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**REVIEW**

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# HEPATITIS C VIRUS AND HEPATOCELLULAR CARCINOM

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**ABSTRACT:** Different etiological factors such as hepatitis viral infection, alcohol, aflatoxin and chemical carcinogens were mentioned in relation to HCC. However, the global distribution of HCC is strongly linked to the prevalence of hepatitis virus infection. The exact pathogenic mechanisms involved in viral-associated HCC are unclear although direct and indirect mechanisms are possible. Direct carcinogenicity is less certain in HCV-Induced HCC since it is a typical RNA virus and therefore the integration of viral genome into host cell chromosomes has not been shown to occur. However, the presence of two conserved potential nuclear localization signals and a DNA binding motif in the HCV core protein suggest a possible functional role as a regulatory element. Moreover, some studies demonstrated that this protein interacts with certain cellular proto-oncogenes at the transcriptional level, resulting in the promotion of cell proliferation and thus affecting normal hepatocyte growth. Therefore the pathogenesis of HCC may be attributed at least in part to the upregulation of hepatocyte growth induced by HCV core protein and other viral proteins like NS3 and NS5. However, the process of malignant transformation represents a dynamic interplay between classes of genes; oncogenes, tumor suppressor genes, mismatch repair genes, genes controlling apoptosis and cell cycle regulatory genes. In conclusion, since the exact mechanism of action of HCV in the context of HCC is still poorly understood, clarification of the molecular basis of viral replication in hepatocytes, the possible genetic and cytogenetic abnormalities that may be induced by the virus were emphasized in this review. Moreover, early detection of hepatocellular changes by molecular biomarkers may help to detect individuals at high risk of development HCC, thus allowing more effective intervention for cure or prevention

*Key words: HCC, HCV, MMR, FHIT, p53, C-erb-B2, MDM-2, p21waf, p21Ras, NS3, apoptosis.*

The process of malignant transformation represents a dynamic interplay between 5 classes of genes; oncogenes, tumor suppressor genes, mismatch repair genes, genes controlling apoptosis and cell cycle regulatory genes.

Although hepatocellular carcinoma (HCC) is relatively uncommon in North America and Western Europe (0.2-2%), it represents 20-40% of cancer cases in countries endemic for viral hepatitis. The greatest numbers of cases are found in Taiwain, Mozambique, Southeast, China and countries bordering the Mediterranean where the annual incidence rates approach 150/100000.

Different etiological factors such as hepatitis viral infection, alcohol, aflatoxin and chemical carcinogens were mentioned in relation to HCC. However, the global distribution of HCC is strongly linked to the prevalence of hepatitis virus infection (1). The exact pathogenic mechanisms involved in viral-associated HCC are unclear although direct and indirect mechanisms are possible. Apoptosis is recognized as an important function in the cell turnover in normal and neoplastic tissues and has been shown to play a significant role in normal liver and in several types of hepatobiliary disease. It takes place naturally in the liver at a very low rate. It has been shown that increased hepatocyte proliferation induced by several factors is commonly followed by increased apoptosis. There is evidence that apoptosis does not occur at random in the liver as cells in putative preneoplastic foci of rat liver show a much higher rate of apoptosis than normal hepatocytes. Apoptosis is frequently detected in HCC and it is not related to the type and grade of the tumor. It was mentioned that there is a highly significant positive relation between the apoptotic rate in HCC and both the proliferative activity and p53 protein expression. A similar phenomenon was observed in putative cancer precursors, which supports the role of p53 in regulating apoptosis in prencoplastic and neoplastic liver lesions. Cytotoxic T lymphocytes and lymphokine induced apoptosis of infected hepatocytes during the course of chronic viral hepatitis is thought to be important for both disease termination and prevention of hepatocellular transformation.

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*Abbreviations: MMR – mismatch repair; FHIT – fragile histidine tirad; p53– oncosuppressor gene; C-erb B2– protein receptor with tyrosine kinase activity similar to epidermal growth factor receptor; MDM-2 – one of the apoptosis regulating proteins; p21-Waf – cyclin dependent kinase inhibitor (involved in cell cycle regulation; p21 Ras – oncogene; NS3 – nonstructural 3 protein coded by HCV*

Direct carcinogenicity is less certain in HCV-Induced HCC since it is a typical RNA virus and therefore the integration of viral genome into host cell chromosomes has not been shown to occur. However, the presence of two conserved potential nuclear localization signals and a DNA binding motif in the HCV core protein suggest a possible functional role as a regulatory element. Moreover, some studies demonstrated that this protein interacts with certain cellular proto-oncogenes at the transcription level, resulting in the promotion of cell proliferation and thus affecting normal hepatocyte growth. Therefore the pathogenesis of HCC may be attributed at least in part to the upregulation of hepatocyte growth induced by HCV core protein (2). It has also been reported that the core protein activates the c-myc promoter and Rous sarcoma virus long terminal repeat, and suppresses the Rb, interferon, B-actin genes and HIV-1 long terminal repeat (3). It is also able to repress the transcription activity of the p53 promoter when tested in COS7 and HeLa cells (4), inhibit cisplatin and c-myc-mediated apoptotic cell death under certain conditions and transform primary rat embryo fibroblasts with a co-operative H-ras oncogene (2). In contrast, other studies (5) were not able to detect transformation of primary rat fibroblasts with HCV core protein from two different genotype (la and lb) in co-operation with H-ras oncogene, even though the core protein was successfully expressed 20 days after transfection. However, the core protein was able to induce the transformation of rat-1 cells with various efficiencies depending on the expression level of the core protein. This clearly indicates that HCV core protein has an oncogenic potential to transform rat-1 cell line, but is not sufficient to either immortalise primary R-EFs by itself or to transform primary cells in conjunction with the H-ras oncogene. As HCC develops over a period of more than 20 years (6), it is reasonable to speculate that HCV core protein is just one of several factors required for carcinogenesis or has a weak oncogenic activity which is sufficient to stimulate only a part of a complex, multistep pathway, or both (5). Furthermore, the core protein had a key role in protecting various cells from apoptosis mediated not only by anti-Fas but also by TNF-α. As normal hepatocytes, all human hepatoma cell lines tested express detectable amounts of Fas on their surfaces (7). Fas is a 43 Kd cell surface glycoprotein belonging to TNFR superfamily. Cross-linking of the Fas antigen by either the recently cloned Fas Ligand (FasL) or by anti-Fas antibodies *in vitro,* triggers apoptosis of Fas-expressed cells. Previous reports suggest that Fas might be important in the regulation of apoptosis in hepatocytes as it is expressed in normal mouse and human liver. Also, the intraperitoneal injection of an anti-Fas antibody causes severe apoptosis in the liver and finally, it has been shown that during chronic hepatitis the expression of Fas is unregulated in the hepatocytes nearby the infiltrating lymphocytes. This protection is carried on via a mechanism dependent on the activation of NF-KB in certain cells.

Apoptotic cell death due to viral infection can be induced by the host immune response through the function of cytotoxic T lymphocytes (CTL) and natural killer cells, or by viral proteins themselves, and apoptosis has been suggested to be a common pathway of virus clearance by host organisms (8&9).

As a part of the defense mechanism of host organisms, cells infected by viruses are induced to initiate apoptotic cell death by signals delivered from CTL (10). On the other hand, a number of viruses have been reported to cause infected cells to escape from this apoptosis to maintain persistent infection (9). In case of HCV infection, it was suggested that apoptosis in hepatocytes, especially that mediated by Fas, plays an important role as the main mechanism of viral clearance (11), which would result in the liver damage observed in chronic hepatitis.

The HCV genome encodes a polypeptide precursor consisting of about 3,010 amino acid residues. This precursor protein is cleaved by the host and viral proteases to generate at least 10 functional protein units: the core, envelope 1 (El), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (12). Ray et al. (2) reported that the core suppresses apoptosis induced by cisplatin in human cervical epithelial cells, by c-myc overexpression in Chinese hamster ovary cells, and by TNF-α- in human breast carcinoma cell lines. Fujita et al. (13) also suggested that NS3 protein inhibit actinomycin D-induced apoptosis in NIH 3T3 cells.

One of the antiapoptotic effects of the core was exerted through the activation of NF-KB in certain cells. However, deletion analysis indicated that at least the C-terminal region of the core is important for that function. Although a simple explanation for this is that the C-terminal portion of the core forms the NF-KB activation domain, the real reason seems to be more complicated.

The NF-KB signalling pathway is a key component of the cellular response to a variety of extracellular stimuli, including TNFα, interleukin-1 (IL-1) and phorbol ester (14). This transcription factor, known to regulate a large number of genes involved in inflammatory response, cell proliferation, and apoptosis, is composed of homo- and heterodimers of Rel family proteins. These family proteins include at least the following five distinct members: C-Rel, p50, p52, p65 (RelA), and RelB; of these, the p50/p65 heterodimer is the most abundant and ubiquitous (14). In the uninduced cells, NF-kB is sequestered in the cytoplasm by binding to a labile IkB family protein with a regulatory and inhibitory function, of which IKB-α and IKB-β appear to be the key members (15). Upon induction by several agents, including virus, inflammatory cytokines, and stresses, the intracellular signalling pathways that generally converge on IkB rapid phosphorylation and/or modification and subsequent degradation in the

proteosome are activated, thus allowing NF-kB complexes to enter the nucleus and activate the target genes. After degradation, the IKB-α is rapidly replenished by NF-kB-mediated transcription of IKB-α gene, which then constitutes the autoregulatory loop of NF-kB-IkB activation. Of note, unlike IKB-α, which elicits only transient NF-kB activation, the IKB-β degradation causes a sustained activation of NF-kB due to a large lag period Of IKB-β resynthesis. Recent studies have identified two cytokine-inducible IkB kinases (IKK), termed IKKα and IKKβ, which appear to form heterodimers in the large multiple complex (700 kDa) and catalyse IkB site-specific phosphorylation (16). In spite of this tight regulatory loop for NF-kB activation, this transcription factor is activated by different viral proteins with oncogenic potential, such as human T-cell leukemia virus type 1 Tax (17), Epstein-Barr virus latent membrane protein 1 (LMP-1) (18), the X protein of hepatitis B virus (19).

The second branch of the stress response is the JNK pathway, which targets to the activation of transcription factor AP-1, ATF-2, and E1K-1 (20). The signal transduction cascade of JNK activation is well defined and involves small GTP-binding proteins (Cdc42 and Rac), p21 activated protein kinase, and mitogen-activated protein kinase kinase members (MEKK1 and MEKK4) (21). Many stimuli that induce NF-kB, such as TNF-α, UV irradiation, and lipopolysaccharide, also activate the JNK cascade, thereby raising the possibility that the two pathways share common signal transduction components. Supporting this notion is the fact that TRAF2 and MEKK1 are two critical components of both the JNK and NF-KB stress response pathways (22), although contradictory findings were also reported (23). Despite these discrepancies, these two signal pathways diverge at a discrete level. For example, while JNK and its target c-Jun are critical mediators of apoptosis induced by TNF-α or ceramide, the NF-KB in general has an anti-apoptotic effect. Finally, it is concluded that HCV core protein inhibits the onset of apoptotic cell death, and at least one of the important pathways for this includes NF-kB activation by the core protein. This antiapoptotic effect induced by the core might be advantageous for HCV by allowing the host hepatocytes to survive apoptosis, resulting in sustained infection. Further studies are necessary to determine the molecular mechanism by which the core enhances NF-kB activity and to find the other antiapoptotic pathway mediated by the core independently of NF-kB, because this might allow development of effective strategies for the prevention of chronic sustained viral infection. In addition, the HCV core protein forms a complex with apolipoprotein the entire lipid droplet, which in turn may contribute to the liver steatosis in HCV-infected chimpanzee or humans (24). The interaction between the HCV core protein and the tumor necrosis factor receptor (TNFR)-related lymphotoxin-β receptor (LT-PR) was also demonstrated. This interaction modulates one of the biological activities, i.e., cytolytic activity, of LT-OR triggering by its recombinant ligand in HeLa cells but not in HH-7 cells. Moreover, the HCV core protein also interacts with TNFR 1, although its effect on TNF-induced cytolytic activity still remains controversial. Like the TNF ligand receptor family, the LT-OR is also engaged in activation of the transcription factor NF-kB and c-Jun in some cell types. Conceivably, the interaction of HCV core protein and TNFRI may potentiate their NF-kB or c-Jun N-terminal kinase (JNK) signalling pathways (24).

On the Other hand, the non-structural protein 3 (NS3P) of HCV genome was linked to the neoplastic transformation of normal hepatocytes in chronically infected patients. The NS3P-transfected NIH3T3 cells grow rapidly, proliferate serum-independently, lose contact inhibition and induce significant tumor formation in nude mice (25). Furthermore, sequence analysis showed unique changes at the vicinity of the catalytic sites of the NS3P clones isolated from HCC tissues but not from the non-tumors hepatic tissues (26). However, the exact mechanisms involved in this process are unidentified yet especially in the Egyptian population where genotype 4 represents the commonest type. Therefore, our lab investigated the correlation between NS3P, DNA ploidy and the expression level of *p53, p21 $^{waf}$, p21ras, mdm2* and *c-erbB2* in HCV-associated HCC and normal tissue distant to the tumor (NDT). This was done in an attempt to understand the possible pathogenetic mechanisms contributing to the development and progression of HCC in chronic HCV infected patients.

The high incidence of NS3P expression reported in our study supports previously published data and denotes the importance of this protein in the development of HCC in chronic HCV patients. This incidence is comparable to Feng et al. (27) who detected NS3P expression in 62% of HCCs and 83% of NDT. In our study, we did not find a correlation between NS3P and any of the studied genes in HCC however, there was a strong correlation between this viral protein, *p21 $^{waf}$* and *c-erbB2* in NDT.

Our results regarding c-erbB2 overexpression are consistent with those of Zekri et al. (28) who showed that *c-erbB2* is highly upregulated in HCV-associated HCC and CAH cases with no significant difference in the expression level between the two groups. This indicates that, alterations affecting *c-erbB2* start to manifest at an early stage of hepatocyte transformation. We proposed that, HCV infection might induce *c-erbB2* overexpression, which stimulates signal transduction enhancing the proliferative activity and liability for random mutations and possible malignant transformation of hepatocytes.

The correlation between HCV infection and down regulation of *p21 $^{waf}$* was detected in previous studies (29,30). Kwun et al. (29) demonstrated that, NS3P

specifically repress the promoter activity of *p21 waf* in a dose-dependent manner especially when combined with HCV core protein. This repression was completely lost when the *p53* binding sites on *p21 waf* promotor were removed indicating that, this function is achieved via modulating the activity of *p53*. However, there are several other mechanisms for *p21 waf* regulation (30,31).

Zhou et al.(32) demonstrated that, *c-erbB2* overexpression activates the phosphorylation of phosphatidylinositol-3-kinase (PI-3K)/Akt which associates with *p21 waf* resulting in cytoplasmic sequestration of the latter protein. Since the cell-growth-inhibiting activity of *p21 waf* is strongly correlated with its nuclear localization, cytoplasmic sequestration will block its function leading to cellular proliferation (33). In addition, cytoplasmic *p21 waf* forms a complex with apoptosis-signal-regulating kinase 1 (ASK1) that inhibits the stress-induced mitogen-activated protein (MAP) kinase cascade with subsequent loss of apoptosis (34). Our results provide support for this theory, since cytoplasmic immunostaining for *p21 waf* was recognized in 80% of the cases, which overexpressed *c-erbB2*. Our study also show higher expression of *p21 waf* in NDT than in HCCs. The same finding was reported by Zhu et al. (35) who showed that the expression of *p21 waf* was lower in HCC than in paratumoral liver tissues. However, in our work *p53* overexpression was detected in 63% of cases with absent nuclear *p21 waf* indicating that, the *p53*-dependent pathway is still considered the main pathway for *p21 waf* regulation (31,35). Inactivation of *p53* was previously reported in HCC and benign hepatic lesions (27,37,38). Our results regarding the expression level of *p53* in the studied groups are in agreement with Livni et al. (37) and Feng et al., 1998 (27) who reported *p53* overexpression in 67% of HCC and the nontumorous tissues within the adjacent regenerative nodules but not in the normal hepatic tissues. However, the expression of *p53* was higher in HCC than in NDT and was significantly associated with the degree of tumor differentiation. They also reported a significant correlation between *p53* overexpression and NS3P in NDT however; we did not find such a correlation neither in HCC nor in NDT. This could be partially attributed to the difference in the HCV-genotype between our series and theirs since the predominant subtype in our cases was type 4 but in their study it was type 1.

Our study provides evidence that; complexing with mdm2 protein is a highly suggested mechanism for *p53* inactivation. Although amplification of the *mdm2* gene was shown to be a rare event in hepatic tumors, the high expression level of *mdm2* reported in the present study in HCC and NDT (59.4% and 44.4% respectively) indicates that it is a possible candidate for HCV-induced hepatocarcinogenesis. Schlott et al. (39) were able to detect *mdm2* overexpression in HCC cases and those with focal nodular hyperplasia, however, point mutations were only found in HCC cases only. They also demonstrated that, the c-terminal RING domain is involved in repression of cyclin A gene, which controls the transition from G1 to S phase.

The correlation between *mdm2* overexpression and the presence of cirrhosis denotes an important role for this protein in the early stages of hepatocyte transformation in chronic HCV patients. Furthermore, the correlation between *mdm2*, *p53* and *c-erbB2* in HCC arising on top of cirrhosis suggests the presence of a dynamic interplay between these genes in HCV-associated HCC.

The *p21ras* is a highly possible target for HCV since it represents the second most commonly affected gene in HCC cases (73.3%) investigated in the present work. To our knowledge, our results in this context are novel, since there are only few reports available regarding *p21ras* status in HCC, the majority of them were performed on cell lines or animal models (40,41,42,43). Our results clearly show that, *p21ras* alterations are not only a frequent event in HCC but they are also of a predictive prognostic value since *p21ras* overexpression was associated with large-sized tumors, the presence of multiple intrahepatic nodules and the presence of nodal metastasis. Similarly, *c-erbB2* overexpression was strongly correlated with the presence of multiple intrahepatic nodules and cirrhosis. Whereas absence of *p21 waf* and *p53* overexpression were highly correlated with the presence of intrahepatic and venous metastases. Our results in this regard are in agreement with those of Qin et al. (31) and Zhu et al., (35) who mentioned that *p21 waf* was lower in tumors with intrahepatic metastases than in those without metastases, and that absence of *p21 waf* expression was highly correlated with *p53* mutations. Furthermore Qin et al. (31) demonstrated that a high protein expression is significantly associated with solitary nodules. Our results are also comparable with Naka et al. (44) and Qin et al. (31) who showed that, cases exhibiting aberrant *p53* protein and reduced *p21 waf* expression are those with multiple nodules, advanced stage, high grade and large-sized tumors. Also, Heinze et al. (45) proved in their study that, patients with overexpressed *p53* and *c-erbB2* usually have reduced survival time.

Our data regarding the absence of any significant correlation between the ploidy status of tumors and the clinicopathological features of patients are in agreement with Terris et al. (46) and Ng (47) who showed that assessment of the ploidy status of HCC cases does not provide additional prognostic information. However, we found a significant difference in the DNA content between HCC and NDT foci denoting the importance of DNA content assessment in monitoring CAH patients. Our results in this regard are in agreement with Zeppa et al. (48) and Attallah et al. (49) who mentioned aneuploidy as an important marker in cirrhotic livers that could be used to monitor the development of carcinoma. They also mentioned that abnormal DNA content is an

independent prognostic parameter in predicting survival in HCC patients. These controversy in results could be attributed to the difference in the etiological factor in different studies (HCV, HBV or aflatoxin B1 associated HCC) or to the difference in the way of measuring the DNA content (flowcytoetry Vs image analysis). Another explanation was provided by Oriyama et al. (50) who mentioned the presence of marked intratumoral heterogeneity of DNA ploidy status. This heterogeneity may develop along with changes in the growth pattern and dedifferentiation or by confluence of nodules originating from different tumor cell clones.

The presence of a percentage of patients whose NDT samples revealed aneuploid populations raises the question whether those patients constitutes a high risk subset that is more prone to progress into HCC. The answer for this question could be obtained from a larger follow-up study of CAH and cirrhotic patients who will be monitored by frequent DNA content analysis and correlation with other features of disease progression.

We conclude that, NS3P may exert its hepatocarcinogenic effect in an early stage of hepatic transformation being higher in NDT than in carcinomatous foci. At first, there are alterations affecting the expression of *c-erbB2* and *p21*$^{waf.\ which}$ provide a proliferative advantage to the chronically infected cells through promoting cell growth and loss of apoptosis. This enhanced proliferation and prolonged survival will allow for the acquisition of additional mutations in other genes e.g. *p53, p21ras* and *mdm2*.... etc. Accumulation of genetic aberrations will finally lead to the malignant phenotype. The correlation between *c-erbB2, p53* and *mdm2* and their strong association with HCC arising on top of cirrhosis suggests a dynamic interplay between these genes especially in HCV-associated HCC that is often preceded by cirrhosis. Furthermore, the significant association reported in the our study between some gene products and the clinicopathological features of patients is of utmost importance since they add some new, biological prognostic factors to the list of the existing prognostic parameters. These new, individually based markers may help in improving the dismal outcome of HCC patients by accurately categorizing high and low-risk patients and by patronizing therapy on an individual basis. They can also help in monitoring chronic hepatitis patients and identifying certain groups who are prone to develop HCC.

Cytogenetic and comparative genomic hybridization studies of HCC-derived cell lines demonstrated recurrent unbalanced translocations involving 3p 14-21 and loss of the DNA copy number of regions 3p 12-14. Both alterations overlap the location of the FHIT gene and therefore it was proposed that the FHIT gene might be implicated in a subset of liver cancers (51, 52). The FHIT gene, a member of the human histidine triad gene family is located on the short arm of chromosome 3 in a region carrying multiple tumor suppressor genes involved in cancer development (53, 54). It is composed of 10 exons covering 1.1 kb of mRNA which spreads out over 1 Mb of genomic DNA, including FRA3B and the t (3; 8) (p14.2; q24.1) translocation site (54). The open reading frame of the FHIT gene is located in exons 5 to 9, whereas the first four exons and exon 10 are not translated. Although homozygous deletions (HZD) of the FHIT gene has been reported in several cancer-derived cell lines (53, 55), its exact role in the process of tumorigenesis is still unknown.

We amplified the functional area only of the FHIT gene from exon 5 to 9 and we were able to find a good positive correlation between HZD and mRNA expression in the majority of cases in which both normal and truncated transcripts were recorded. An important point in our work is that, we studied each exon separately in order to determine the exact site of deletion in HCV-associated HCC cases. Most of the previously published studies used either extra- or intragenic microsatellite polymorphic markers within the FHIT locus to assess the gene status. Therefore, these studies were not able to identify the exact exon affected and therefore, depended upon sequence analysis of the truncated mRNA which might be deleted or integrated within normal FHIT transcripts.

In the our study, aberrant FHIT transcripts were detected in 2 HCC cases. Virtually most of the abnormal transcripts represented precise splicing changes with deletions of various exons. The mechanisms responsible for the formation of the aberrant FHIT transcripts are unknown but may reflect DNA deletions and rearrangements within the FHIT locus. RT-PCR amplification from five HCC cases with aberrant transcripts yielded both normal- and abnormal-sized products whereas 2 cases revealed the abnormal transcript only. Sequence analysis confirmed the loss of exons without other sequence abnormalities. Our results regarding the presence of normal and aberrant transcripts are in agreement with those of Ohta et al. (54) and Negrini et al. (56) who reported the same findings in monoclonal cell lines and in HCC cases.

The mRNA used in this work was extracted from tissue homogenates, and therefore, it was not possible to assess whether the same cells produced the normal and abnormal messengers. However, the coexistence of normal and aberrant transcripts in the same cell makes the biological significance of the aberrant transcripts uncertain. Because a start codon is present on exon 9, transcripts lacking exons 5 and 6 (or 5-8) might encode a 13-amino-acid peptide at the COOH-terminus of the FHIT protein but this phenomenon was not reported in our HCC cases. However, Kisielewski et al. (57) found a putative truncated pFHIT in cell lines and one primary tumor of the head and neck using Western blot. Considering that the functional FHIT protein is a homodimer, it is conceivable that a defective peptide might dimerize and negatively affect the wild-type FHIT protein.

Liver cirrhosis is considered a precancerous condition, and FHIT abnormalities in the cirrhotic tissue could indicate generalized genomic instability favoring the emergence of neoplastic clones. To our knowledge, the present study represents the first report regarding FHIT gene abnormalities in cirrhotic and CAH patients. Multivariate analysis was used to assess the relationship between HZD in each exon and the presence of cirrhosis and/or CAH. Our results show that, HZD of exon 5 and 9 are significantly associated with HCC arising on top of cirrhosis (p=0.041 and 0.006 respectively) whereas, exons 8 and 9 are significantly associated with the presence of CAH (p=0.029 and 0.034 respectively). These findings could suggest a possible role of the FHIT gene in certain types of chronic liver disease and consequently in the early stages of hepatocarcinogenesis.

This is in agreement with Gramantieri et al. (58) who reported LOH at D3S1300 and D3S1234 in HCC and cirrhosis. Their LOH analysis showed a single band both in HCC and cirrhosis however; aberrant transcripts were also detected in nonneoplastic tissues. Therefore, the authors could not exclude the possibility that both tissues lost the same allele and due to the minimal amount of tissue available in the majority of cases studied, any further analysis on genomic DNA was not performed.

Chen et al. (59) investigated FHIT changes in 18 cases of HCC and normal liver tissues. They identified aberrant FHIT transcripts in a high percentage (44.4%) of non-tumorous liver parenchyma and consequently concluded that the presence of aberrant FHIT transcripts might not be related to liver carcinogenesis. However, this conclusion is not definite since it was based on a small number of cases. In a similar study performed on 31cases of colorectal cancer, Thiagalingam et al. (60) found normal-sized RT-PCR products of FIHT with a normal sequence in 29 of the cases. They consequently suggested that either FHIT is not involved or is functionally inactivated at the translational level in some tumors, it is located adjacent to another target gene or its alterations can be related to the cancer-specific genetic instability of this region.

We also observed aberrant FHIT HZD in NDT obtained from six patients. In four cases, the NDT showed the same aberrant FHIT HZD of the matched HCC samples, whereas in the remaining two cases aberrant HZD were found but in different exons.

On the other hand, none of the NDT samples showed deletion at exon 5. However, frequent homozygous deletion was detected in HCC cases this could be related to the facts that exon 5 represent the initial code for FHIT gene transcription. Huebner et al. (61) found that within the FRA3B region, the characteristic induced chromosome gaps can occur across the entire region, but 60% of the gaps were centered on a 300 kb region flanking FHIT exon 5, which is the first protein-coding exon. Also most of these transcripts lacked exon 5 in which translation initiation codon is located. Replacement of FHIT expression in shit-negative cancer cells abrogates their tumorigenicity in nude mice. Analysis of the approximately 300-k DNA sequence encompassing FHIT exon 5 in the FRA3B epicenter has provided clues to the mechanism involves recombination between LINE 1 elements with deletion of the intervening sequence, often including FHIT exons.

In three recent studies (28, 52, 59) aberrant FHIT transcripts were detected by RT-PCR in HCC as well as in nonmalignant hepatic tissue. Similarly, absence of FHIT protein was reported in 50% of primary HCC derived from Qidong, China, was characterized by chronic HBV infection or exposure to certain hepatocarcinogens. The result of the present study supports the findings of these previous studies. The aberrant FHIT transcripts in nonmalignant liver tissue may also reflect early damage caused by HCV.

It was previously shown that, alterations in the FHIT gene occur early in tumor development, particularly in neoplasms associated with environmental carcinogens (62). Hence, loss of the FHIT locus in nonneoplastic tissue of smokers and ex-smokers is indicative of early carcinogen-induced damage (63, 64). Epidemiological data link HCC with exposure to several carcinogens, chemical agents, and oncogenic viruses. Since, both chemical carcinogens and oncogenic viruses are known to target perferentially fragile sites (65, 66, 67) it seems explainable that FHIT gene abnormalities are common findings in HCV (virus)-associated HCC and that, HCV may cause damage to this locus early in the process of hepatocarcinogenesis. In conclusion, our study demonstrates that HZD and abnormal FHIT transcripts occur in a fairly high proportion of HCV-associated tumors and non tumor liver diseases as well as in normal tissues adjacent to HCC. Therefore, it might represent one of the early changes in the genetic cascade of hepatocarcinogenesis. Hence, the presence of such abnormalities in CAH and cirrhotic patients is alarming and might be considered a negative prognostic factor. However, our results are preliminary and need to be confirmed in a larger study including more cases and with an extended follow-up.

Among the other pathogenic mechanisms mentioned in the context of HCV associated HCC, the consequence of chronic inflammation, cirrhosis and regenerative process in HCV infected patients function as tumor promoter (5). Another possible mechanism is the enhanced proliferative activity of affected cells and the increased frequency of spontaneously occurring random mutations due to chronic necroinflammatory hepatic disease induced by HCV infection. This may be achieved through induction of neu-oncogene overexpression which was found to be highly expressed in CAH and HCC patients leading to stimulation of signal transduction, enhancement of proliferation, mutation and malignant transformation (67). Amplification and/or

overexpression of the neu-oncogene has been implicated in experimental cellular transformation and tumorigenesis in a wide range of human cancers, including carcinoma of the breast, ovary, gastrointestinal tract, salivary gland, kidney, urinary bladder and liver (69).

The neu oncogene encodes a 185-kDa transmembrane protein, which is structurally similar to the epidermal growth factor receptor [70]. The extracellular domain (ECD) of the p185 transmembrane growth factor receptor is released from the surface of human cancer cells that overexpress p185 and can be detected immunologically in the extracellular environment in vitro (71). Similarly, it has been reported that overexpression of the c-erbB-2 oncogene can be detected immunologically in vivo by quantification of the ECD of p185 in the serum of human patients with cancers that are known to overexpress p185 (24).

In a study on human hepatocellular carcinogenesis, it was possible to detect increased serum levels of p185 ECD in individuals who eventually developed cancer 60 months before clinical diagnosis, indicating, that serum neu-oncopeptide may be a useful biomarker for early detection of HCC. It was also reported that elevated serum levels of neu oncoprotein correlate with the increased expression of this oncoprotein in tumor tissue (72), suggesting that the serum level of neu oncoprotein can be used as an indicator of its tissue expression. The distribution of hepatitis C virus (HCV) Genotypes among Egyptian patients positive for anti-HCV was determined and their influence, when combined with neu-oncoprotein overexpression, on the development of hepatocellular carcinoma (HCC). It had been shown that 84.3% of the Egyptian CAH or HCC patients who were positive for HCV had a single genotype including la (12.4%), lb (2.2%) and 2a (11.2%). Genotype 4 (a or c+d) was detected in 57.3% of the patients and only one patient had genotype 10a. On the other hand, 15.7% showed mixed infection. This study revealed a high incidence of genotype 4 in CAH and HCC patients. In addition, there was a significant overexpression of neu-oneoprotein in CAH and HCC patients, which was highly associated with subtypes 1a and 4 infection suggesting that infection with these two subtypes may be considered a risk factor for the induction of neu-over-expression and subsequent development of HCC.

It had also been proved that cells with defects in the DNA mismatch repair system have increased-risk for accumulating mutations in oncogenes and tumor suppressor genes. At least six human MMR genes have been identified as house keeping genes because they are expressed, although variably, in all tissues tested. They are found in every prokaryotic organism and their structure and function appear to be highly conserved. Genomic instability due to defect in DNA mismatch repair is now considered to be an important mechanism for carcinogenesis (73). The hMSH 2 gene (MMR-2) has been cloned as a candidate for HNPCC gene and mutations of this gene have been identified in some malignancies. Inactivation of the MMR-2 gene increases the spontaneous mutation rate and results in both genetic instability of microsatellites and of the oncogenic mutations of critical transforming genes such as K-ras, p53, DCC and APC.

The DNA mismatch repair system (MMR) is expressed in all tissues at various levels. It plays an important role in the maintenance of genomic integrity as it corrects replicative mismatches of the escaped DNA polymerase proof reading (74). Biochemical and genetic studies in eukaryotes have defined at least 5 genes (*MSH2, MSH3, GTBP, MLH1and PMS2*) whose protein products are required for DNA-MMR (75). Direct evidence for the association of genetic instability and mutant mismatch repair genes is derived from the biochemical studies *in vitro* in which nuclear extracts from human tumor cell lines with mutated MMR genes were unable to efficiently repair heteroduplex DNA fragments (76). Hence, it was proposed that, cells with defective MMR mechanisms have a reduction in the fidelity of DNA and can not correct genetic errors that occur during cellular replication (77). Several studies have focused on the impact of defective MMR genes on the pathogenesis of tumors and genes encoding components of the MMR system have been mentioned in relation to several human solid tumors (78, 79, 80, 81). It was shown that, defects in mismatch repair genes lead to a genome-wide instability of the microsatellites (81). When this occurs in oncogenes or tumor suppressor genes, loss of control over cell growth and proliferation may develop (82). Moreover, loss of expression of the mismatch repair genes leads to resistance of tumour cells to the damage induced by some chemotherapeutic agents such as 6-thioguanine, some methylating agents, cisplatin and carboplatin (83,84,85). This acquired resistance could be achieved through several mechanisms including failure to recognize DNA adducts formed by some chemotherapeutic agents or failure to activate signaling pathways that trigger apoptosis (74). However, its role in HCC is incompletely defined (77). In our study, the multiplex RT-PCR assay was used to assess the expression level of 5 MMR genes in HCV-associated HCCs and their NDHTs.

Reduced expression of *hMSH2, hMLH1, GTBP* and *hPMS2* but not *hPMS1* was frequently observed in HCC cases. In general, reduced expression of more than one gene was more frequent than single gene affection. The most frequently affected genes in the present study were MSH2 (71.9%) followed by GTBP (56.7%) and MLH1 (53.3%). This reduced expression could be speculative for somatic mutations affecting these genes in particular and lead to reduced expression.. Literature review shows only very few reports with controversial data in this context. In 1999 Yanno et al.(86) reported the

presence of mutations in *hMSH2* gene in HCC patients and demonstrated that these mutations are closely correlated with the overall survival of patients. In a recent study on the gene expression profile of HCC cases using the cDNA array, reduced expression of MSH2 was found to be a frequent event in HCC and was closely associated with tumor metastasis (87). In contrast, Wang et al., (88) using immunohistochemical techniques, did not find any correlation between the expression level of *hMLH1 or hMSH2* and the incidence or prognosis of HCC patients. They concluded that, defects in *hMSH2, hMLH1*do not contribute significantly to hepatocellular carcinogenesis. This controversy in the results of different studies may be attributed to the difference in the detection method of MMR genes defects, the presence of different HCV-genotype of viral infection, epigenetic factors or other factors including race, geographical area, genetic profile and other environmental hazards. It was shown that, some mutations can cause premature translation termination that mediates degradation of mutant mRNAs. Moreover, hypermethylation may be another mechanism of gene silencing where hypermethylated genes have reduced mRNA levels.

It is also likely that inactivation of transcription factors may reduce the expression of MMR genes with the occurrence of gradual shutdown as tumor progresses (79).

In addition, several studies demonstrated that the affected MMR genes and the pattern of defects differ according to the type of studied tumor Karl et al. (89). Whereas in the current stuay the most frequently affected genes were *MSH2*, *GTBP* and *MLH1*, Soliman et al. (90) using the same panel of MMR genes and the same technique demonstrated that *hPMS2* was the most frequently affected gene in Egyptian colorectal cancer (CRC) cases. However, Thomas et al. (91) studied germline mutations of the *MMR* genes in African/ Americans with CRC and found an association between germline mutations of *hMLH1 and hMSH2* and the development of CRC in African Americans. Therefore, it seems possible that, defects in MMR genes could be induced by the interplay between several genetic and non-genetic factors and even in the same tumor type we may find different patterns of gene defects.

A novel finding in our work is the frequent involvement of *GTBP* since this gene has not been mentioned before in relation to hepatocellular carcinogenesis. Moreover, in the majority of cases which should reduced expression of more than one gene *GTBP* was usually a partner. Therefore, we assume that genetic defects leading to loss of function of *GTBP* might represents an important step in the chain of MMR gene defects involved in hepatocyte transformation especially in the presence of HCV infection. This assumption is confirmed in the present study by finding a significant difference between the expression level of *GTBP* in tumor and normal hepatic tissues obtained from the same patients. An additional evidence is provided by he high association reported in the present study between reduced expression of *GTBP* and the presence of HCV in HCC cases.

On the other hand, *hMLH1* was the third most frequently affected gene and was commonly associated with reduced expression of *MSH2*. Moreover, reduced expression of *hMLH1* in NHTDT was comparable to that reported in tumor tissues in cases from which paired samples of normal and neoplastic tissues were taken (43.2% *vs* 53.3%). These findings indicate that *hMLH1* is a likely candidate gene in hepatocarcinogenesis and it possibly exerts its role in the early steps of hepatocyte transformation. Our data in this regard confirm the results of Macdonald et al. (77) who detected LOH at *hMSH2* and/or *hMLH1* in malignant, premalignant and adjacent normal hepatic tissues. The high association reported in the current study between reduced expression of *hMSH2* and *hMLH1* ($p=0.03$) suggests the presence of a cooperation between these two genes at a certain stage of hepatocarcinogenesis. Moreover, there was a significant association between this reduced expression of *PMS2* gene. and absence of cirrhosis in HCC patients ($P=0.0197$) since in this group of patients with reduced expression of *PMS2* was second to *MSH2*. This provides a possible pathogenetic pathway for the development of HCC in non-cirrhotis patients via failure to repair damaged DNA. Subsequently, it could be proposed that defects in *hPMS2* is likely associated with growth advantage and proliferative stimulation which in the absence of effective DNA repair mechanisms may lead to malignant changes in the non-cirrhotic patients. The significant association between reduced expression of *GTBP* and *hPMS2* in HCC especially in non-cirrhotic cases ($P=0.003$), could be explained by the presence of a cooperation between these two genes in the development of HCC.

The significant association reported in our study between the presence of HCV in HCC cases and reduced expression of *hPMS2* and/*or GTBP* ($P=<0.001$ and 0.002 respectively) suggests that these genes could be targets for HCV in the genetic cascade controlling HCV-associated hepatocarcinogenesis. Alternatively reduced expression or inactivation of these genes may interfere with (prevent) efficient repair of DNA as a result of HCV infection. It is now well-known that, HCV-associated HCC involves alterations in the concerted action of proto-oncogene, growth factors and tumor suppressor genes. The presence of 2 nuclear localization signals and a DNA binding motif in the HCV core protein suggests a possible functional role for HCV as a gene regulatory element (90).

Moreover, some studies suggest that this protein interacts with certain cellular proto-oncogenes at the transcriptional level, resulting in the promotion of cell proliferation which in the presence

of DNA damage and/or in absence of efficient DNA repair mechanisms affects normal hepatocyte growth and differentiation. Therefore, the pathogenesis of HCV may be attributed, at least in part, to the up regulation of hepatocyte growth induced by HCV core protein and the loss of DNA repair (42).

To the best of our knowledge no previous study revealed the relation between MMR defects and HCV infection in the process of hepatocarcinogenesis. Numerous studies have shown that over-expression of growth factor receptors is associated with altered cellular response to DNA damage and DNA repair. In breast and ovarian cancer cells, over-expression of cerbB-2 gene product increased sensitivity to drugs through inhibition of DNA repair (92, 93, 94) also, we previously reported overexpression of *c-erbB2* in HCC and chronic active hepatitis patients in association with HCV genotype-4 which is the predominant genotype in Egyptian patients (28). It has been clearly indicated that modulation of *c-erbB2* inhibits DNA repair either directly or indirectly (95). Finally, the high mutation rate occurring in case of MMR gene defects in the coding or regulatory sequences of other genes leads to more and more genomic damage with an increased probability for neoplastic transformation (Fink et al., 1998). This finding may in part explain the high resistance of HCC to the most known regimen of chemotherapy. Since it has been previously reported that there is a close correlation between the MMR genes defect and resistant to chemotherapy.

*finally*, the present study represents a step forward for understanding the genetic events that induce HCC in HCV infected patients. We here show that reduced expression of *MSH2, GTBP, MLH1* and *PMS2* is a frequent event in HCC. Both *GTBP* and *PMS2* are possible candidates for hepatocarcinogenesis HCC in HCV infected patients. However, the mechanism(s) involved in this process have to be clarified. Reduced expression of *hMLH1* occurs in the early stages of hepatocarcinogenesis as evidenced by finding no significant difference in the expression level of *hMLH1* between tumors and NAHTs. reduce reduce expression of *hPMS2 was* most frequently found in non- cirrhotic HCC associated with HCV infection.

Although the results of the present study are encouraging, the rarity of data concerning the role of MMR gene defects in HCC development and the stage at which these defects become manifest necessitates confirmation of these results in a larger study to establish the correlations and the clinical significance of these results. Also, reduced MMR capabilities must contribute to acquired resistance to chemotherapeutic agents. In such cases, evaluating MMR genes in tumors by multiplex RT-PCR could help to predict response to aggressive chemotherapy.

In addition, a high rate of p53 mutation in HCC was reported in several studies (50-60%) indicating a role in the development and/or progression of HCC. Recent data display a direct interaction of p53 with specific MMR-2 (hMSH-2) promotor DNA binding activity, which could affect the cell cycle and the repair mechanism. However, there is a discrepancy in the incidence of p53 mutations reported which could be explained by the difference in dietary aflatoxin intake and the prevalence of HBV and HCV infection in different geographical areas.

In conclusion, since the exact mechanism of action of HCV in the context of HCC is still poorly understood, clarification of the molecular basis of viral replication in hepatocytes, the possible genetic and cytogenetic abnormalities that may be induced by the virus should be emphasised. Moreover, early detection of hepatocellular changes by molecular biomarkers may help to detect individuals at high risk of development HCC, thus allowing more effective intervention for cure or prevention.

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