EDITORIALS

Much ado about Nutlin

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Human hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and is particularly common in the Asia-Pacific region.¹ Because no effective therapies are available, its overall prognosis is poor. Molecular analyses from HCC samples, either in the form of unbiased approaches using expression profiling strategies or more targeted approaches to elicit specific alterations in signaling pathways, have been intensely pursued in an attempt to identify the key processes that contribute to HCC and to stratify individuals at high risk of recurrence, but also in the hope of designing more effective targeted therapies.

Since its discovery 30 years ago, the tumor suppressor p53 has been the subject of active study because of its importance in human cancers. Defects of p53 (either mutations or disrupted gene activation pathways) are commonly found in human HCC. The contribution of p53 to chromosomal instability (CIN) in hepatocarcinogenesis has been shown in human samples^{2–4} as well as in mice exposed to diethylnitrosamine (DEN).⁵ CIN can lead to mutations, deletions, translocations and polyploidy of chromosomal material. In human HCC, chromosomal abnormalities include 1, 4q, 8, 9p, 11, 13, 16q and 17p.^{5–7} Also, in >80% of hepatitis B virus-associated HCC, viral DNA sequences integrate at multiple sites to cause chromosomal rearrangements and deletions.⁸ Many affect chromosome 17, in the vicinity of p53.⁸

Tumor suppressor p53 is activated (levels increase and protein moves to the nucleus) by cell stresses, particularly in response to DNA damage.^{4,9} Activated “p53 effector pathways” include DNA repair and genomic stability, cell cycle arrest (through p21, to enable time for DNA repair) and deletion of DNA-damaged cells, either actively by apoptosis or passively by senescence.⁹ Together, p21 expression, induction of apoptosis and degradation of anti-apoptotic Bcl-XL provide a molecular fingerprint of p53 biological actions.¹⁰ Among numerous studies of differentially expressed genes in human HCC, the striking themes associated with poor survival are upregulation of mitosis-promoting/cell proliferation genes and downregulation of p53.^{11,12} p53 is also the most common loss of heterozygosity (LOH) site.^{2,3,11,12} It seems likely that inactivation of p53 by mutation, deletion or upregulation of pathways for its proteasomal degradation contributes importantly to the molecular pathogenesis of HCC,^{9–11} for example, by facilitating expansion of preneoplastic lesions. We recently showed the potency of p53 as a “brake” against HCC. In ataxia-telangiectasia mutated *–/–* mice treated with DEN, p53 is upregulated early in response to ataxia-telangiectasia-related protein, a pathway for sensing of DNA strand

breaks. In these mice, no animal developed HCC or even preneoplastic foci by 15 months, in marked contradistinction to >80% of wild-type (wt) mice.¹³ Thus, interventions to stabilize or restore wt p53 would be attractive HCC therapeutic options.

The cellular level and activity of p53 are under tight control both under physiological conditions and during stress. Post-translational modifications can stabilize and activate p53.¹⁴ Under normal conditions, p53 levels are maintained by the mouse double minute-2 (mdm2)-p53 autoregulatory loop, (¹⁵, Figure 1a). Genetic disruption of the mdm2 gene is embryonically lethal in mice, and rescue from lethality is ensured when p53 is concomitantly deleted.¹⁶ Genotoxic and other stressful stimuli not only induce an accumulation of p53, but also the phosphorylation of mdm2, thereby enhancing p53's nuclear localization, ubiquitination and subsequent degradation.¹⁷ Of note, mdm2 is overexpressed in many tumors and effectively impairs p53 function by binding directly to p53; this promotes its ubiquitination and targeting to the 26S proteasome for protein degradation.¹⁷

Protein–protein interactions have long been considered challenging targets for therapeutic intervention, because their opposing surfaces are often too large or flat for effective disruption by small molecules. The more successful chemical antagonists take advantage of specific interactions within well-defined pockets on the surfaces of one or both protein partners.¹⁸ The discovery that p53-mdm2 binding was dependent on only *three* p53 amino acid

Figure 1 Schematic diagram summarizing the p53-mouse double minute-2 homologue (mdm2) autoregulatory loop and mechanism by which mdm2 antagonists operate. (a) Mdm2 and p53 mutually regulate their levels in unstressed proliferating cells. Mdm2 expression is controlled by a p53-dependent promoter, and is upregulated when p53 levels increase. In turn, mdm2 binds p53 and inhibits its transcriptional activity; mdm2 also serves as an E3 ubiquitin ligase for p53, thereby facilitating its ubiquitin-dependent degradation by the 26S proteasome. (b) Small molecule antagonists of mdm2, such as the Nutlins, bind to the p53 pocket, thereby inhibiting the p53-mdm2 interaction and release p53 from negative control. Thus, p53 stabilizes, accumulates in the nucleus and activates p53 dependent signalling pathways. Ub, ubiquitin.

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residues interacting with a discrete mdm2 pocket stimulated efforts to identify potential small molecule inhibitors.¹⁹ The first potent and selective antagonists of p53-mdm2 were the Nutlins.²⁰ They represent a class of *cis*-imidazole analogues that bind to the p53 pocket on the surface of mdm2 in an enantiomer-specific manner. The three reported Nutlins, -1, -2 and -3, show potency against p53-mdm2 binding in the 100–300 nmol range with 150- to 200-fold range in affinity between enantiomers. They inhibit the p53-mdm2 axis by mimicking the interaction of the three critical amino acid residues with the hydrophobic activity of mdm2.²⁰ In addition to their high potency *in vitro*, they penetrate cell membranes, activate p53 and inhibit cell proliferation at a range of 1–3 μ mol. Released from its negative control in the presence of Nutlin (Fig. 1b), p53 is stabilized and accumulates in cells leading to the activation of target genes; for example, p21 and mdm2. This effect, however, is dependent on the presence of wt p53, because cells in which p53 is deleted or mutated do not respond to Nutlin treatment.²⁰ In addition to parenteral routes, Nutlins can also be given orally, which is highly desirable for their applicability in animal models and in the clinic.²⁰

In this issue of the *Journal*, Wang *et al.*²¹ utilized Nutlin-3 against three human HCC cell lines with wt (HepG2), mutant (Hep3B) and null p53 (Huh7). This selective mdm2 antagonist induced growth arrest in all three cell types *in vitro* with significant abrogation of the pro-proliferative genes, cyclin D1/cdk4, cyclin E/cdk2 and E2F transcription factor. Cell cycle arrest was mediated by upregulation of p21 only in p53-intact HepG2 cells, whereas p27, another downstream target of p53, was expressed by all three tumor cell lines. Regardless of p53 status, Nutlin-3 treatment triggered increased apoptosis in all tumor cells, as well as increased expression of Bax, Noxa and PUMA.²¹

p73 is a member of the p53 family, sharing similarities in protein structure and activity. Like p53, p73 is activated in response to stress and the oncogenes, E1A and myc.²² Once activated, p73 (like p53) can induce apoptosis and cell cycle arrest. p73-dependent apoptosis is primarily regulated by its ability to transcriptionally activate pro-apoptotic genes including Bcl2 family members Bax, PUMA, Noxa, Bad, the death receptors TRAIL-R1, TRAIL-R2, TNF-R1, as well as caspases 3, 6 and 8.^{22,23} In order to ascertain whether p73-mediated apoptosis was operative in the tumor cell lines lacking intact p53, the authors carried out p73 siRNA knockdown in Hep3B and Huh7 cells. This led to a significant diminution of cell death compared with mock-construct controls.²¹ These findings suggest that Nutlin-3-induced apoptosis might be mediated by both p53 and p73. Furthermore, carrying out of co-immunoprecipitation studies in HepG2 cells showed that Nutlin-3 treatment caused an impressive reduction in both p53-mdm2 and p73-mdm2 complexes, with a persistent effect in the latter after p53 knockdown.

Despite the promising findings in this paper, there are several major issues associated with p53 activation as a therapeutic strategy in HCC and other cancers for that matter. First, it has been postulated that p53 activation requires not only stabilization and accumulation of the protein, but also post-translation modifications, such as phosphorylation, acetylation and sumoylation, in response to genotoxic stress.²⁴ Although small molecule antagonists of mdm2 prevent p53 from binding to mdm2, they do not technically affect post-translational modification.²⁰ Thus, p53 that is stabilized by interference with Nutlins might well still be func-

tional. Second, although approximately 70% of human HCC bear aberrant p53, it is unclear if wt p53 still exists and remains functional in these tumors, or whether the downstream signaling pathways of p53 are intact. Although defects in the signaling upstream of p53 can be compensated by other molecules, such as HAUSP and COP-1 (which also stabilize p53 and p73),²⁵ there are few compensatory mechanistic alternatives for defects downstream of p53; Nutlins would be rendered ineffective in the latter context.

While the work by Wang *et al.*²¹ is of major clinical relevance, the true impact of their observations await further validation in other HCC experimental models that are more akin to human HCC. Although the investigations have mechanistically shown the importance of the p53 and p73-mdm2 mediated pathways in a targeted intervention for HCC, what remains unresolved are: (i) the role of p53, p73 and mdm2 in the “at risk” cirrhotic liver; and (ii) nor have the pathogenic contributions of p53 and mdm2 as key steps early in liver carcinogenesis been addressed. The present study underscores the fundamental importance of understanding the molecular pathways underlying hepatocarcinogenesis, utilizing appropriate systems and models to test targeted interventions. While we can rejoice in the encouraging results of using Nutlin-3 against HCC cells in culture, more definitive experiments are necessary to show a discernible benefit before its utility in human HCC can be tested.

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Solid pseudopapillary tumor and wnt signaling pathway . . . Way to go!?

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Solid pseudopapillary tumors (SPT) are uncommon tumors, constituting 1–2% of all pancreatic exocrine tumors and 5% of cystic

pancreatic tumors.¹ First described by Franz in 1959,² several large series have recently been presented in the literature, which perhaps indicates the increased awareness and ability to characterize this tumor. The classic scenario is of a young woman presenting with non-specific symptoms. The tumors, though dramatically large on presentation, are indolent with low-grade malignant potential and with excellent response to surgical resection.³

Imaging and histology are the two keystones on which the diagnosis rests. Despite progress in imaging, SPT have to be differentiated from cystic neoplasms and the intraductal papillary mucinous neoplasms of the pancreas. On the other hand, although the microscopic appearance of SPT is characteristic, it is a challenge to differentiate these tumors from pancreatic neuroendocrine tumors, acinar cell carcinoma, metastatic renal cell carcinoma and metastatic melanoma, especially in the limited material available by way of needle biopsy (percutaneous or endoscopic ultrasound-guided).

The definitive diagnosis of SPT, especially in limited material as in needle biopsies is based on characteristic immunohistochemistry findings. A typical SPT demonstrates CD10 (neural endopeptidase) immunoreactivity in 80% of cases, progesterone receptor positivity, focal synaptophysin staining, beta-catenin and E-cadherin expression. The beta-catenin expression is 100% in SPT with cytoplasmic and nuclear staining. As regards E-cadherin, there is loss of membrane staining with the antibody to the extracellular domain whereas there is nuclear staining with the antibody to the cytoplasmic domain.^{3,4} In contrast, in pancreatic neuroendocrine tumors, there is characteristic membrane staining of E-cadherin with absent nuclear staining of beta-catenin, which differentiates these tumors from SPT.⁴

E-cadherin and beta-catenin are important molecules in the Wnt signaling pathway and the distinct over-expression profile of beta-catenin seen provides irrefutable evidence of this pathway in the tumorigenesis of SPT. Moreover, mutations of the beta-catenin gene have been identified in most SPT. These mutations are at or around the GSK-3 beta phosphorylation sites, which abrogate subsequent ubiquitin-mediated degradation of beta-catenin protein; this allows for cytosolic accumulation with consequent nuclear shift and an increase in transcriptional activity.^{5,6}

The question is whether this key information is limited for use in diagnosis alone, or whether we can move further to explain and understand the tumor phenotype and behavior, or even to develop and evaluate targeted therapy for this tumor. Beta-catenin protein is an important linking molecule between E-cadherin and cytoskeleton. Thus, mutation of beta-catenin and loss of membrane E-cadherin leads to disruption of adhesion junctions and eventual disorganized growth that forms the characteristic pseudopapillary pattern of SPT.⁷

Based on this molecular profiling and characterization, it would be beneficial as a next step to identify candidate molecularly targeted therapies for SPT. A novel methodology is to create a virtual prototype tumor cell using all the known signal pathways. This biological system would then enable virtual screening of molecularly targeted drugs alone or in combination, coupled to the capability to predict quantitatively cellular pathways and markers like apoptosis, survival, angiogenesis, metastasis and tumor metabolism.^{8,9}

Using the knowledge about beta-catenin over-expression and activity as being the key differentiator for SPT, one can create a virtual customized tumor profile of SPT, where beta-catenin is

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