

Review

MDM2–p53 Pathway in Hepatocellular Carcinoma

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Abstract

Abnormalities in the *TP53* gene and overexpression of MDM2, a transcriptional target and negative regulator of p53, are commonly observed in cancers. The MDM2–p53 feedback loop plays an important role in tumor progression and thus, increased understanding of the pathway has the potential to improve clinical outcomes for cancer patients. Hepatocellular carcinoma (HCC) has emerged as one of the most commonly diagnosed forms of human cancer; yet, the current treatment for HCC is less effective than those used against other cancers. We review the current studies of the MDM2–p53 pathway in cancer with a focus on HCC and specifically discuss the impact of p53 mutations along with other alterations of the MDM2–p53 feedback loop in HCC. We also discuss the potential diagnostic and prognostic applications of p53 and MDM2 in malignant tumors as well as therapeutic avenues that are being developed to target the MDM2–p53 pathway. *Cancer Res*; 74(24); 7161–7. ©2014 AACR.

Introduction

The increase in cancer burden in the world, from 12.7 million new cases in 2008 to a predicted 22.2 million by 2030 (1), makes cancer a critical global problem. Of all human cancers, hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and is the third leading cause of cancer death globally (2). HCC is commonly correlated with viral infection (hepatitis B and C), alcohol consumption, and aflatoxin B1 exposure. Moreover, HCC is often accompanied by cirrhosis and hepatic insufficiency, which makes the treatment of HCC more difficult than for many other forms of cancer. Currently, surgical resection is the most commonly practiced therapy for HCC.

p53 acts as a tumor suppressor by initiating cell-cycle arrest, apoptosis, and senescence in response to cellular stress to maintain the integrity of the genome. Fifty percent of overall human tumors carry mutant p53, and many p53 mutants facilitate oncogenic functions such as increased proliferation, survival, and metastasis or exert a dominant-negative regulation over remaining wild-type (WT) p53 (3). p53 is primarily regulated by the E3 ubiquitin ligase MDM2 (Murine Double Minute 2; usually denoted as Mdm2 in mice and HDM2 in humans; MDM2 is used hereafter for simplicity). MDM2 binds

p53 at its transactivation domain blocking p53-mediated transcriptional regulation, while simultaneously promoting its polyubiquitination and proteasome-dependent degradation. Interestingly, p53 enhances *MDM2* transcription through p53-specific response elements in the promoter region of *MDM2*, thus forming an autoregulatory feedback loop, which is critical to control the balance of p53 and MDM2 (Fig. 1). Inhibition of MDM2–p53 binding could reactivate p53 in cancer cells with WT p53 and may offer an effective therapeutic approach for millions of patients with cancer (4). Furthermore, the top two risk factors of HCC are metabolic disease (such as fatty liver) and viral infection (such as hepatitis B and C), both of which cause cirrhosis before HCC (5, 6). As one of the hallmarks of cancer (7), the changes observed in cancer cell metabolism and bioenergetics are current hotspots in cancer research (8), and the ability of p53 to regulate metabolism has also been attracting more attention over recent years (9). Therefore, the connection between p53 stress response and the disordered metabolic process leading to HCC, as the liver is the primary metabolic organ, is a potential avenue for development of targeted therapies against HCC. In this review, we focus on the role of the MDM2–p53 pathway in HCC, but the basic principles discussed here can also be applied to other forms of cancer.

p53 misregulation in hepatocellular carcinoma

p53-mediated apoptosis depends primarily on death stimuli that target the mitochondria either directly or indirectly through the proapoptotic members of the Bcl-2 family such as Bax and Bak, which both exhibit reduced expression in HCC with mutated *TP53* (10). Interestingly, the normal liver is relatively resistant to p53-mediated cell death, and the link between apoptosis and the translocation of p53 to the mitochondria following DNA damage is rarely observed (11). In cultured HCC cells, p53 activation preferentially triggers cell-cycle arrest rather than apoptosis, and the mitochondrial-dependent p53 program of apoptosis is also often blocked in hepatocytes (12). One potential mechanism responsible for this

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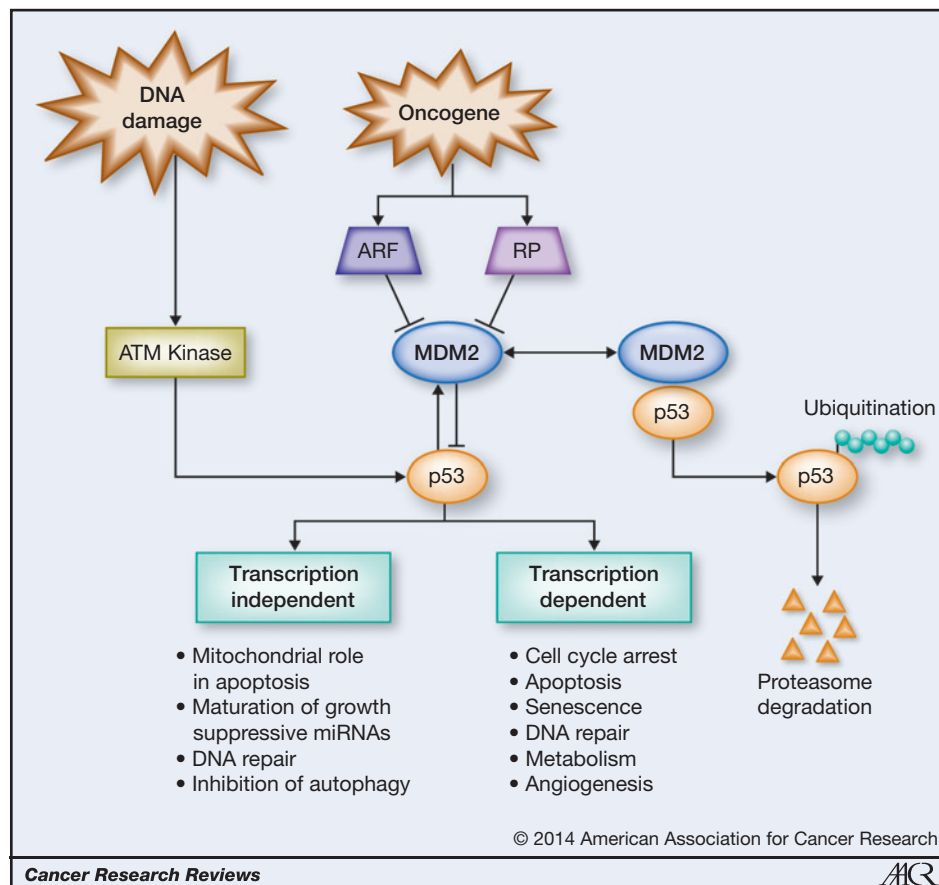


Figure 1. The MDM2-p53 autoregulatory feedback loop. MDM2 inhibits p53 by stimulating its degradation, blocking its transcriptional activity and promoting its nuclear export. In turn, p53 activates the expression of MDM2. DNA damage induces p53 phosphorylation, which prevents the MDM2-p53 binding and leads to p53 stabilization. Oncogenes such as *c-Myc* can activate p53 through p14ARF-MDM2-p53 pathway.

change is that p53 activation results in the enhanced expression of hepatic insulin-like growth factor-binding protein-1 (IGFBP1), which antagonizes the mitochondrial p53 program and inhibits apoptosis (13). It is clear that p53 plays a role in mitotic fidelity and DNA ploidy conservation in hepatocytes of both the normal and regenerative liver. In quiescent livers, hepatocytes exhibit higher ploidy levels in the absence of p53, and this phenotype is further exaggerated when the tissues undergo regeneration after partial hepatectomy (14). p53 not only restricts malignant transformation by triggering a cell-autonomous program of cell-cycle arrest or apoptosis, but it also does so in a non-cell autonomous manner through the release of senescence-associated secretory phenotype to inhibit tumorigenesis by promoting a tumor-suppressive microenvironment. Ablation of the p53-dependent senescence program in hepatic stellate cells under chronic liver damage increases liver fibrosis and cirrhosis, which are associated with reduced survival; furthermore, loss of p53 enhances the transformation of adjacent epithelial cells into HCC (15). In conclusion, p53 plays important and unique roles in normal liver cells and HCC, and it is important to further explore the alterations and mechanisms behind this regulation.

Alterations in the MDM2-p53 pathway in HCC

Alterations in the MDM2-p53 pathway are common in HCC (16-18), and single base substitutions in *TP53* occur in approx-

imately 25% of HCC, suggesting a relevant role for p53 in HCC (19). Mutations of *TP53* in HCC occur primarily in the DNA-binding domain of p53, resulting in a lower affinity to bind the sequence-specific response elements of its target genes, which also decreases p53-mediated induction of *MDM2*. Consequently, the misregulation of MDM2 results in elevated levels of mutant p53 in many tumors cells or tissues (20). MDM2-p53 regulation in the liver seems to differ from other tissues as it is reported that in MDM2 null mice, liver was the only tissue where accumulation of mutant p53 R172H was not detected (21). It is unknown why MDM2 null mice do not express mutant p53 (p53R172H) in the liver; however, several possible mechanisms could contribute to this effect. First, numerous studies including the study by Terzian and colleagues have demonstrated that p53 expression levels in the liver are typically much lower than that of other tissues. Tissues such as spleen, thymus, bone marrow, and small intestine are commonly utilized in the study of mouse p53 because of the readily detectable p53 levels. Second, the regulation of p53 by MDM2 may be different in liver tissue compared with other tissues. For instance, liver is considered a radiation insensitive tissue compared with other tissues, in which radiation induces strong p53 response. It has also been theorized that the MDM2-p53 feedback loop functions differently in liver tissue than in other tissues, and this difference may contribute to the subdued p53 (or p53R172H in this case) accumulation in the absence of

MDM2. Furthermore, unlike in other tissues, p53 seems to be insensitive to the high levels of aneuploidy that accumulate in the liver. Such "abnormal" characteristics of p53 make the liver a unique tissue in terms of p53 regulation, and further studies of the mechanisms behind this tissue specificity are needed to fully understand the complex role of mutant p53 in HCC.

Stresses associated with *in vitro* or *in vivo* systems may also affect the feedback control of the MDM2-p53 loop and its function. In HCC, this loop can be affected at multiple levels (22): (1) frequent p53 mutations occur in aflatoxin-induced HCC (>50%); (2) frequent p53 mutations occur in 20% to 40% of HCC not associated with aflatoxin exposure; (3) micro deletions of p14ARF (alternative reading frame product of *CDKN2A* locus, p19Arf in mouse) occur in 15% to 20% of HCC with WT p53 but rarely occur in HCC with mutant p53; (4) increased MDM2 expression has been observed in HCC; and (5) the vast majority of HCC overexpress gankyrin, which inhibits both retinoblastoma protein (Rb)-checkpoint and p53-checkpoint functions; (6) WT p53 can be inhibited in trans by p53 mutants under conditions of high mutant p53 expression (23). Under normal conditions when key sites in MDM2 and p53 are not phosphorylated (24, 25), an increase in MDM2 expression leads to the direct inhibition of p53 transcriptional activity and facilitates tumorigenic cell growth through the evasion of cell-cycle checkpoint control. Specific hotspots in MDM2 and p53 are associated with environmental carcinogen exposure and the development of HCC. For example, the 309T > G polymorphism (SNP 309, rs2279744), which is located in the intronic p53-responsive promoter of the *MDM2* gene, has the effect of increasing MDM2 protein levels and has been shown to be associated with the early onset of HCC in patients with chronic hepatitis C virus (HCV) infection (26). Yoon and colleagues evaluated the association of MDM2 and p53 polymorphisms with the early onset of HCC in Korean patients with chronic hepatitis B virus (HBV) infection. This study found that not only is the MDM2 SNP 309, but also the p53 codon 72 R > P polymorphism associated with the development of HCC in Korean patients with chronic HBV infection (27). Somatic mutations of R > S at the third base in codon 249 of p53 have also been shown to relate to HBV hepatitis B infection, and exposure to aflatoxin B1 (28). These HCC-associated alterations provide potential targets for earlier detection of multiple cancers, and more specifically, may also help doctors to more accurately diagnose patients with HCC in the clinic.

Mechanisms of targeting the MDM2-p53 pathway in HCC

The following section addresses the specific mechanisms that are known to facilitate HCC development by altering the MDM2-p53 pathway, which may also be applicable to other forms of cancer (Fig. 2). Our laboratory has previously shown that p53 can be stabilized by disruption of ribosome biogenesis, as several ribosomal proteins (RP) that bind to MDM2 are released from the nucleolus when ribosome biogenesis is inhibited (29). In the regenerating rat liver after partial hepatectomy, there is also evidence that a downregulation of rRNA synthesis can stabilize p53 through the inactivation of MDM2-mediated p53 degradation by the binding of RPs released from the nucleolus (30). Hepatitis B virus X-protein (HBx), which

binds to p53 and localizes it to the cytoplasm, has been shown to play an important role in the development of HCC. Doxorubicin treatment has been shown to increase p53 levels in cells containing HBx protein; additionally, doxorubicin treatment restores p53-mediated transcriptional activity by reducing MDM2 levels and increasing the nuclear accumulation of p53 (31). Similarly, inactivation of the tumor suppressor KLF6 has been reported to occur in response to HCV infection and KLF6 expression has been shown to inversely correlate with HCC prognosis (32). KLF6 is a member of the Krüppel-like C2H2 zinc finger family, which has been shown to be involved with cell-cycle regulation, signal transduction, and cell differentiation. Tarocchi and colleagues reported that reduced KLF6 expression causes MDM2 to increase along with p53 reduction, and this imbalance in the MDM2-p53 pathway is further associated with decreased survival in patients with surgically resected HCC; conversely, overexpression of KLF6 leads to reduced MDM2 expression and a corresponding increase in p53 expression in HCC cell lines (33). Jung and colleagues found that the Enigma LIM domain protein, which is involved in signal transduction through protein kinases, can increase MDM2 ubiquitin ligase activity and p53 degradation (34). Furthermore, Enigma can be stimulated by serum response factor (SRF), which is also overexpressed in HCC and leads to further MDM2 stabilization and p53 degradation (34, 35). Min and colleagues showed that one p53 target gene, phosphatase of regenerating liver 1 (PRL-1), that is overexpressed in a variety of cancers through an unknown mechanism, strongly down-regulated p53 levels and inhibited p53-mediated apoptosis by inducing MDM2 phosphorylation through Akt signaling, which forms another feedback loop contributing to HCC development (36). Inhibitor of growth 1 (ING1) has been reported as a type II tumor suppressor that affects cell function by altering chromatin structure and regulating transcription (37). Zhu and colleagues found that ING1 acts as a tumor suppressor by inhibiting hepatoma cell proliferation through the induction of apoptosis and cell-cycle arrest. These tumor suppressor functions are likely mediated by two possible mechanisms: an increase in p14ARF expression to inhibit MDM2, or an increase in p53 acetylation and activation (38). It has been demonstrated that iron status influences p53 activity by downregulating MDM2 expression and that this decrease in MDM2 expression plays a protective role in HCC development (39). Interestingly, there is a potential feedback loop between p53 activation and iron concentration because p53 has been shown to contribute to growth arrest by reducing iron uptake and intracellular iron concentration through interaction with iron-responsive element-binding proteins (40). Sirtuin-3 (Sirt3) is a member of the mammalian sirtuin family that is localized to the mitochondria and contributes to the control of metabolic activity and is further associated with the deregulation of cancer cell metabolism, commonly referred to as the Warburg effect (41, 42). Sirt3 protein expression is shown to be downregulated in human HCC tissue; furthermore, overexpression of Sirt3 inhibited HCC cell growth and induced apoptosis in HepG2 and HuH-7 cell lines by upregulating p53 protein activity (43). As summarized in Fig. 2, the balance between MDM2 and p53 is disrupted in HCC. Each of the factors shown performs a unique

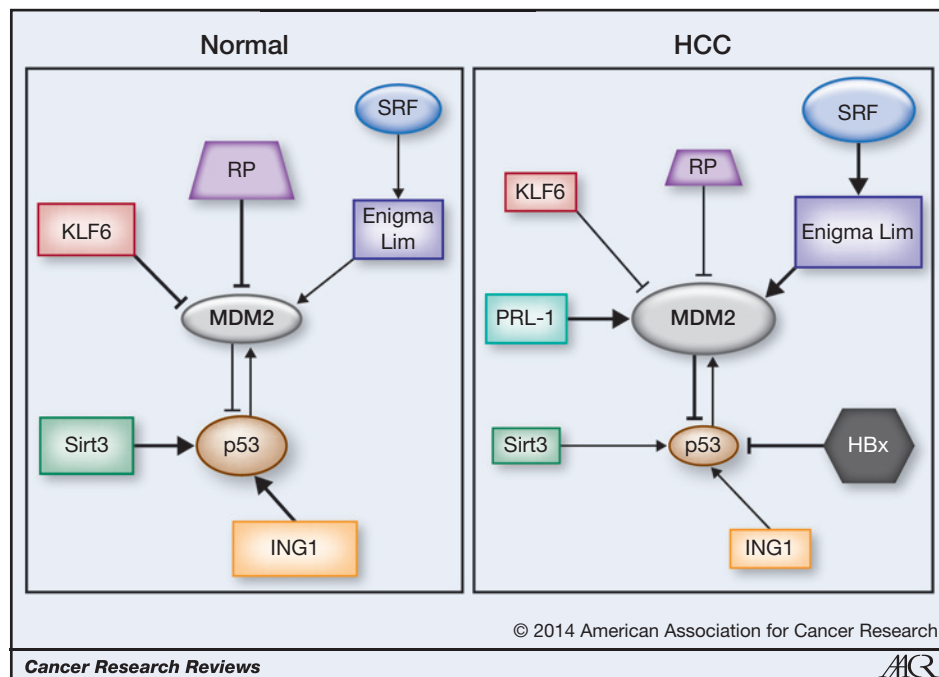


Figure 2. MDM2–p53 pathway alterations in HCC with WT p53. In normal tissues, the expression of MDM2 and p53 is balanced, whereas in HCC cells, the balance is disrupted, where the expression of MDM2 can be high and p53 can be low. This imbalance can be attained through the higher expression of SRF, Enigma Lim, HBx, and PRL-1 in combination with lower expression of KLF6, Sirt3, and ING1. Furthermore, upregulation of rRNA synthesis can inhibit p53 due to reduced ribosomal protein availability for MDM2 binding. (Larger shapes depict higher expression of the indicated proteins and thicker lines depict upregulation of indicated pathways.)

role in facilitating cancer development, but dysfunctional p53 is consistently shown to be the core contributor to the development of HCC. More systematic and comprehensive studies are needed to provide a better understanding of these mechanisms and other currently unknown mechanisms that contribute to HCC development through the manipulation of p53.

Potential of the MDM2–p53 pathway in the diagnosis and prognosis of hepatocellular carcinoma

The connections between the MDM2–p53 loop and HCC development suggest that a better understanding of this pathway could be a valuable tool for the diagnosis of malignancies or prognoses for patients with HCC (44). Specifically, serum concentrations of p53 could be a convenient and useful noninvasive screening test for HCC. There is a reported correlation between high levels of p53 expression in aggressive HCC phenotype to both early recurrences of HCC and poor clinical outcomes (45). A case-controlled study demonstrated that the levels of anti-p53 antibodies triggered by accumulation of mutant p53 were also associated with increased malignancy, which indicates that serum p53 protein levels and antibody concentration may be used as early serologic markers in the diagnosis of HCC (46–48). Moreover, the levels of p53 and MDM2 in HCC tissue were shown to be significantly higher than those in the adjacent hepatic tissues. Zhang and colleagues demonstrated that p53 and MDM2 are overexpressed in all 181 pairs of HCC tissues compared with the adjacent hepatic tissues in their study (49). Patients with a low pathologic grade HCC had a higher tendency to express p53 in tumor cells than the patients with high pathologic grade HCC; similarly, decreased MDM2 expression in HCC was also shown to be a predictor of better survival of patients after tumor resection (49). These studies indicate that p53 and MDM2

expression levels could be useful indicators for predicting the prognosis of HCC. Currently, the approaches utilized in the early diagnosis of HCC are limited. The observed alterations in the MDM2–p53 pathway may provide more effective and accurate methods to diagnose early-stage HCC, which would enable earlier surgical resections for patients and significantly improve the clinical outcomes even in the absence of a novel targeted therapy against the pathway.

Therapeutic avenues of targeting the MDM2–p53 pathway in malignant tumors

The critical role of p53 in tumor development and progression has made p53 an exciting target for anticancer drug design (50). Therapies that focus on restoring p53 function in tumors have been shown to be deleterious to cancer cells that express both mutant and WT p53 (12). The main strategies for the treatment of these cancers aim to deliver exogenous therapeutic WT p53 or to restore WT p53 function from inactivation by using the following methods: (1) chemotherapy and radiotherapy, (2) overexpression of ADP-ribosylation factor proteins that block p53 degradation pathways, (3) disruption of the MDM2–p53 interaction, and (4) introduction of molecules that stabilize the active conformation of the p53 protein (51). To investigate the importance of the MDM2–p53 loop in radiation-induced cell death in HCC, Koom and colleagues used two HCC cell lines expressing different levels of MDM2 and two adenoviral vectors containing WT or MDM2 binding deficient human p53. The study demonstrated that the antitumor effect was the highest for WT p53 plus radiotherapy in the low-level MDM2 cells, whereas the tumor suppressor effect is mimicked by overexpressing MDM2 binding deficient p53 in the MDM2-overexpressing cells (52). The study also demonstrated that disrupting binding between p53 and MDM2 can effectively kill

tumor cells that overexpress MDM2. Specifically, exogenous WT p53 induces not only apoptosis, but also causes a down-regulation of genes involved in angiogenesis, which makes tumors more sensitive to chemotherapy (53). Xue and colleagues used RNAi to conditionally regulate endogenous p53 expression in a mouse model of liver carcinoma to determine whether brief reactivation of endogenous p53 in p53-deficient tumors can produce complete tumor regression. Interestingly, the primary cellular response to p53 reactivation was not apoptosis, but involved the induction of a cellular senescence program that was associated with differentiation and the upregulation of inflammatory cytokines (12). This study illustrated how the cellular senescence program can act together with the innate immune system to potentially limit tumor growth, and also proved that p53 loss is required for the maintenance of mouse liver carcinomas.

Activation of WT p53 in tumors through manipulation of the MDM2-p53 pathway could be achieved by four main techniques: (i) using mimetics of a negative regulator of MDM2, such as p14ARF (54); (ii) by reducing MDM2 levels with antisense oligonucleotides or siRNA (55); (iii) blocking the interaction between MDM2 and p53 with small molecules, synthetic peptides, or monoclonal antibodies; and (iv) blocking the MDM2-mediated ubiquitination of p53 (56). p14ARF inhibits MDM2-dependent degradation and transcriptional silencing of p53; therefore, p14ARF mimetics could be used to activate the p53 stress response pathway. Midgley and colleagues screened a series of overlapping synthetic peptides derived from the p14ARF protein and found that a peptide corresponding to the first 20 amino acids of p14ARF is sufficient to bind MDM2, which induces p53 protein stabilization and activates p53-dependent transcriptional regulation (54). MDM2 can also be inhibited by antisense anti-MDM2 oligonucleotide and the *in vivo* antitumor activity of such an oligonucleotide occurs in a dose-dependent manner (55). Small molecules that disrupt MDM2-p53 interaction such as nutlin-3, MI series (57, 58), benzodiazepinedione (59), and RITA (reactivation of p53 and induction of tumor cell apoptosis; ref. 60) can be used to treat cancer through increased stabilization of p53. Moreover, MDM2 E3 ubiquitin ligase inhibitors such as HLI98 (61), Tenovin-1/Tenovin-6, and JJ78:12 (62) can similarly disrupt p53-MDM2 complexes and exert an antitumor effect by stabilizing p53 protein. Ribosomal proteins, such as L11 and L23, are known to induce p53 by inhibiting MDM2-mediated p53 degradation (63, 64), which suggest that modification of the RP-MDM2-p53 pathway may also reduce the proliferation of tumor cells with WT p53. Better systems for the delivery of

exogenous p53 or small peptides are being developed for more efficient transduction of tumor cells. It is expected that some of these small molecules will progress to clinical use, alone or in combination with other established therapeutic interventions, and these studies will inform the design of novel approaches that specifically target the MDM2-p53 pathway. The clinical application of MDM2-p53 modulating drugs is under intense development. Safety and efficacy of newly designed drugs capable of modulating either WT or mutant p53 are now being evaluated.

Future Studies and Conclusion

Future studies should focus on the regulation of metabolism by p53 especially in the reversible fatty liver stage, which will help to elucidate the central molecular mechanisms at this pre-HCC stage and potentially contribute new ideas on how clinicians may be able to stifle HCC in its vulnerable early stages. The effect of the MDM2-p53 loop in cancer progression is complex, and despite the high number of studies that have already been completed, it is clear that a better understanding of tumor cell-specific signaling pathways that modulate the MDM2-p53 pathway is required for the improvement of clinical outcomes. In this review, we have summarized the vital roles that the MDM2-p53 pathway plays in the development of HCC, the specific mechanisms that are known to facilitate HCC development by altering the MDM2-p53 pathway, and the current research initiatives aimed at using this knowledge in the clinic. By focusing on HCC, we hope to give a detailed account of the molecular mechanisms affecting the MDM2-p53 pathway and the important role that balanced MDM2-p53 expression plays in HCC that can be translated to other forms of cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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