle and has the ability to cause the HCC development through these different signalling pathways (Ali, 2014).

Hbx role in DNA repair

DNA repair is one of the most important processes in the cell in order to avoid mistakes during cell replication. DNA damage is one of the key molecular mechanisms of HBV that contributes to Hepatocellular carcinoma (HCC). HBx plays a vital role in the dysfunction of DNA repair by interacting with the transcription factor IIH (TFIIH), which is a multiprotein complex and it is considered an integral component of DNA repair pathway (Liang, 2009). HBx has the ability to interfere the function of TFIIH leading to increase sensitivity in ultraviolet (UV) and to the reduction of DNA repair capacity. Other studies showed that HBx inhibit the BER pathway, which is a key enzyme in the base excision repair. HBx also contribute to the reduction of another HOGG1 and hMYHa, which are two important DNA repair enzyme for oxidative DNA damage, in mRNA level which eventually lead to the formation of HCC (Liu, Koh and Lee, 2016), (Ali, 2014).

HBx in apoptosis

Another important mechanism that HBx uses that contributes to the development and progression of HCC is by affecting the apoptotic pathway. Many studies have been shown that HBx can inhibit apoptosis (anti-apoptotic features). A great example involves a complex formation and inhibition of p53in the cytoplasm as a result p53 cannot enter into the nucleus. This leads to the dysfunction of the p53 as a result it cannot regulate the effector molecules like Bax, p21 or Fas, which are key factors in apoptotic pathway (Liang, 2009). Therefore, the cell survival increases. HBx also affects the P3K-Akt-Bad pathway, which is another pathway used by p53 in order to cause apoptosis, it achieves that by inhibiting the caspase 3 function by induction of phosphatidyl inostitol-3 kinase and Akt pathway. Caspase 3 is mainly related to the H-ras oncogene which regulates the cell division and this results to abnormal cell proliferation. Another way the HBx affects apoptosis is through p38/MAPK pathway by elevating the levels of anti-apoptotic protein called survivin which lead again to abnormal cell proliferation which in turn cause the development of liver cancer (Liu, Koh and Lee, 2016), (Ali, 2014).

The effect of HBx on miRNAs

MicroRNAs are short noncoding RNAs which have the ability to regulate gene expression. They play part in vital pathobiological processes and abnormal regulation or inhibition of their expression can affect many important pathways including p53, RAS/MAPK, P13K/ATK/mTOR, WNT/β-catenin and TFG-β (transforming growth factor β), which can lead in carcinogenesis. There is strong correlation between Hepatitis B virus and miRNA (Liang, 2009). Many studies have revealed that specific miRNAs have been demonstrated to be upregulated in the development of Hepatocellular carcinoma caused by HBV infection these miRNAs include the three most common miR-143, miR-34 and miR-19. They also considered to cause more aggressive and type of HHC. Furthermore, Hepatitis B protein HBx have been shown to play a vital role in the HBV-related HCC by affecting the regulation of miRNAs (Wang, Wu and Huang, 2017). Through the years, scientists wanted to discover how the HBx can affect the miRNA expression during the development of HCC. Recently scientists discovered three molecular mechanisms in which HBx regulates the expression of miRNAs (Ali, 2014). One of the mechanisms that HBx use to change the expression of miRNAs is to affect their production process through reducing the cofactor of Drosha called DGCR8. Another study indicates that HBx induced the expression of the regulatory Y11 which can also inhibit the activity of DGCR8. Another mechanism is that HBx downregulates Let-7a and leads to an increased abnormal cell proliferation (Wang, Wu and Huang, 2017). Additionally, HBx downregulates another miRNA called miR-152, which in turn upregulates the DNA methyltransferase 1 (DNMT1), which is a specific enzyme that cause the methylation of various tumour suppression genes leading to the development of HHC (Ali, 2014). Other studies demonstrate that another equally important miRNA

is the miR-221, which is also has strong correlation with HBV-related HCC (Liu, Koh and Lee, 2016. miR-221 have been shown to be downregulated during acute HBV infection whereas in chronic it does not have any significant change in its expression and it also have been found to be upregulated in HHC patients (Wu et al., 2013). HBx protein also have the ability to control the miRNA expression and effects directly miRNA-related targets, such as miR-145. Recent studies have shown that HBx protein inhibits the expression of miR-145, which results in the release of the CUL5 (Cullin-5) which plays a significant role in the cell life cycle (Wang, Wu and Huang, 2017). Additionally, HBx produces high the levels of miR-7, -21 and -107, which directly inhibits the function of maspin, protein and leads to the development of HCC. The inhibition of maspin protein also cause chemoresistance which makes the several treatment options unsuccessful. As a conclusion, HBx has the ability to downregulated the expression some targeted miRNAs and upregulated the expression of other targeted miRNAs, however the result is the same as in both conditions as they cause the development of HCC either through abnormal cell proliferation, inhibition of cell apoptosis or excessive cell differentiation (Wang, Wu and Huang, 2017), (Liu, Koh and Lee, 2016), (Ali, 2014).

Finally, all these findings are great tools for the design of novel and more effective therapeutic strategies which will target specific mechanisms of the Hepatitis B virus which are used in order to achieve replication and the development of HCC. (Slagle and Bouchard, 2018)

Main part

From Acute to Chronic HBV Infection.

Hepatitis B virus infection is one of the major causative factors of the Hepatocellular carcinoma (HCC) development and it is considered one of the major health problems worldwide. Some patients can develop acute (short-lived) or/and chronic (long lasting) HBV infection. Most of the times newly infected patients with Hepatitis B virus especially adults with clean medical history achieve complete clearance of the virus within a period of few months. However, other patients may start with acute HBV infection but due to fact that their immune system is not able to kill the virus or because they are already infected with another virus usually HIV develop a chronic HBV infection. Acute infection lasts less than six months whereas chronic infection lasts more than six months or even a lifetime. Whether the HBV infection will be characterised as an acute or chronic infection is depended on the host's immune system. Chronic infection has different clinical features as some of the patients are asymptomatic carries of the HBV but with normal hepatic histology while other patients have severe

liver complications including liver cirrhosis or they develop hepatocellular carcinoma (HCC). According to many studies chronic infection is the main causative factor for severe liver damage (liver cirrhosis) and eventually for the development of Hepatocellular carcinoma (HCC). Chronic infection is more common in younger age, especially new-borns or children under the age of 5. It also known that patients with chronic infection may not develop any symptoms and the virus will be undetected for decades until a person develop severe symptoms that indicate liver damage. There are four phases of chronic infection but not all the CHBV patients go through all of them. The four phases include the immune tolerance stage, immune clearance stage, inactive HBsAg carrier stage and reactivation stage. The reactivation stage is considered one of the most dangerous stage because the immune system of the patient is already weakened and it is not able to fight against the virus, which often leads to serious complications. The reactivation stage may occur more than once, especially in immunocompromised patients or patients with coinfection with other disease for example HIV. This situation makes the treatment even more difficult and also increase the duration of the infection. Many studies also found that certain medicines have the ability to reactivate HBV infection as a result patient with HBV must be careful with other medications they may need.

Newly infected patients or patients with early childhood-acquired HBV infection go through the immune tolerance stage which is characterized by increase HBeAg and serum DNA level, but the levels of the hepatic enzyme aminotransferase is at normal levels. Patient in this stage also have no significant or even no inflammation on their liver biopsy. This is a result of immune tolereance to HbeAg. It is also stated that HBeAg promote HBV chronicity as it functions as an immunoregulatory protein. These clinical features are rarely observed in adult patients and in patients with chronic HBV infection. HBV patients in this phase are considered at low risk of develop serious complications such as cirrhosis or development of HCC. In this stage antiviral therapy is not necessary but the patients should under observation in order to monitor the progression of their infection.

Chronic HBV infection stages

Immune clearance stage:

In this stage the host's immune system there is a nonspecific increase of inflammation in the liver or/and a reduction in the concentration of HBeAg in the serum. This may be a result of the promoter region or pre-core region mutants. One of the main characteristics of this phase is the persistent increased levels of the ALT hepatic enzymes as well increase viral DNA levels. Even though these levels are higher during the immune tolerant phase. Liver biopsy during the immune clearance stage will indicate liver damage as a result of necroinflammation of the hepatic cells. Sometimes signs of liver fibrosis may occur due to the tissue damage. Another common characteristic of this stage is the

appearance of spontaneous flares which indicates an increased immune response against the virus, which are usually accompanied with an increase of HBV DNA level. In immune clearance stage some patients develop symptoms of acute HBV infection whereas others are asymptomatic carriers. This depends on the levels of the HBV DNA in the host and the levels of the liver damage. The prognosis of acute flares is relatively good as they can be detected early due to the fact that they accompanied with high levels of ATL hepatic enzymes and thus the chances of complications are reduced. Recents studies showed that the duration of this stage is strongly connected with the development of complications such as liver cirrhosis or the progress of hepatocellular carcinoma. Usually older patients (after the age of 40) have an increase risk factor of severe complications than patients before the age of 30 even though they have the same clinical features. This stage can be also observed in the other family member of the hepatotropic viruses including Hepatitis A, Hepatitis C and Hepatitis D, especially in patients with an increased risk factor (Tarocchi, 2014).

Inactive HBsAg carrier stage

This stage of the infection is the most common in the patients with chronic HBV infection. It is an inflammatory stage where HBV infection leads to HBeAG seroconversion, which means the development of an increase levels of antibodies in the host's blood that are able to detect the virus. Many studies revealed that after seroconversion the majority of the patients have negative HBeAg test, but they are tested positive for its antibody anti-HBeAg. Most of the patients in this phase have undetectable HBV DNA level where some of them are detected with low HBV DNA levels. The results of the liver biopsy during the inactive HBsAg carrier stage vary as some patients may have mild liver inflammation to negligible fibrosis while other are detected with inactive liver cirrhosis. the results of the liver biopsy are strongly connected with the severity of the infection during the immune clearance stage. The progression of the infection in this stage is usually leads to benign tumours.

Reactive stage

Finally, the reactive stage is a very important stage because the host's immune system is already weak, and many patients already have liver damage which makes the situation worse. This stage may cause severe complication such as sever liver damage and HCC development. Chronic infection patients divided into categories the chronic HBeAg-negative patients (inactive)and the chronic HBeAG-positive (active) patients. Many studies showed that reactivation stage has some difference between the two groups. For example, the hepatic enzyme ATL and the HBV DNA levels are lower in HBeAG-negative patients than the HBeAg-positive patients. Also, the fibrotic activity is higher in

the HBeAg-negative patients. However, necrotic inflammation is similar in both HBeAg- positive and -negative patients.

HBx and innate immunity

There are several mechanisms that the mammalian immune system uses in order to successfully detect and clear a viral infection, the innate and the adaptive immunity. The innate immunity is the first line of immune defence in order to limit an infection and achieved that by detecting the viral pathogens. On the other hand, adaptive immunity is able to detect recognizing peptide antigens through the receptors expressed on the surface of B and T cells (Croagh and Lubel, 2014). The HBV protein called HBx has various strategies for affecting the host's immune system in order to promote the viral replication as well as the development of Hepatocellular carcinoma (HCC). As already mentioned innate immune response is responsible for the detection and elimination of the foreign substances (viral antigen), one of the main characteristics of the innate immune system is the production of cytokines, type I interferon-α/β also called INF-α/β and the activation of Natural killer (NK) cells. Many studies have shown the correlation between the Hepatitis B X protein (HBx) with MAVS (mitochondrial antiviral signalling), as a result it promotes the degradation of MAVS by the the MAVS protein Lys136 in order to avoid the induction of INF-β. Additional analysis of MAVS on clinical samples showed that MAVS is downregulated in the HBV-induced HCCs and that HBV replication also inhibits the function of INF in HepG2.2.15 liver cells. Finally, according to many studies the viral protein HBx reduce and weakens the innate immune response and promotes the development of HCC. These results suggest some of the main functions of the viral HBx protein which cause the dysfunction of the host's immune system, the progression of the HBV infection as well as the HBV-induced hepatocellular carcinogenesis (Zhang, Wang and Ye, 2014).

HBx and adaptive immunity

CD8+ T cells are the main responsible for the HBV infection clearance in the host, dysfunction or even lack of CD8+ T cells can lead to the progression from acute to chronic HBV infection or/and to the Hepatocellular development. Many studies showed that HBx has the ability to decrease the production of interferon-γ and the apoptosis of CD8+ T cells. One of the most important factors in carcinogenesis is that immune cells (immunocytes) can affect the function of the adaptive immune system. The disulphide-linked heterodimeric cytokine Interleukin-12 (IL-12) has an immunostimulatory function and recent studies suggested that simultaneous expression of HBx and Interleukin-12 can cause an increased accumulation of CD8+ T cells. This accumulation of CD8+ T

cells results in the inhibition of stromal cell development including the vascular endothelial cells (Croagh and Lubel, 2014). Furthermore, flow cytometric analysis in patients who received the HBV vaccine showed an increased CD8+ T cells lymphocyte production in contrast with the patients that did not receive the vaccine. Other studies in vivo, showed that the reduction of CD8+ T cells had an antitumor immune activity. Finally, the results showed the strong connection of the HBx-related adaptive immunity in the development and the progression not only of the HBV infection itself but also in the development of hepatocellular carcinoma (Zhang, Wang and Ye, 2014).

Hbx enhances cell transformation

Many studies investigated that one of the main factors of hepatocarcinogenesis is the HBx protein, using HBx-transgenic mice scientists demonstrated that HBx gene expression can cause alterations in the normal cell growth and function. The main cells that are affected by HBx are the hepatocytes which showed an abnormal cell cycle proliferation in the presence of HBx (Croagh and Lubel, 2014). One of the most important property of HBx is that it has the ability to inhibit G1/S transition of the hepatocyte cell proliferation which results in the liver dysfunction and eventually in cell apoptosis in the HBx -transgenic mice. HBx gene affects the cell life cycle and development in normal cells as well. Many in vivo and in vitro studies revealed the carcinogenic properties of HBx. Furthermore, HBx can cause cancer by increasing the transcription of NFkB, AP-1 and surviving. Another characteristic of HBx is that it can act as a pro-apoptotic factor and cause abnormal and uncontrolled cell death but at the same time it can act as an anti-apoptotic factor and cause the stimulation of cell proliferation. According to these results HBx is considered one of the most important factors of HBV infection progression and also an activator of hepatocarcinogenesis (Zhang, Wang and Ye, 2014).

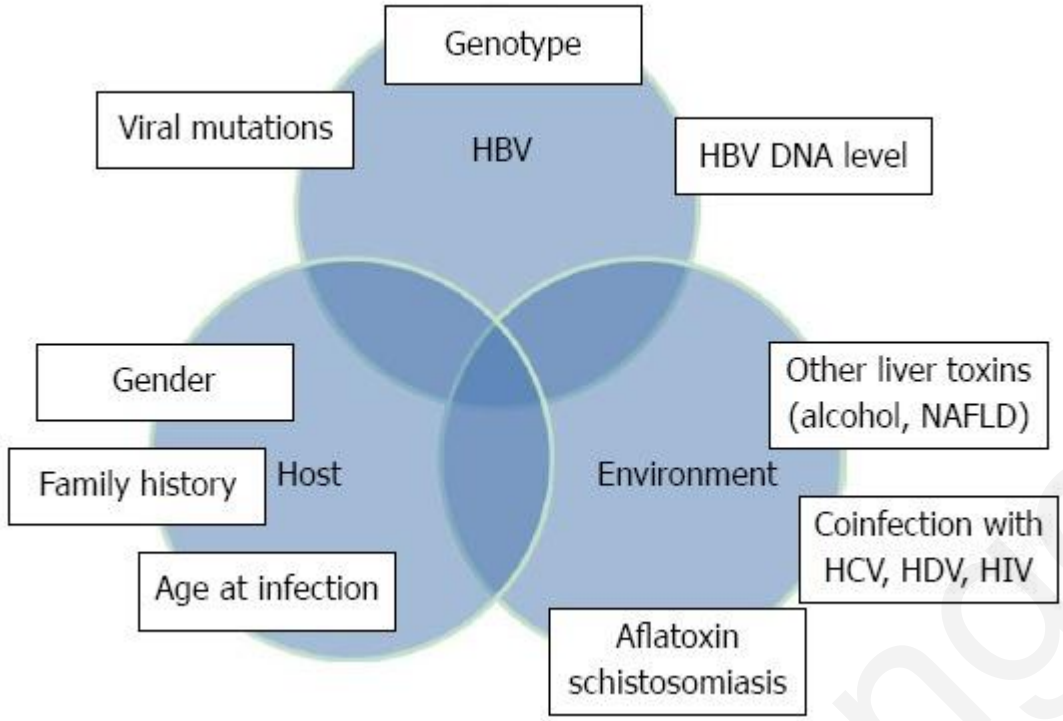

Figure 3: A schematic that shows the factors that can lead to chronic HBV infection (Croagh and Lubel, 2014).

HBV cause oxidative DNA damage

Many studies showed that there are strong interactions between viruses and their viral products such as proteins and several DNA damage response (DDR) pathways. HBV infection is a great example of these interactions. The Hepatitis B virus has been proven to interact with DDR proteins in order to replicate and generate hepatic carcinogenesis. HBV has the ability to hijacks cellular factors such as DNA damage response in order to achieve some vital steps of its life cycle. HBV proteins, especially HBx, have the ability to deregulate certain DDR pathways either directly through binding to DDR proteins or indirectly through the control f different intracellular DNA damage response pathways that affect DNA repair mechanism. The HBx vrial protein as it is already mentioned has a multifunctional role and according to many studies HBx is expressed from the very early stages of the infection. HBx is considered as a key factor of the progression of the HBV infection. This study demonstrates the importance of HBx protein in the different DDR pathways. One of the most important and well-known interactions of HBx is the interation with DDB1 protein. This interaction is implicated in important processes including sensitization of hepatic cells to ultraviolet radiation, apoptosis of the hepatocytes and S phase progression.

In *Hagen et al., 1994* study scientists demonstrated the presence of oxidative DNA damage during chronic active Hepatitis B infection and they hypothesised that oxidative DNA damage may be an important causative factor for hepatocellular carcinogenesis. Using a transgenic mouse model which overexpressed a specific HBV large envelope protein. They observed that this specific protein causes neuroinflammatory liver disease which eventually leads to hepatocellular carcinogenesis. They also observed that this mouse strain develops characteristics of human active chronic HBV infection including hepatocellular necrosis (severe liver damage) and hepatocellular hyperplasia. They used a certain histological marker NTT in order to indicate the presence of reactive oxygen species in the livers of the transgenic mice. They observed an overproduction of the HBV large envelope protein that accumulates in the endoplasmic reticulum which leads hepatocellular necrosis and inflammation, which in turn results in the development of small preneoplastic hyperplastic foci in the mice liver. This preneoplastic hyperplastic foci leads to the development of benign hepatic tumours and then eventually in the formation of malignant lesions that destroy the liver of the mice and cause their death. They wanted to check the correlation between the inflammation caused by the HBV result to increase levels of oxidative DNA damage, so they checked the levels of oxo^{8}dG in different liver tissues of the non-transgenic and transgenic mice. They found that there is an increase level of oxo^{8}dG in liver cells with severe injury, especially in cells with microplastic nodular hyperplasia (MNH), which indicates that oxidative DNA damage occurs in sever tissue damage. Furthermore, many studies showed that the oxidative DNA damage increases during aging. In *Hagen et al., 1994* study scientists confirmed that as they also observed an increased level of oxo^{8}dG in older transgenic mice in contrast with younger transgenic mice.

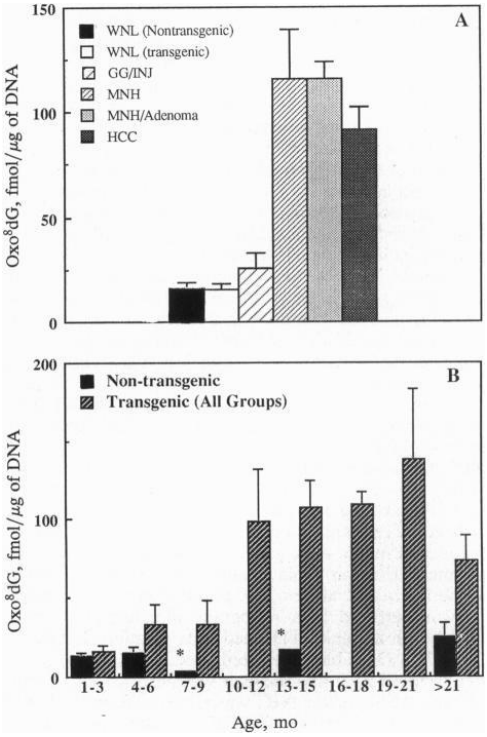

Figure 4: Oxidative DNA damage in transgenic mice. **(**A)This figure shows the rate of the oxidative DNA damage in wild type (non-transgenic) and transgenic mice which with severe liver injury. Oxo8dG is significantly increased in mice with signs of microscopic nodular hyperplasia (MNH), adenoma and HCC. (B). This figure shows the rate of oxidative DNA damage according to age (Hagen et al., 1994).

Furthermore, many studies have shown the correlation between HBV and more specifically its product HBx protein with the production of reactive oxygen species. According to *Ha, 2010* scientists showed that the cell lines that expressed HBx protein had increased ROS levels.

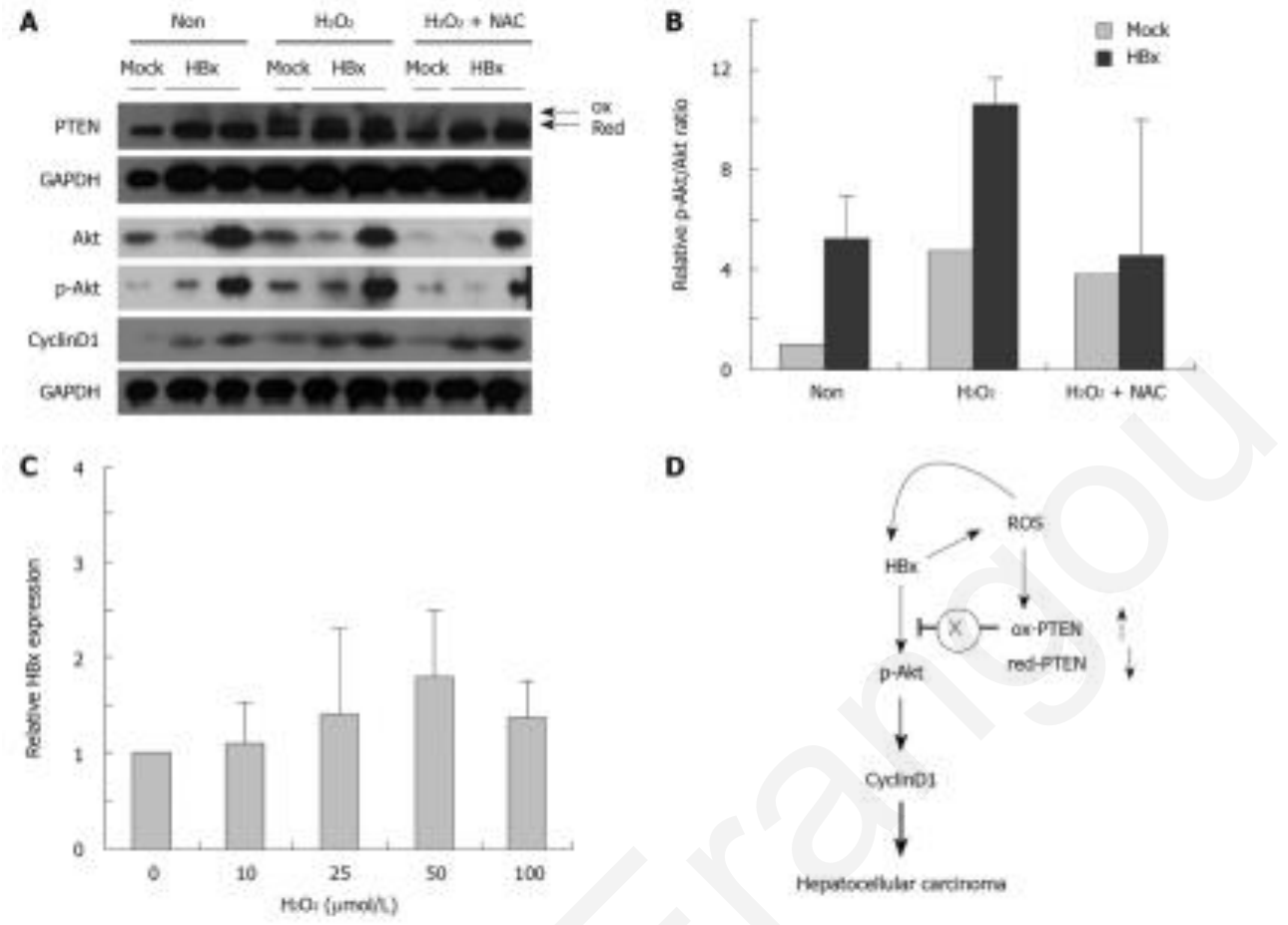

Figure 5: Effects of ROS on Akt pathway and HBx expression. (A, B): Shows the activation of AKkt pathway, phosphatase and tensin homolog induced by the treatment with H_2O_2. (C): Shows the effects of ROS in the HBx protein expression (D): A proposed schematic showing the effects of ROS in the activation of Akt pathway through the PTEN oxidation in HBx-induced HCC (Ha and Yu, 2010).

In this study scientists observed an increased cell proliferation in the transgenic mice, which concluded that cell necrosis and abnormal cell proliferation in a high oxidant environment is strongly correlated with neoplastic transformation in these mice and consequently to patients with chronic Hepatitis B virus infection. This abnormal cell proliferation leads to the unwinding of the double-stranded DNA and also to the removal of histone which have a protecting role. As a result, allows the exposure of DNA to reactive oxygen species. Furthermore, abnormal and uncontrolled cell division leads to reduce repair of any DNA damage, which cause excessive generation of mutation to the primary cells as well as in the daughter cells. Thus, chronic cell damage from the HBV infection have similar effects on DNA mutagenesis with ionizing radiation, as a result it confirms that HBV infection is one of the key factors for HCC development (Ha and Yu, 2010).

HBx protein in apoptosis through different cell signalling pathways

Apoptosis is a vital biological process and it is a form of programmed cell death which occurs in multicellular organisms. Apoptosis is characterized by various biological and biochemical events which lead to important cell alterations and eventually in cell death. Both excessive apoptosis and not apoptosis at all are strongly connected with carcinogenesis, including hepatocellular carcinogenesis. Many studies have proved that the HBV viral protein HBx has proapoptotic and antiapoptotic properties. A study in primary rat hepatocytes showed the multifunctional viral HBx protein regulates various important cellular signal transduction pathways including the apoptotic pathway. This study demonstrates that HBx has the ability to modulate apoptosis in primary rat hepatocytes through the regulation of both NF-kB signalling pathway and the mitochondrial permeability transition pore. Firstly, the cells were stained with early and late apoptotic markers, annexin V and 7-AAD respectively and the scientists observed low levels of apoptosis in those cells (primary rate hepatocytes). The finding after FACS analysis indicate that HBX inhibits spontaneous apoptosis. Then the scientists wheather HBx has the ability to inhibit TNF-α-induced apotptosis. TNF-α is a cytokine which is highly expressed during the chronic HBV infection. The scientists found that apoptosis is significantly increased in the primary rat hepatocytes through the addition of both cytokine TNF-α and the protein synthesis inhibitor CHX. They observed that TNF-α alone decrease the levels of apoptosis in the control rat hepatocytes while in the HBX-expressing rat hepatocytes TNF-α-Induced apoptosis was inhibited. As a result, HBx has the ability to inhibit apoptosis in primary rat hepatocytes through TNF-α cytokine. The scientists wanted to further examine that HBx has the ability to inhibit apoptosis in primary rat hepatocytes, they observed the levels of cleaved caspase 3. Cleaved caspase 3 is considered an inhibitor of late-stage apoptosis and play an important role in the programmed cell death. The scientists observed reduced levels of apoptosis in HBx-expressing hepatocytes, in contrast with the control hepatocytes. Furthermore, they found that Bx inhibited the TNF-α induced elevate in the levels of cleaved caspase 3. Finally, they observed la decrease levels of cytosolic cytochrome c in the HBx-expressing hepatocytes in contrast with the control hepatocytes in assays with apoptosis, which indicates the antiapoptotic effect of HBx. The antiapoptotic properties of HBx was more visible in the treatment of TNF-α. Their results showed that HBx inhibit both spontaneous apoptosis ad TNF-a -induced apoptosis in primary rat hepatocytes. (Clippinger, Gearhart and Bouchard, 2009).

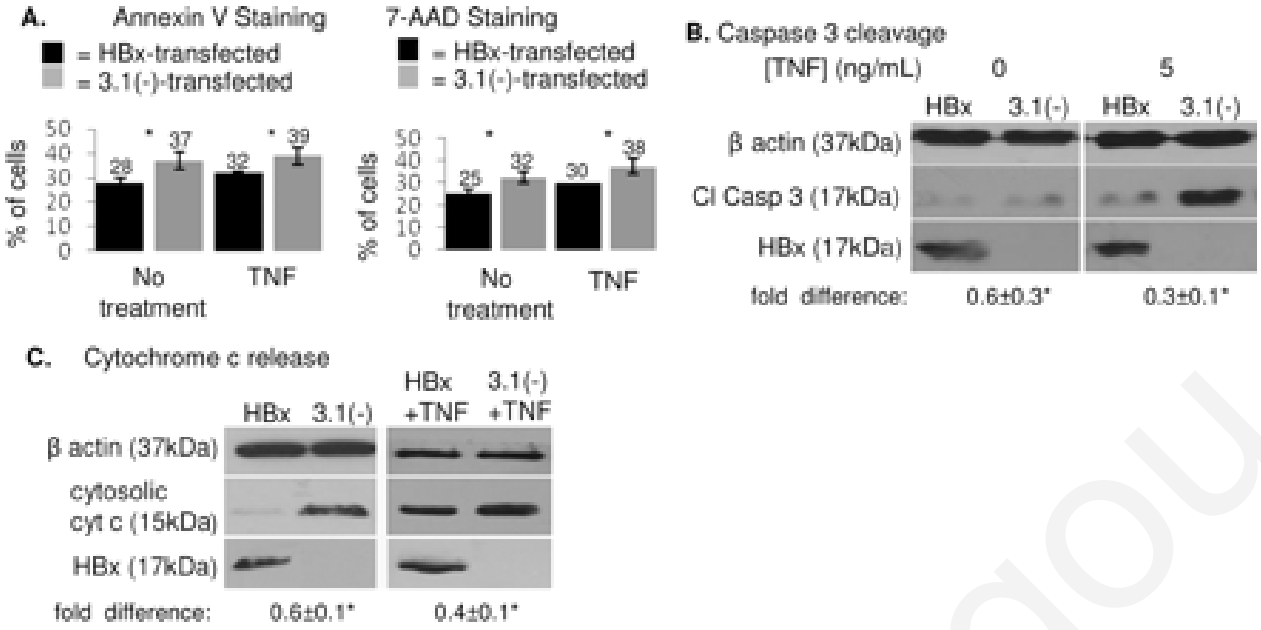

Figure 6: HBx viral protein prevents spontaneous and TNF-α-induced apoptosis in primary rate hepatocytes. The primary rat hepatocytes were treated with TNF-α 30 hours after transfection. (A). Shows the statistical analysis of hepatocytes after they were stained with annexin V and 7-AAD. (B). Western blot analysis of anti-β-actin, anti-cleaved caspase 3, Cl Casp 3 and cleaved caspase 3. (C). Western blot analysis showed the expression of β-actin and cytochrome c (Clippinger, Gearhart and Bouchard, 2009).

The scientists wanted to understand better the mechanism that is used by HBx to induce apoptosis, so they treated HBx-expressing hepatocytes with an inhibitor protein synthesis called cycloheximinde (CHX). They observed that HBx induces caspase 3 cleavage, which means that apoptosis is strongly connected with a continual protein synthesis and that inhibition of protein synthesis leads to stimulation of apoptosis by HBx. Since cycloheximide (CHX) treatment has the ability to inhibit the production of NF-kB-regulated proteins and due to the fact that HBx protein is responsible for the activation of NF-kB scientists wanted to examine the role of NF-kV activity and HBx-mediated apoptosis. They found that HBx caused apoptosis in hepatocytes that were cotransfected with an inhibitor of Nf-kB signalling activity called IKB supperrepressor. Their results showed that HBx inhibition of apoptosis is directly connected with the activation of NF-kB (Clippinger, Gearhart and Bouchard, 2009).

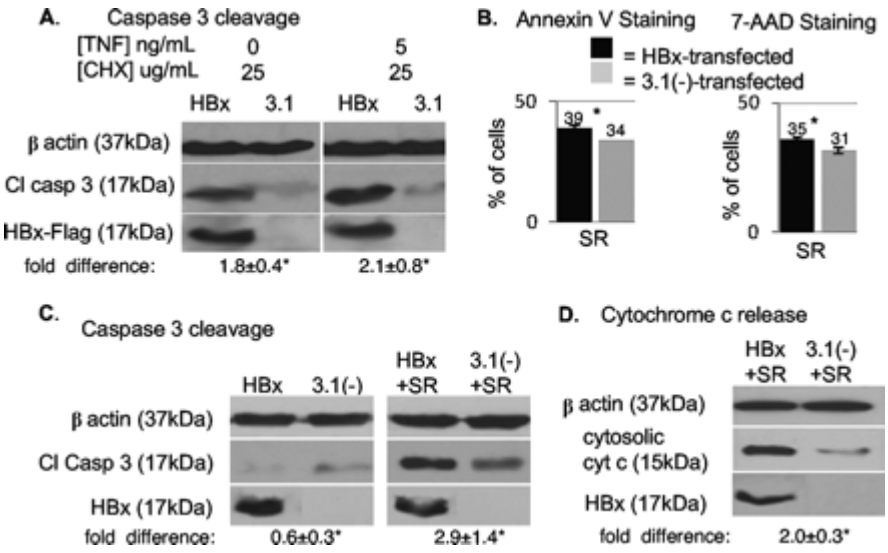

Figure 7: HBx protein causes apoptosis in the absence of NF-kB signalling pathway activity. (A). Shows HBX-expressing that were treated with the inhibitor protein synthesis CHX. (B). Shows a FACS analysis of the rat primary hepatocytes which are stained with annexin V and 7-AAD. (C). Shows a western blot analysis for β-actin, cleaved caspase 3 and HBx viral protein. (D). Shows a wetsrn blot analysis in hepatocytes that were cotransfered with FL1-154 HBx and IKB-SR (Clippinger, Gearhart and Bouchard, 2009).

Finally, many studies showed that HBx can interact the with components of the MPTP and control MPTP activities and mitochondrial membrane potential, the scientists wanted to examine whether HBx has the ability to induce apoptosis in the absence of NF-kB activity. Thus, they examined the possibility that HBx may cause apoptosis through MPTP. Their results showed that HBx is not only dependent to the activation of NF-kB to induce apoptosis, but it can also cause apoptosis by the regulation of the MPTP (Clippinger, Gearhart and Bouchard, 2009).

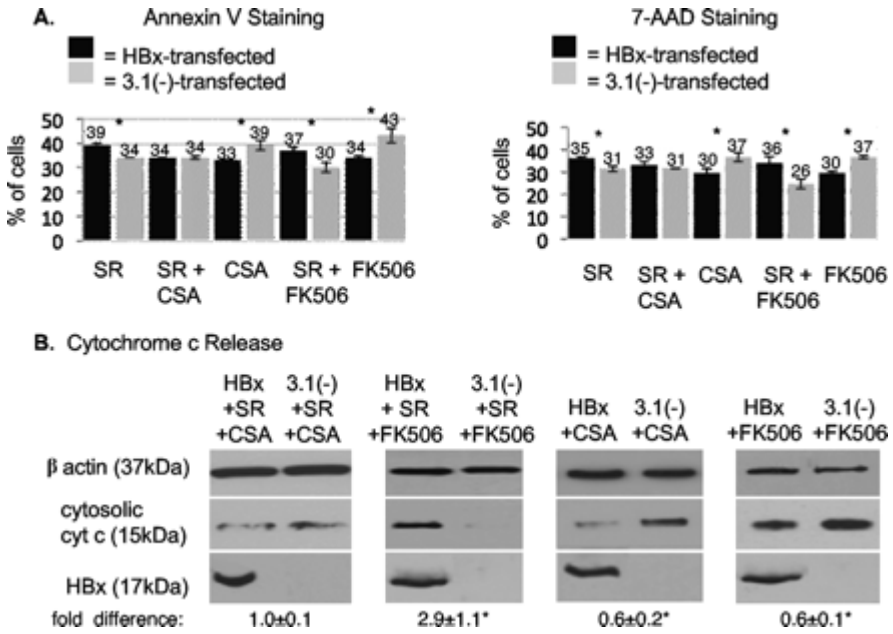

Figure 8: HBx protein in the absence of NF-kB signalling pathway activity via an MPTP-dependent mechanism (Clippinger, Gearhart and Bouchard, 2009).

Their results (Clippinger, Gearhart and Bouchard, 2009) demonstrate the multifunctional properties of HBx. They found that activation of NF-kB signalling pathway through HBx protein inhibits the activation of apoptotic pathways whereas when the HBx-induced activation of NF-kB signalling pathway is inhibited HBx can induce apoptosis. They also observed that by inhibiting the activity of the mitochondrial permeability transition pore has the ability to block the HBx activation of apoptosis. Therefore, their results propose that HBx has proapoptotic or antiapoptotic effects on primary rate hepatocytes. These effects are strongly connected with the activation status of NF-kB signalling pathway as well with the effects of the HBx in the modulation of mitochondrial permeability transition pore. Finally, their results showed that the effects of the viral protein HBx on the apoptotic pathways are directly connected with the development of hepatocellular carcinogenesis in HBV patients.

Hepatitis B virus effects apoptosis, mainly its viral product HBx regulatory protein. HBx and its mutants are highly expressed in the HBV genes. The HBX protein has two main functional domains called C- and N- terminus and any deletion in the full – length HBx or its mutants may cause alterations in the functional domains and affects the regulation of cell replication, cell survival (apoptosis), transactivation and protein-protein interactions. According to many studies HBx protein has the ability to activate or inhibit cellular apoptosis, rarely it has not any significant effects on apoptosis and some studies suggested that it has the ability to sensitize liver cells to other growth factors that are responsible for cell survival. These observations lead to the fact that HBx protein affects various important steps of the viral life cycle or even plays an important role in the progression

of the chronic HBV infection (Slagle and Bouchard, 2018). HBx protein has a dual role in regulating apoptosis. Some studies showed that HBx has an anti-apoptotic role by activating the transcription factor NFkB which is an activator of anti-apoptotic signals where other studies investigated that HBx has a pro-apoptotic role as it has the ability to inhibit NFkB. Further studies in primary hepatocytes showed that HBx has the ability to activate AKT which is another activator of anti-apoptotic signals. This anti-apoptotic role of HBx leads to abnormal hepatocyte proliferation and promotes cell survival which in turn cause increased levels of HBV replication. In contrast with other studies where showed that HBx protein induced apoptosis by inhibiting AKT and this inhibition stimulates the HBV replication in hepatocytes. Scientists concluded that HBx pro-apoptotic role is achieved either through the activation of NFkB or the activation of AKT (Slagle and Bouchard, 2018). Additional studies revealed that HBV and especially HBx protein increase the expression of Bax which is a pro-apoptotic factor and decrease the expression of anti-apoptotic Bcl-cL (Zhang et al., 2019), which has the ability to sensitize hepatocytes to pro-apoptotic signals without inducing apoptosis directly. Also, HBx protein leads to an increase inhibition of Bax by the HBx mutants HBx (31-154) and HBx (61-254) which have C-terminal deletion. In conclusion, full – length HBx and all its mutant have directly effect on the expression of certain cell cycle regulators and each cell cycle regulators has different response in the full-length HBx or its mutants. For example, Bax was better modulated by the mutant HBx (61-124) and p53 by the mutant HBx (1-94). Scientists ended up that the pleiotropic effect of HBx on different cellular reregulation is due to the co-existence of different mutants (forms) of the HBx (*Tu et al. [20]).*

HBx protein can also affect apoptosis pathway through mitochondria, which can regulate the cell survival. According to recent studies HBx expression increase the levels of reactive oxygen species (ROS) which usually derive from mitochondria, decrease the expression of oxidative phosphorylation enzymes in mitochondria and induce pro-apoptotic signals in hepatocytes (81). Another way that Hepatitis B virus and its protein HBx can induce apoptosis is through the induction of mitophagy in liver-derived cells (primary hepatocytes). In this case activation of mitophagy acts as a cell protector and as a result the virus can replicate into the cells. General, Hepatitis B virus is considered as a noncytopathic which means that the cells are not killed during the virus replication or infection. The persistent HBV replication is due to the activation of anti-apoptotic signals from HBX protein and when HBx activates pro-apoptotic signals they kill the infected cells. However, future studies on the anti- and pro- apoptotic signals are necessary in order to determine the exact effects in the HBV lifecycle or the influence in the stages of HBV infection (Zhang et al., 2019).

Scientists then wanted to check the effects of wild type HBx protein and its mutants in apoptosis. Firstly, they confirmed that HBx and its mutants are highly expressed in the HBV genes. According

to past studies HBx protein has two main functional domains called C- and N- terminus and any deletion in the full – length HBx or its mutants may cause alterations in the functional domains and affects the regulation of cell replication, cell survival (apoptosis), transactivation and protein-protein interactions. In this study, *Al-Anazi et al., 2018*, the scientists demonstrate the carcinogenic potential of the full -length (wild type) HBx protein in contrasts with its mutants. It also highlights the alterations caused by the HBx protein and its mutants in the expression of various effector molecules which are involved in the cell cycle proliferation and survival (Tarocchi, 2014). They found that HBx has the ability to deregulate cell cycle checkpoints through expression or proteolytic degradation of cyclin-dependent kinase inhibitors, which results in the abnormal and uncontrolled cell proliferation. P27 and p21 are cell cycle regulators, which are active during the G1 phase of the cell cycle and inhibit in the G1/s phase transition and they inhibit the function of the cyclin E-cdk2 complex by directly binding to it. This study showed that both wild type of HBx and all its mutants have the ability to downregulate p21 expression as well as the p53 expression. This results in the inhibition of the p21 and suggest that there is a strong correlation between p21 and p53. Furthermore, they showed that p53 was also downregulated by the full-length of HBx and all its mutants and that HBx (61-124) has amino acids (58-119) that plays an important role in signal transduction. While the missing C- an N- terminus seems to be the main causative factor for its enhanced activity in downregulation of p53. Many studies showed that HBx. This study also reveals that the downregulation of p53 may also be a result of the increased HBx binding to Myc (p53 promoter) (*Al-Anazi et al., 2018)*.

Another anti-apoptotic effect of HBx is that it inhibits the caspase 3 activity, which is a cysteine – aspirate protease and its main function is to cleave various substrates such as PARP which are responsible for cell apoptosis. *Al-Anazi et al., 2018* demonstrated that all HBx mutants had similar inhibitory effect on the PARP expression, especially the Hbx (61-124) mutant. Their results showed that C-terminal deletion mutations of HBx has a decrease in the inhibition of caspase activity. Furthermore, HBx blocks p53-mediated upregulation of Fas, which has the ability to activate caspase 3 activity. They also observed that the HBx mutants decrease the regulation of Fas-mediated caspase 3 activity which in turn increase the inhibition of cleaved PARP and caused the inhibition of pro-apoptotic signals. As a result, wild type HBx protein and its mutants have direct effect on the expression of the cell cycle regulatory molecules. C- terminal deletions showed an increased carcinogenic potential of HBx while N- terminal deletions played a vital role in the upregulation of cell cycle checkpoint regulators. However, more studies are necessary in order to understand in detail the exact mechanisms of HCC development by HBx and its deletion mutants (*Al-Anazi et al., 2018)*.

HBx protein induced inflammatory response in human LO_2 hepatocytes through the regulation of GPR43

The *He et al., 2020* study showed that G coupled-protein receptor 43 (GPR43) which is also known as free fatty acid recptor 2 (FFAR2) has a regulatory role in the cytotoxic effects of HBV through transfecting the viral protein HBx into the human LO_2 liver cells (hepatocytes). This transfection of HBx into the suppresses the LO_2 hepatocytes reduce the expression of GPR43. *He et al., 2020* was the first study to demonstrate this role of GPR43. According to many studies HBx protein has the ability to activate and release proinflammatory cytokines such as Interleukin-6 (IL-6), MCP-1 and high mobility group box 1 protein (HMGB1) (He et al., 2020). It is already known that HBx also promotes oxidative stress through the production of Reactive Oxygen Species (ROS). In this study (*He et al., 2020)* scientists used human LO_2 cell line and a certain GPR43 agonist called (S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenyl thiazole-2-yl) butanamide (PA). Firstly, they found that HBx is highly expressed in both mRNA and protein levels in the human LO_2 hepatocytes.They found that the HBx protein dramatically decrease the expression of GPR43 in human LO_2 hepatocytes, which leads to the hypothesis that there is a connection between GPR43 expression and HBx-mediated pathogenesis of HBV-induced severe liver damage and eventually carcinogenesis (He et al., 2020).

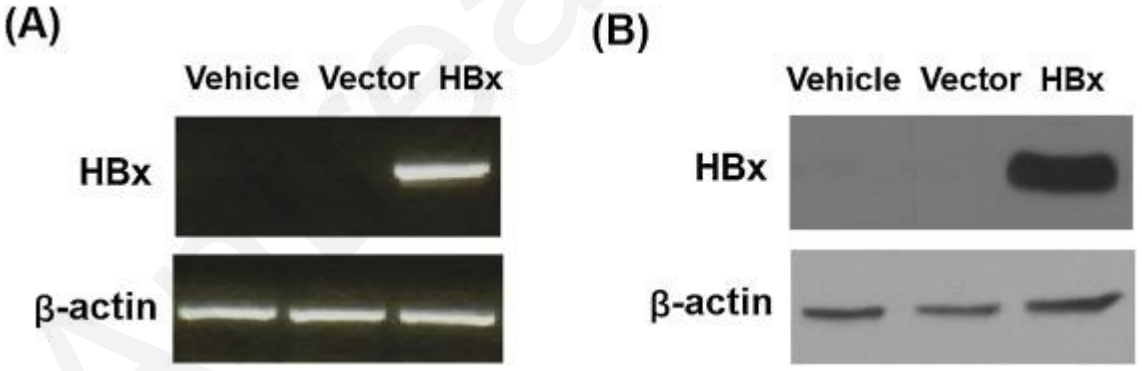

Figure 9: HBx protein is highly expressed in human primary LO_2 hepatocytes. (A). RT-PCR analysis of HBx protein. (B). Western blot analysis of HBx protein (He et al., 2020).

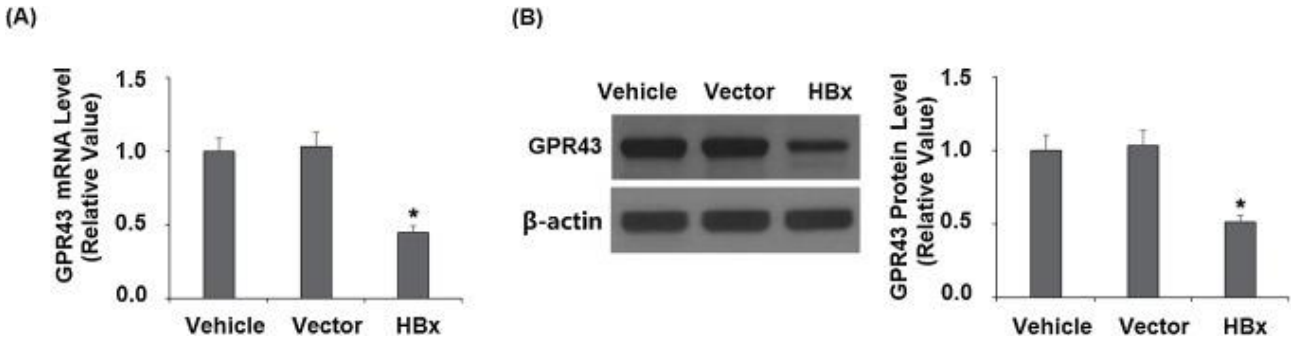

Figure 10: This figure shows the that HBx protein dramatically decreased of GPR43 expression in human primary LO2 hepatocytes. (A). shows the mRNA level expression of GPR43; (B). shows the GPR43 expression in protein levels (He et al., 2020).

Agonism of GPR43 ameliorates HBx-induced oxidative stress

The important role of the oxidative stress in the HBV infection has been already demonstrated by previous studies, as well as the major role of the viral HBx protein in the induce of oxidative stress in hepatocytes. HBx has been proven to induce oxidative stress mainly through the upregulation of ROS and NOX-4 expression. Oxidative stress it is known to cause damage and changes in lipids, proteins and DNA which lead to genetic mutation, elevated mitochondrial replication, viral integration and eventually carcinogenesis. This study (*He et al., 2020)* showed the contribution of GPR43 in HBx-mediated oxidative stress. The scientists showed the levels of the reactive oxygen species, the antioxidant glutathione (GSH) and NOX-4 in the human LO_2 hepatocytes with HBx in the presence or lack of presence of GPR43 agonist PA. They found a significant reduction of ROS and GSH levels in the presence of the GPR43 agonist PA (He et al., 2020).

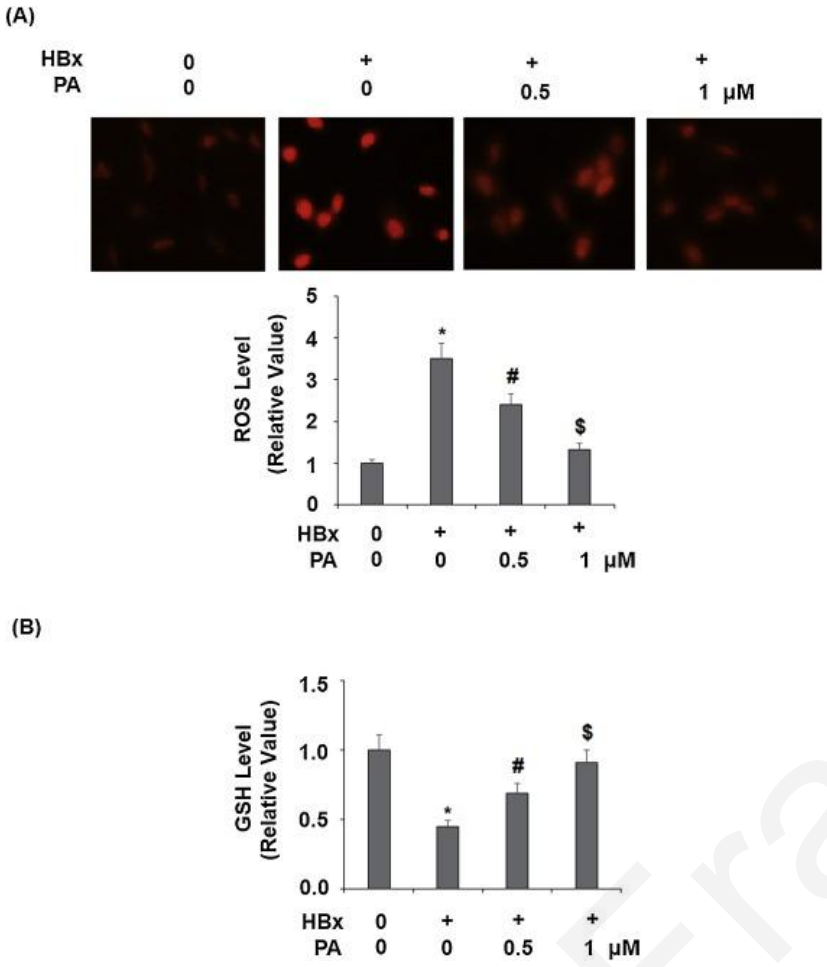

Figure 11: This figure presents that the activation of GPR43 with its specific agonist PA [(S)-2-(4-chlorophenyl)-3,3-dimethyl-N-(5-phenyl thiazole-2-yl) butanamide] prevents the HBx-induced oxidative stress. Human primary LO_2 hepatocytes were transfected with HBx plasmid. After 24 hours of transfection, the cells were treated with the agonist of GPR43 PA (0.5, 1 μM) for 24 hours. (A). Shows the levels of intracellular ROS; (B). Shows that the levels of GSH decreased after the PA treatment (He et al., 2020).

Furthermore, this study (*He et al., 2020)* showed that the increased production of ROS due the viral HBx protein activates different inflammatory signalling pathways in order to promote the release of proinflammatory cytokines which are responsible to mediate and regulate immunity and inflammation. Some cytokines that are release are IL-6, MCP-1, CXCL2 and HMGB-1, especially Interleukin-6 (IL-6) has the ability promote different intracellular signalling pathways which play a crucial role in the development and progression of hepatocellular carcinogenesis. The other cytokine MCP-1 has been proven to promote inflammation in the liver and more specifically to hepatocytes. As a result, it induces the accumulation of macrophages, fibrosis and steatosis which then contributes to the development of HCC. HBx also elevates the expression of the chemokine CXCL2 which is

strongly connected cancer metastasis in patients with HCC. It is considered as one of the future therapeutic targets for HCC. Finally, they observed that the overexpression of IL-6 and CXCL2 due to HBx protein activates the NF-kB pathway. Finally, their results suggested that the agonist GPR43 (PA) has the ability to decrease the expression of these cytokines and chemokines, which also decrease the inflammatory response in HBV infection. These findings revealed a key factor for the restriction of the HBV infection as well the development of HCC (*He et al., 2020).*

As a conclusion, their findings suggested that agonism of GPR43 significantly improved HBx-induced expression of cytokines and chemokines which results in decrease level production of oxidative stress. They also investigated the role of GPR43 in HBx-induced cellular toxicity. Finally, they investigated that HBx protein activates IkBa/NF-kB signalling pathway through the release of proinflammatory cytokines and chemokines (*He et al., 2020).* Their results are useful tools for future therapeutic target against HBV infection and eventually to the prevention of HCC development. However, further research is necessary in order to learn in depth the exact molecular mechanisms that help GPR43 agonism to have anti-inflammatory, oxidative stress preventing and antiviral properties (*He et al., 2020).*

HBx and NF-kB

NF-kB is one of the most important transcription factors and it has the ability to regulate the expression of different genes that are responsible for the activation of both innate and adaptive immune response. NF-kB controls important functions such as DNA transcription, cytokine production and cell survival. Cytokines are main signalling molecules and more specifically large proteins, such as peptides and glycoproteins, that are secreted by certain immune cells. They are responsible for regulating and mediating inflammation and immunity. According to recent studies activation or abnormal regulation of NF-kB is the main cause of many liver diseases such as hepatic inflammation and also plays a vital role in the development of Hepatocellular carcinoma (HCC). It has been proven that the viral protein of Hepatitis B, HBx is strongly connected to the activation of NF-kB and it is implicated in the development of HCC. The activation of NF-kB by the protein HBx plays an important factor in the regulation of different genes that are responsible for the control of cell development and growth as well as the development of primary hepatocellular carcinoma *(Lim et al., 2013).* The activation of NF-kB from HBx was one of the first properties that were identified about HBx. However, the exact molecular mechanism that HBx uses in order to activate NF-kB is still not well understood. One of the most common hypotheses is that HBx protein stimulates NF-kB signalling through the inhibition of IkB and p105 and it achieves that through the activation of Ras-Raf-MAPK pathways and through the induction of oxidative stress. Another way that HBx activates NF-kB is by the activation of different oncogenes such as AIB. In this study *(Lim et al., 2013)*

scientists found a novel mechanism by which HBx induced NF-kB signalling activation. This novel mechanism is the interaction of HBx with p22-Flip. P22-Flip forms a ternary complex with HBx and NEMO for the HBx-induced NF-kB activation. Firstly, the scientists identified the expression of p22-flip in the hepatocytes which suggested the role of p22-Flip for the NF-kB activation in the liver cells, which is closely related with that of immune cells *(Lim et al., 2013)*.

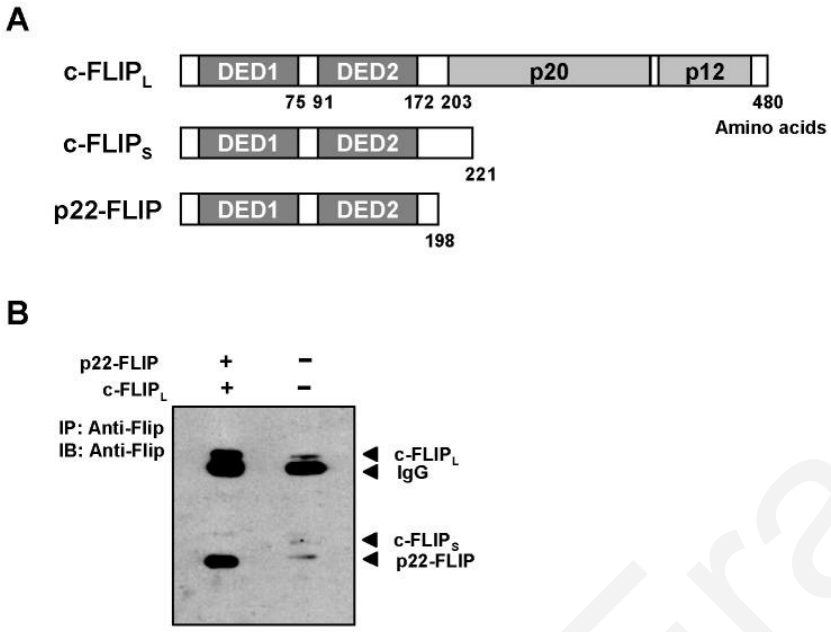

Figure 12: The presence of endogenous p22-Flip in human hepatocytes.

(A). This schematic shows the structure of c-Flip$_L$, c-FLIP$_S$, and p22-FLIP. (B). Western blot shows the detection of endogenous p22- Flip in Huh7 cells *(Lim et al., 2013)*.

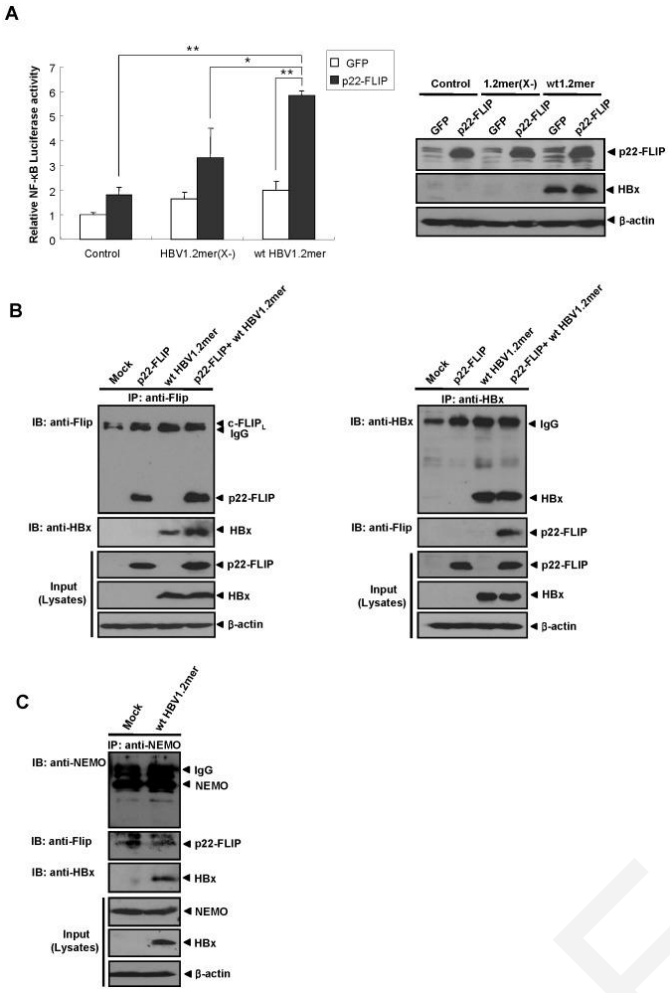

Figure 13: p22-FLIP enhances HBx-induces NF-kB signalling activation through the interaction of the viral protein HBx. (A). Shows the that p22-FLIP synergistically promotes HBx-mediated NF-kB activation in the presence of HBV DNA (full genome). The left panel shows the NF-kB activity 48 hours after infection in Huh7 cells. The right panel shows the western blot analysis of the expression of p22-FLIP and HBV genome-driven HBx. (B) shows the interaction of p22-FLIP with the viral protein HBx in hepatic cells. (C) Shows the formation of the ternary complex between p22-FLIPP, HBx and NEMO *(Lim et al., 2013).*

Many studies revealed that c-$FLIP_L$ overexpressed in various cancer including hepatocellular carcinoma. In this study scientists wanted to check effects of the overexpressed c-$FLIP_L$ on the expression of p22-FLIP. They hypothesized that c-$FLIP_L$ has the ability to convert into p22-FLIP by the cleavage by procaspase 8 during carcinogenesis. Furthermore, c-$FLIP_L$ was detected in 83% of human HCC cells and it was completely absent from the health (normal) hepatic cells. It was also observed that the human hepatocellular carcinomas showed resistance to death receptor-mediated apoptosis. This study also showed the synergistic activation of NF-kB by a ternary complex of p22-FLIP, HBx protein and NEMO (NF-Kappa-B essential modulator), which helps the virus to escape the host's immune system. Finally, their findings gave a better understanding of HBx-induced NF-

kB activation from the ternary complex played an important factor in the progression of the HBV infection and the development of HCC. However, further research is necessary to understand the fate of HBV infected cells *(Lim et al., 2013).*

Another study (He et al., 2020) revealed the NF-kB signalling pathway is indeed activated from the persistent HBV infection, which contributes in carcinogenic alterations. Scientists also suggested that the HBV take advantage of the anti-apoptotic function of NF-kB in order to enhance the progression of the infection and the excessive replication of the infected hepatic cells. The results of this study (He et al., 2020) indicate that the agonism of GPR43 by PA inhibits the phosphorylation of p38 protein and IkBa, which are the main inhibitors of the NF-kB activation. GPR43 also decreases the nuclear accumulation of p65 protein which contributes to the reduction of NF-kB activation (He et al., 2020).

In this study, scientists found the endogenous expression of p22-Flip in human hepatocytes. They scientists indicate the role of p22-FLIP in the activation of NF-kB pathway in the hepatic cells which has common characteristics with the activation of NF-kB in the immune cells. The exact mechanisms of the NF-kB signalling pathways by p22-FLIP in the hepatic cells are still unclear. However, the scientists in this study demonstrated a novel mechanism by which p22-FLIP forms a synergistically hyperactivation of NF-kB with the HBx viral protein and the NF-kappa-B essential modulator (NEMO) in the hepatocytes. Their results from the knock-down experiment suggest that the endogenous p22-FLIP has a strong connection with the HBx-induced NF-kB activation. Finally, they observed that Hepatis B virus has the ability to stimulate c-FLIPL- or p22-FLIP-mediated NF-κB signals in hepatic cells by cooperating with the viral protein HBx. This indicate the major role of HBx in the progression of the HBV infection (Lim et al., 2013). However, further studies are necessary in order to understand in depth the mechanisms of HBx-induces NF-kB by p22-FLIP as this may be a potential therapeutic target.

Future Therapeutic strategies

The need of new and efficient therapeutic strategies is increased as the Hepatitis B infection remains one of the most serious and life-threating viral infections. Especially due to the connection of correlation of the chronic HBV infection to the development of severe liver damage and hepatocellular carcinoma. The current treatments, which are only use for chronic HBV infection, target to limit the carriers with active chronic HBV. Drugs used for HIV infection and herpes viruses also have been found to help against HBV infection (Slagle and Bouchard, 2018). However, these medications are not effective in clearing the virus from the host, but it only suppresses the active

infection and decrease the symptoms. Thus, the safest and more efficient strategy remains the prevention which is achieved through the control of the risk factors. One way of prevention of the HBV infection is through mass vaccination in children and also in third world countries. It is also helpful that pre-existent chronic HBV patients to continue with the antiviral therapy as it minimizes the changes of developing severe complications such as HBV-related HCC. The use of protection during any sexual intercourse as well as the compliance of the transfusion rules (every blood should be tested before transfusion) . There are also centres where drug users can be supplied with clean needles and drug-injection equipment which prevents users sharing the same equipment. This can minimize the spread of the infection in people with already weak immune system. Apart from prevention, new specific antiviral medication should be designed in order to target exact mechanisms that are used from the virus to induce HCC. One of the most common antiviral therapeutic strategy, which is used in other viral infections such as HIV, is to target the viral life cycle. By reducing the level of replication or by avoiding the virus entering the host's cell it will minimize the replication of the virus and thus, the progression of the infection. Additionally, antiviral drugs that target the development from acute to chronic infection are necessary as it is one of the most important problems (Torresi and Locarnini, 1999). These antiviral drugs, may have the form of therapeutic vaccines, should support the immunity of the host in order to avoid the excessive and uncontrolled viral replication. Since HBx protein plays a vital role in the progression of the infection and the development of HCC through different important cellular signalling pathways, including NF-kB Ras/Raf/MAPK, Wnt-b-catenin and much more. Therefore, therapeutic strategies targeting the viral protein HBx could be effective in to inhibit any stage of either viral replication or HCC development.

Finally, recent studies both in vivo and in vitro revealed that gene therapy is one possible therapeutic method against HBV. Gene therapy includes the use of antisense oligodeoxynucleotides and RNA, ribozymes, dominant negative mutants and therapeutic HBV vaccines. Last but not least combination of antiviral drugs with vaccination and nucleoside analogue therapy is another strategy for HBV treatment (Lim et al., 2013). However, further studies are required in order to better understand the molecular mechanisms of HBV and more importantly HBx-induced HCC. This should be the key for the development of novel and targeted therapeutic methods against HBV (Tarocchi, 2014).

Discussion

Hepatitis B is an infectious liver disease caused by the Hepatitis B virus; this virus mainly affects the hepatocytes. HBV can cause both acute which lasts up to six months and chronic infection which lasts more than six months. Hepatitis B virus is a member of Hepadnaviruses, it is the smallest enveloped DNA (Tarocchi, 2014). HBV infection is a major health problem worldwide and almost 2 billion people have been exposed to the virus. Some of the HBV patients do not develop any significant symptoms, especially in the beginning of the infection (Yang and Roberts, 2010). However, others can develop mild to severe symptoms of hepatic failure including abdominal pain, jaundice (yellow colour in the skin and the white of the eyes), high fever and vomiting. Other symptoms may include dark urine, loss of appetite and fatigue. Usually the symptoms occur three months (90 days) after he infection from the virus. Patients with chronic HBV infection do not develop any symptoms and they can remain asymptomatic carriers even for decades depend on their immune system response. If chronic HBV patients develop symptoms though they are similar to those of acute infection (Yang and Roberts, 2010). There are several blood tests in order to diagnose the infection. Regarding to the treatment, there is no available treatment for patients with acute HBV infection, especially if the symptoms are mild. To those patients' doctors recommend rest, sufficient nutrition and plenty of fluids. Patients with severe symptoms is recommended to have medical care in order to minimize the progression of the infection. However, there are several medications for patients with chronic HBV infection and many drugs are still in the development. There is also available vaccination which offers adequate protection against the HBV infection. Hepatis B virus is mainly transmitted from mother to child during birth, through infected blood during blood transfusion or by sharing needles or any kind of drug-injection equipment. Another common transmission way is through unprotected sexual intercourse. Many studies have proved a strong connection between chronic hepatitis B virus infection and the development of hepatocellular carcinoma (HCC). Hepatocellular carcinoma is the fifth most common cancers and each year there are almost 700000 new cases and more than 50% of them are due to HBV infection. Chronic HBV infection has four stages but not all of the patients go through all four stages. The four stages include immune tolerance stage, immune clearance, inactive HBsAg carrier stage and reactivation stage. There are several factors which contribute to the transformation of acute to chronic infection. First of all, the host's immune system whether is strong or weak, most of the times patients with weak immune system due to other infections such as HIV are not able to clear the HBV infection as a result it becomes chronic. Secondly, the gender and the age that the patients are infected play an important role in the transformation from acute to chronic infection. Another important factor is the HBV itself, the kind of the viral mutations such as mutations in the C- or N- terminal and the levels of the HBV DNA in

the host also contribute to the transformation. Last but not least, environmental factor including coinfection with other viruses such as HCV or HIV and liver damage due to other toxins from alcohol or NAFLD also contribute to the development of chronic infection. Numerous studies showed the molecular mechanisms of the HBV which are used in order to develop HCC. Some of these mechanisms include the accumulation of DNA (genetic) damage due to persistent inflammation and the induction of oxidative stress (Tarocchi, 2014). It has been proven that chronic HBV infection cause persistent inflammation which lead to the activation and interaction of different signalling pathways such as NF-kB which play vital role in the cell proliferation and apoptosis. This results in increases and continuous liver damage and eventually in the development of HCC. One of the most important factors of HCC develop is the integration of HBV-DNA into the host's genome, which can be achieved through insertional mutagenesis, the alteration of gene expression and chromosomal instability. The integration of the viral DNA into the host is also considered as a primary stage in carcinogenesis. According to man y studies the insertion of the viral DNA into the host occurs randomly whereas other studies showed that HBV integrate its DNA close or inside certain genes that are responsible for the cell life cycle, proliferation and cell death. For example, telomerase reverse transcriptase (TERT), Fibronectin 1 (FN1) and cyclin E1. These results suggest that these specific target genes of HBV play an important role in the development of hepatocellular carcinogenesis. Some studies also showed that apart from genetic effects HBV also has epigenetic effects by the alterations of the genomic methylation status. Furthermore, many studies indicate the role of the viral protein HBx in the hepatocarcinogenesis. HBx is a multifunctional protein and it has the ability to regulate several of cellular activities including transcription, cell cycle progression, DNA damage repair system, cell proliferation and apoptosis. It does not the ability to act on cellular promoters through protein to protein interactions and regulates signalling pathways in the cytoplasm. HBx mainly localizes in the cytoplasm however, it can also be found in the nucleus and in the mitochondria. This cellular distribution is one of the main factors where HBx is characterized as a multifunctional protein. HBx plays a vital role not only in the progression of the infection but also in the development of HCC. To investigate the role of HBx some studies checked the effects of the wild-type HBx and all its mutants (Wang et al., 2014). The main conclusion was that lack of an X open reading frame does not induce hepatocellular carcinoma in the transgenic mice. In addition, HBx mutants, especially those with mutation in the C-terminal (COOH-terminal) increased the development of hepatocellular carcinoma in mice. This review showed some of the most important molecular mechanisms of Hepatitis B virus which are used to induce hepatocellular carcinogenesis. In addition, some studies have showed that HBx protein target to p53 and p53 family and inhibit their function which results in alterations in the cell cycle (apoptosis) and DNA repair. The inhibition of p53 by HBx can lead to hepatocellular carcinogenesis. Finally, HBx has the ability to cause

angiogenesis in HBV-induced HCC through the upregulation of the vascular endothelial growth factor (VEGF) and proangiogenic growth factor called angiopoietin 2 (ANG2) (Wang et al., 2014).

One of the most important factors of HCC development is that HBV inhibits apoptosis through the viral protein HBx, which regulates different cell signalling pathways, including the mitochondrial apoptotic pathway. The mitochondrial apoptotic pathway can be activated by HBV through the BAX insertion into the mitochondrial membrane. Furthermore, HBx has the ability to activate the NF-kB signalling pathway. One study showed that HBx activates NF-kB signalling pathway through the upregulation of TBK1 expression. TBK1 also known as TANK-binding kinase 1 plays an important role in innate immunity antiviral response but it also plays a major role in cell proliferation, apoptosis and autophagy. This study demonstrated that the upregulation of TBK1 by HBx plays important role not only in the replication of virus and the progression of the HBV infection but also in the development of HCC (Kim, Lee and Jung, 2009). Furthermore, Hepatitis B virus can cause hepatocellular carcinoma through the induction of oxidative DNA damage. Many studies have showed that HBV has the ability to increase the oxygen reactive species (ROS) which lead to lipid, protein and DNA damage, which lead to cell aging and eventually in the formation of hepatocellular carcinoma (Wang et al., 2014). Cell aging is directly connected with mutagenesis, which is one of the most important factors in carcinogenesis. ROS activate various cellular signalling pathways including the NF-kB signalling pathways. According to Hagen et al., 1994 ROS also affect the AKT signalling pathway which is responsible in various important biological processes including cell proliferation, development and cell life cycle. This study showed that excessive ROS levels in the hepatocytes caused by the HBx protein activate the AKT signalling which caused uncontrolled cell proliferation. This uncontrolled cell division allow mutagenesis and disturb the normal function of DNA repair mechanism which promotes the development of HCC (Wang et al., 2014).

Another way that HBV causes hepatic carcinogenesis is through the induction of inflammatory response in LO2 hepatocytes via HBx regulation of GPR43. This study showed that HBx protein decrease the expression of GPR43. GPR43 has the ability to transfer the viral protein HBx protein into the hepatocytes. They also showed the contribution of GRP43 in HBx-mediated oxidative stress. They found that lack of presence of GRP43 agonist PA increase the levels of ROS whereas there was a significant reduction of ROS and GSH in the presence of GRP43 agonist PA. Their result showed agonism of GRP43 dramatically decrease the levels of oxidative stress and this can be used a potential therapy against HBV infection.

Furthermore, many studies showed that HBV uses HBx in order to activate NF-kB signalling pathway either through the inhibition of IKB and p105 and it achieves that by the activation of Ras-Raf-MAPK pathway or by forming a ternary complex with p22-FLIP and NEMO (NF-Kappa B essential

modulator). This is novel mechanism of the ternary complex of HBx, p22-FLIP and NEMO helps the HBV to escape the host's immune system which plays a vital role in the progression of acute HBV infection to chronic HBV infection, which in turn cause the development of HCC. However, due to the fact that this a new mechanism and it is not well studied, further research is necessary in order to understand the effects on hepatocytes. By understanding this mechanism will be useful in the formation of new therapeutic strategies against HBV.

As a conclusion, due to the fact that HBx is essential for the viral replication, the progression of the infection and also it considered as a key factor for the development of hepatocellular carcinoma, in the future it would be helpful to design antiviral drugs that target HBx protein and its functionality. Further studies are necessary for the better understanding of the molecular mechanisms of HBV and also its viral product HBx in order to design more effective and targeted therapeutic methods which will minimize the progression of the infection and eventually the development of the hepatocellular carcinoma.

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