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HIPPO TUMOR-SUPPRESSOR PATHWAY – novel implications for the treatment of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) remains the main cause of death in cirrhotic patients and the third most common cause of cancer related death worldwide (CA Cancer J Clin 2005;55(2):74-108). Curative treatment options are restricted to less than 30-40% of patients due to disease progression at diagnosis (Lancet 2003; 362:1907-1917), demanding better surveillance programs for early tumor detection and the development of both chemopreventive treatments and novel treatment strategies. A multikinase inhibitor sorafenib greatly succeeded in the treatment of advanced HCC and became the only FDA-approved therapy, clearly demonstrating that molecular targeted therapy is a realistic treatment option in this cancer (N Engl J Med 2008;4:378-390). This success has facilitated many preclinical and clinical trials investigating novel small molecules and monoclonal antibodies for the treatment of HCC (Hepatology 2008;48:1312-27). In the future, more precise and comprehensive characterization of molecular classes, genetic and epigenetic changes, and altered cell signaling pathways should be taken into account to choose the right treatment scheme for each patient, leading to a much more personalized medicine in HCC (Semin Liver Dis 2010;30(1):35-51).

Wnt- β -catenin, p53, IGF, Ras-MAPKK, PI3/Akt/mTor and c-Met are well studied signaling pathways involved in the development and progression of HCC. Recently, genetic and biological studies confirmed the importance of the novel Hippo tumor-suppressor pathway in regulating cell proliferation, apoptosis, organ size and tumorigenesis in mammals (Cancer Cell 2008;13:188-192). This relatively new pathway was first described in *Drosophila* in 1995 by identifying the tumor suppressor Warts (WTS), followed by the establishment of

several other core Hippo pathway components (Fig. 1) (Curr Opin Cell Biol 2008;20(6): 638-46). In mammalian cells NF2, FRMD6 and FAT4 are upstream regulators of the MST1/2 kinases that physiologically form a complex with WW45 upon activation to then phosphorylate and activate the downstream kinases LATS1/2. This core complex together with MOB1 phosphorylates and inhibits the transcription co-activators YAP1 and TAZ by causing their cytoplasmic translocation (Nat Rev Mol Cell Biol 2007;8(8):613-21; Nat Rev Cancer 2007;7(3):182-91).

Many of these Hippo pathway members have been implicated in the genesis of cancer. *NF2* is mutated or silenced in familial (neurofibromatosis type 2) and sporadic human tumors, *MOB1* deletion has been shown in human melanoma and mouse mammary gland carcinoma. *LATS1* deficient mice develop ovarian tumors and soft tissue sarcomas and both *LATS1* and 2, have been shown to be silenced by methylation in an aggressive subtype of breast cancers. Furthermore, *LATS2* seems to be regulated by two micro-RNAs (miRNA), commonly overexpressed in testicular cancer (Nat Rev Cancer 2007;7(3):182-91). Contrary to these tumor suppressors, *YAP1* is a candidate oncogene due to their function as growth promoters and induction of EMT, cell migration and invasion, respectively. The amplification of *YAP1* in human cancers including liver cancer was first described in 2006 (PNAS 2006;103:12405-12410; Cell 2006;125:1253-1267) and its overexpression in mouse liver induced a dramatic increase in organ size and HCC formation (Cell 2007;130:1120-1133; Curr Biol 2007;17:2054-2060). Prostate, colon, breast, ovarian, lung cancer and medulloblastoma show increased expression of YAP1 and thereby support its oncogenic function. Hypermethylation and silencing of two candidate MST1 regulators RASSF1A and NORE1A/B as well as decreased activity of MST1 was detected in human HCC samples, suggesting an important role in the apoptotic cascade (Gastroenterology 2006;130(4):1117-1128), but only one clinical study of 177 HCC samples detected YAP1 expression in 62% of the cases, associated with poorer tumor differentiation and high serum α -fetoprotein (AFP) levels (Cancer 2009;115:4576-4585).

We will summarize and comment herein the recent work of Zhou et al. who confirmed the role of Mst1/2 kinases as tumor suppressors by showing that combined *Mst1/2* deficiency leads to loss of the inactivating phosphorylation of YAP1, massive liver overgrowth and the development of HCC *in vivo* (Cancer Cell;16:425-438). The authors generated germline and liver specific conditional *Mst1/2* double knock-out mice to assess the function of the pathway. Germline *Mst1^{-/-}Mst2^{-/-}* were embryonic lethal, while *Mst1^{-/-}Mst2^{+/-}* mice developed HCCs due to *Mst2* loss of heterozygosity in the tumors as early as at 7 months of age. Interestingly, the authors observed high levels of a cleaved, activated MST1/2 in form of a previously described 34 kDa fragment in normal livers from wild type mice, whereas in spleen only full-length MST1/2 were detectable. This suggests a tissue-specific mechanism of MST1/2 regulation *in vivo* and a constitutive activation of both kinases in quiescent adult liver. In order to explore simultaneous deficiency of *Mst1/2* in the liver, the authors next introduced a *Mst2* conditional knockout allele onto the *Mst1* and 2 germline null background and detected massive organ overgrowth, increased hepatocyte proliferation and multifocal HCCs. *Mst1/2* null livers were protected from apoptosis by injection of the proapoptotic anti-Fas antibody. In a hepatocyte specific Albumin-Cre model, *Mst1^{-/-}Mst2^{F/F}* mice also developed massive liver overgrowth and HCCs at 3 months of age, suggesting that MST1/2 suppress the development of HCC.

To address the influence of *Mst1/2* loss on contact inhibition of proliferation, an *in vitro* model of mouse embryonic fibroblasts (MEF) was used, showing that MST1/2 are not required for LATS1/2 and YAP1 phosphorylation in response to cell density (Fig.1). *Mst1* restoration in a HCC cell line derived from the mouse model was able to inhibit the tumorigenic phenotype, leading to MOB1 and YAP1 phosphorylation in the absence of

altered LATS1/2 phosphorylation, decreased YAP1 nuclear residence, increased fraction of HCC cells in G1, increased early and late apoptotic cells, decreased c-myc expression, reduced cell proliferation and colony formation. These results were also confirmed when *Yap1* depletion in vitro completely restored the phenotype, underlining its critical role as an oncogene in HCC development.

Finally, MST1/2 and YAP1 signaling components were examined in 21 matched human HCC and normal liver samples, showing a loss of negative regulation of YAP1, most likely due to decreased MST1/2 activity.

Comment

Molecular mechanisms driving cancer are extremely complex. In oncology, only few cancers or subtypes have shown dependency of specific genomic hits (CML) or oncogenic addiction loops (HER2-breast cancer subclass). The classical dissection of known pathways and their implication in cancer subtypes is being completed in most cancers, but new signaling cascades are becoming relevant in the oncolgenic field. The advent of whole-genome deep sequencing, on the other hand, is adding novel information on the number and type of mutations occurring in a given cancer, in some instances more than 20-30. Simultaneously, methylome studies and miRNA data are providing clues of additional mechanisms not previously understood. Thus, how to reconcile all this information is becoming a major challenge for translational research, and thus far strategic approaches such as systems biology are still not ready for a comprehensive answer. We herein are commenting on a novel pathway in the field, the Hippo tumor-suppressor pathway, a rather novel and not well-understood signaling cascade in carcinogenesis, for HCC. This cascade might have clear pathogenic implications in hepatocarcinogenesis, and their drivers might represent novel targets for molecular therapies in the coming decades. For the first time, the authors are able to show that the kinases MST1/2 are tumor suppressors that lead to massive liver overgrowth and the development of HCC in mouse liver when deleted. This effect is mediated by tissue specific and LATS1/2 independent loss of YAP1 phosphorylation, promoting its previously described oncogenic activity (Cell 2007;130:1120-1133). Interestingly, two studies published shortly after confirmed this basic finding in similar models (PNAS 2010;107(4):1431-1436; PNAS 2010;107(4):1437-1442). Song et al additionally found no enlargement of lungs, kidneys and intestine after deleting *Mst1/2* in these organs of mouse pups, underscoring the tissue-specific function of these kinases. On the contrary, they also detected cholangiocarcinomas (CC) among the liver tumors and showed an increased number of CK-19 and AFP expressing cells, suggesting that these tumors may have originated from progenitor cells. They also demonstrated that loss of *Mst1/2* renders the liver resistant to TNF α -induced apoptosis and therefore promotes tumorigenesis (PNAS 2010;107(4):1431-1436). Lu et al. generated *WW45* conditional mutant mice that developed liver overgrowth and liver tumors (HCC and CC) to a similar extent as the *Mst1/2* knock-out mice. In this study, microarray analysis of mutants revealed a network of Hippo signaling genes with specific enrichment for genes involved in immune and inflammatory responses. Finally, they showed that Hippo signaling regulated progenitor cell expansion in the liver by repressing oval cell activation.

Although these two latter studies mostly confirm the results of Zhou et al., many questions still need to be answered: What is the mechanism of MST1/2 inactivation in human HCC? Can the “Hippo” gene signature identify a potential patient subclass with altered Hippo signaling? How is the Hippo pathway altered in cirrhosis patients or in those with chronic infection by Hepatitis C or B virus? What are the upstream regulators of LATS1/2 in response to cell contact and by what mechanism does *SAVI* deletion contribute to tumor formation? What role does the other transcriptional co-activator TAZ play in this pathway?

And finally, what potential targets for anti-tumoral therapy can be identified and translated to a clinical setting? Obviously, the understanding of this novel pathway is still at its very beginning and clearly deserves more attention in the field of HCC research.

Just recently CD44 has been identified as an upstream regulator of Merlin in glioblastoma and CD44 antagonists successfully blocked tumor growth in a preclinical model (Cancer Res 2010;70:2455-2464). Erlotinib treatment abrogated the downstream proliferative effects of YAP1 expression in MCF10A breast epithelial cells indicating that the activation of EGFR signalling is an important non-cell-autonomous effector of the Hippo pathway (Nat Cell Biol 2009;11(2):1444-50). Besides those two examples, no therapeutic agents directly targeting the established Hippo pathway components have been identified or tested to our knowledge. Nevertheless, several known molecular pathways seem to be affiliated with the Hippo pathway: cross-talk with the JNK SAPK pathway (Mol Cell Biol 2009;29(24):6380-90), the PI3/Akt pathway (PLOS One 2010;5(3):e9616), the Notch pathway (PLOS One 2008;3(3):e1761) and the Hedgehog signaling (Genes Dev 2009;23:2729-41) still require confirmation for HCC, but will eventually increase the complexity of the disease itself as well as any possible treatment approach. Therefore more preclinical and clinical studies are needed to detect and confirm the importance and implications of this novel pathway in liver cancer. Integrative analysis of whole genome gene and miRNA expression, methylation analysis and mutation data will help to further characterize the Hippo signaling cascade in HCC and to build the basis for the development of novel treatment strategies.

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