Supplementary material

ADDITIONAL PATHOGENETIC MECHANISMS PROPOSED FOR THE DEVELOPMENT OF NAFLD-RELATED HCC

A critical but relatively new-identified link between cellular senescence and non-alcoholic fatty liver disease (NAFLD) also play a role in NAFLD-related hepatocellular carcinoma (HCC)[1]. Senescence was thought to act protectively against the development of HCC. Obesity and NAFLD associated-activation of hepatic stellate cells and their differentiation into myofibroblasts could lead to excessive liver damage, stimulation of oncogenic pathways and expansion of the malignant clones of liver cells[2]. Senescence of those cells mitigates their harmful effects and acts as a tumor suppressor mechanism[3]. Intriguingly, senescence is a double-edge sword since it is strongly associated with DNA damage, activation of oncogenes and aggravation of hepatic fibrosis due to recruitment of fibroblasts by senescent cells[3]. Importantly, a complex of cytokines, metalloproteases, chemokines, growth factors and matrix remodeling factors constitutes the senescence associated secretory phenotype (SASP) which further provokes the inflammation, induces senescence to neighboring cells and facilitates tumorigenesis[4,5].

Moreover, the chronic hyper-caloric-mediated overproduction of reactive oxygen species (ROS) along with increased endoplasmic reticulum stress leads to hepatocyte cell death. Upon hepatocytes apoptosis, the compensatory inflammation attracts adaptive and innate immune cells which facilitate the downregulation of hepatic genes involved in β-oxidation and lipolysis[6]. Those processes along with chronic hepatocyte damage led to aggravated cell death, DNA damage, further activation of innate immunity with subsequent activation of hepatic stellate cells (HSCs), that favor fibrosis development and pre-malignant lesions[7,8]. When antitumor immune surveillance is not adequate, those pro-carcinogenic lesions can ultimately lead to HCC arising.

In addition the altered composition of gut microbiota and their increased translocation contribute to the hepatic inflammatory response and may facilitate HCC onset[9]. Findings from both animal and human studies demonstrated that

gram-negative bacteria producing lipopolysaccharide are accumulated inside the intestines of obese humans and rodents[10]. The enriched bacterial community prevalence in gut microflora triggers the release of pathogen-associated molecular patterns which are recognized by Toll-like receptors leading to initiation of inflammatory cascade inducing the production of Tumor Necrosis Factor-a, interleukin-1 and interleukin-6[11]. Consistent to that, Yoshimoto *et al*[12] demonstrated that high-fat diet led to enriched presence of gram-positive bacteria in gut microbiota and increased the serum levels of deoxycholic acid in a DMBA-induced HCC murine model while depletion of those bacteria by pharmacological agents, namely vancomycin or ursodeoxycholic acid robustly suppressed HCC development[12].

Most of the current studies have highlighted the critical role of several microRNAs (miRNAs), namely miR-122, miR-34a, miR19a, miR-21, miR-29, miR-23 in NASH-related HCC[13]. A genome wide analysis assessing differentially expressed miRNAs among NASH patients, revealed downregulation of liver miR-122 expression leading to impaired lipid metabolism in NASH patients[14]. Along this line, reduced expression of hepatic miR-122 was also observed in a NASH-related HCC mouse model, demonstrating the role of this molecule in the pathogenesis of NASH-related HCC[15]. Moreover, miR-34a interplays transcriptionally with the tumor suppressor p53 gene, a key gene in the development of HCC, driving the inhibition of glycolysis, exacerbation of hepatic fat accumulation and promotion of oxidative phosphorylation[16]. MiR19a and miR21 and others are involved in the PI3K/AKT/mTOR and PI3/AKT axes, the dysregulation of them mediates a wide spectrum of cellular processes essential for tumorigenesis including proliferation, cell survival and angiogenesis[17]. Steatosis and hepatocarcinogenesis were observed in a PTEN deficient mouse model while downregulation of PTEN along with c-Met upregulation drives to development of poorly differentiated HCC in a Rictor conditional knockout animal model[18,19]. Furthermore, PTEN also act as a negative regulator of insulin signaling via antagonism of the aforementioned PI3K-AKT pathway[20]. Loss of PTEN function or PTEN depletion is associated with hepatic steatosis, steatohepatitis and liver cancer[21]. In accordance with that, mutations in

PTEN have also been found in HCC patients[22]. Reduced expression or even absence of PTEN has been identified in almost 50% of HCC and is associated with poor prognosis[23]. In addition, upregulation of miR-216a and miR-217 mediates the activation of oncogenic pathways of PI3K/AKT and transforming growth factor beta by targeting PTEN and SMAD7 respectively leading to HCC recurrence in a rat model[24]. Besides miRNAs, the long noncoding RNAs (lncRNAs) are also implicated in the development of NAFLD and its progression to HCC and beyond that, they interplay with the liver KCs. Wang *et al*[25] demonstrated that small nucleolar RNA host gene 20 (SNHG20) expression was decreased in NAFLD hepatic tissue, but it was significantly upregulated in the NAFLD-related HCC tissue and in liver-derived KCs[25]. Since SNHG20 acts as a regulator of M1/M2 polarization in KCs[25], silence of SNHG20 in a mouse cell-line of macrophages (RAW264.7) led the shift of KCs towards M1 polarization, a tumor suppressor phenotype, provoking a delay in the progression of NAFLD to HCC[25]. On the other hand, SNHG20 overexpression induced M2 polarization, which was mediated by signal transducer and activator of transcription-6 (STAT-6) pathway activation and facilitated hepatic tumorigenesis[25].

Of importance, obesity, as a common feature of NAFLD, modulates several of the above-mentioned mechanisms, further promoting hepatic tumorigenesis. Excessive adipose tissue and an obese phenotype constitute a chronic low-grade inflammatory environment which seems to further promote HCC development as obesity is closely associated with elevated leptin and diminished adiponectin levels aggravating their actions[26-28]. High saturated diet leads to increased lipid storage in the liver with subsequent increased de novo lipogenesis, production and accumulation of ROS and elevated ER stress while obesity-induced insulin resistance further facilitates HCC development through activation of the previously mentioned oncogenic pathways[29,30]. Of note, new studies have highlighted the burdened effect of obesity and metabolic dysfunction on both histone post-translational modifications and chromatin modifiers, promoting dysregulated transcriptional function. Particularly, the hepatic expression of sterol regulatory element-binding protein-1, a major regulator of lipogenesis, and histone deacetylase 8 (HDAC8), a chromatin modifier were significantly upregulated in a high fructose high

carbohydrate murine model[31]. More importantly, HDAC8 upregulation, via the activation of β-catenin signaling and suppression of Wnt antagonists, led to hepatic tumorigenesis in vivo, indicating the association of chromatin dysregulation with NAFLD-associated HCC[31].

References

1 **Papatheodoridi AM**, Chrysavgis L, Koutsilieris M, Chatzigeorgiou A. The Role of Senescence in the Development of Nonalcoholic Fatty Liver Disease and Progression to Nonalcoholic Steatohepatitis. *Hepatology* 2020; **71**: 363-374 [PMID: 31230380 DOI: 10.1002/hep.30834]

2 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]

3 **Krizhanovsky V**, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008; **134**: 657-667 [PMID: 18724938 DOI: 10.1016/j.cell.2008.06.049]

4 **Coppé JP**, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010; **5**: 99-118 [PMID: 20078217 DOI: 10.1146/annurev-pathol-121808-102144]

5 **Tchkonia T**, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *J Clin Invest* 2013; **123**: 966-972 [PMID: 23454759 DOI: 10.1172/JCI64098]

6 **Wolf MJ**, Adili A, Piotrowitz K, Abdullah Z, Boege Y, Stemmer K, Ringelhan M, Simonavicius N, Egger M, Wohlleber D, Lorentzen A, Einer C, Schulz S, Clavel T, Protzer U, Thiele C, Zischka H, Moch H, Tschöp M, Tumanov AV, Haller D, Unger K, Karin M, Kopf M, Knolle P, Weber A, Heikenwalder M. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell* 2014; **26**: 549-564 [PMID: 25314080 DOI: 10.1016/j.ccell.2014.09.003]

7 **Ohnishi S**, Ma N, Thanan R, Pinlaor S, Hammam O, Murata M, Kawanishi S. DNA damage in inflammation-related carcinogenesis and cancer stem cells. *Oxid Med Cell Longev* 2013; **2013**: 387014 [PMID: 24382987 DOI: 10.1155/2013/387014]

8 **Yuan D**, Huang S, Berger E, Liu L, Gross N, Heinzmann F, Ringelhan M, Connor TO, Stadler M, Meister M, Weber J, Öllinger R, Simonavicius N, Reisinger F, Hartmann D, Meyer R, Reich M, Seehawer M, Leone V, Höchst B, Wohlleber D, Jörs S, Prinz M, Spalding D, Protzer U, Luedde T, Terracciano L, Matter M, Longerich T, Knolle P, Ried T, Keitel V, Geisler F, Unger K, Cinnamon E, Pikarsky E, Hüser N, Davis RJ, Tschaharganeh DF, Rad R, Weber A, Zender L, Haller D, Heikenwalder M. Kupffer Cell-Derived Tnf Triggers Cholangiocellular Tumorigenesis through JNK due to Chronic Mitochondrial Dysfunction and ROS. *Cancer Cell* 2017; **31**: 771-789.e6 [PMID: 28609656 DOI: 10.1016/j.ccell.2017.05.006]

9 **Schuppan D**, Afdhal NH. Liver cirrhosis. *Lancet* 2008; **371**: 838-851 [PMID: 18328931 DOI: 10.1016/S0140-6736(08)60383-9]

10 **Szabo G**, Dolganiuc A, Mandrekar P. Pattern recognition receptors: a contemporary view on liver diseases. *Hepatology* 2006; **44**: 287-298 [PMID: 16871558 DOI: 10.1002/hep.21308]

11 **Bashiardes S**, Shapiro H, Rozin S, Shibolet O, Elinav E. Non-alcoholic fatty liver and the gut microbiota. *Mol Metab* 2016; **5**: 782-794 [PMID: 27617201 DOI: 10.1016/j.molmet.2016.06.003]

12 **Yoshimoto S**, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, Iwakura Y, Oshima K, Morita H, Hattori M, Honda K, Ishikawa Y, Hara E, Ohtani N. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 2013; **499**: 97-101 [PMID: 23803760 DOI: 10.1038/nature12347]

13 **Schulze K**, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, Calatayud AL, Pinyol R, Pelletier L, Balabaud C, Laurent A, Blanc JF, Mazzaferro V, Calvo F, Villanueva A, Nault JC, Bioulac-Sage P, Stratton MR, Llovet JM, Zucman-Rossi J. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015; **47**: 505-511 [PMID: 25822088 DOI: 10.1038/ng.3252]

14 **Cheung O**, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW, Kellum JM, Min H, Luketic VA, Sanyal AJ. Nonalcoholic steatohepatitis is associated with altered

hepatic MicroRNA expression. *Hepatology* 2008; **48**: 1810-1820 [PMID: 19030170 DOI: 10.1002/hep.22569]

15 **Takaki Y**, Saito Y, Takasugi A, Toshimitsu K, Yamada S, Muramatsu T, Kimura M, Sugiyama K, Suzuki H, Arai E, Ojima H, Kanai Y, Saito H. Silencing of microRNA-122 is an early event during hepatocarcinogenesis from non-alcoholic steatohepatitis. *Cancer Sci* 2014; **105**: 1254-1260 [PMID: 25117675 DOI: 10.1111/cas.12498]

16 **Tornesello ML**, Buonaguro L, Tatangelo F, Botti G, Izzo F, Buonaguro FM. Mutations in TP53, CTNNB1 and PIK3CA genes in hepatocellular carcinoma associated with hepatitis B and hepatitis C virus infections. *Genomics* 2013; **102**: 74-83 [PMID: 23583669 DOI: 10.1016/j.ygeno.2013.04.001]

17 **de Conti A**, Ortega JF, Tryndyak V, Dreval K, Moreno FS, Rusyn I, Beland FA, Pogribny IP. MicroRNA deregulation in nonalcoholic steatohepatitis-associated liver carcinogenesis. *Oncotarget* 2017; **8**: 88517-88528 [PMID: 29179453 DOI: 10.18632/oncotarget.19774]

18 **Xu Z**, Hu J, Cao H, Pilo MG, Cigliano A, Shao Z, Xu M, Ribback S, Dombrowski F, Calvisi DF, Chen X. Loss of Pten synergizes with c-Met to promote hepatocellular carcinoma development via mTORC2 pathway. *Exp Mol Med* 2018; **50**: e417 [PMID: 29303510 DOI: 10.1038/emm.2017.158]

19 **Liu Y**, Qi X, Zeng Z, Wang L, Wang J, Zhang T, Xu Q, Shen C, Zhou G, Yang S, Chen X, Lu F. CRISPR/Cas9-mediated p53 and Pten dual mutation accelerates hepatocarcinogenesis in adult hepatitis B virus transgenic mice. *Sci Rep* 2017; **7**: 2796 [PMID: 28584302 DOI: 10.1038/s41598-017-03070-8]

20 **Maehama T**, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 1998; **273**: 13375-13378 [PMID: 9593664 DOI: 10.1074/jbc.273.22.13375]

21 **Watanabe S**, Horie Y, Suzuki A. Hepatocyte-specific Pten-deficient mice as a novel model for nonalcoholic steatohepatitis and hepatocellular carcinoma. *Hepatol Res* 2005; **33**: 161-166 [PMID: 16214396 DOI: 10.1016/j.hepres.2005.09.026]

22 **Khalid A**, Hussain T, Manzoor S, Saalim M, Khaliq S. PTEN: A potential prognostic marker in virus-induced hepatocellular carcinoma. *Tumour Biol* 2017; **39**: 1010428317705754 [PMID: 28621226 DOI: 10.1177/1010428317705754]

23 **Hu TH**, Huang CC, Lin PR, Chang HW, Ger LP, Lin YW, Changchien CS, Lee CM, Tai MH. Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. *Cancer* 2003; **97**: 1929-1940 [PMID: 12673720 DOI: 10.1002/cncr.11266]

24 **Wu K**, Ding J, Chen C, Sun W, Ning BF, Wen W, Huang L, Han T, Yang W, Wang C, Li Z, Wu MC, Feng GS, Xie WF, Wang HY. Hepatic transforming growth factor beta gives rise to tumor-initiating cells and promotes liver cancer development. *Hepatology* 2012; **56**: 2255-2267 [PMID: 22898879 DOI: 10.1002/hep.26007]

25 **Wang B**, Li X, Hu W, Zhou Y, Din Y. Silencing of lncRNA SNHG20 delays the progression of nonalcoholic fatty liver disease to hepatocellular carcinoma via regulating liver Kupffer cells polarization. *IUBMB Life* 2019; **71**: 1952-1961 [PMID: 31408278 DOI: 10.1002/iub.2137]

26 **Stickel F**, Hellerbrand C. Non-alcoholic fatty liver disease as a risk factor for hepatocellular carcinoma: mechanisms and implications. *Gut* 2010; **59**: 1303-1307 [PMID: 20650925 DOI: 10.1136/gut.2009.199661]

27 **Saxena NK**, Sharma D, Ding X, Lin S, Marra F, Merlin D, Anania FA. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res* 2007; **67**: 2497-2507 [PMID: 17363567 DOI: 10.1158/0008-5472.CAN-06-3075]

28 **Dalamaga M**, Diakopoulos KN, Mantzoros CS. The role of adiponectin in cancer: a review of current evidence. *Endocr Rev* 2012; **33**: 547-594 [PMID: 22547160 DOI: 10.1210/er.2011-1015]

29 **Postic C**, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest* 2008; **118**: 829-838 [PMID: 18317565 DOI: 10.1172/JCI34275]

30 **Vinciguerra M**, Carrozzino F, Peyrou M, Carlone S, Montesano R, Benelli R, Foti M. Unsaturated fatty acids promote hepatoma proliferation and progression through downregulation of the tumor suppressor PTEN. *J Hepatol* 2009; **50**: 1132-1141 [PMID: 19398230 DOI: 10.1016/j.jhep.2009.01.027]

31 **Tian Y**, Wong VW, Wong GL, Yang W, Sun H, Shen J, Tong JH, Go MY, Cheung YS, Lai PB, Zhou M, Xu G, Huang TH, Yu J, To KF, Cheng AS, Chan HL. Histone Deacetylase HDAC8 Promotes Insulin Resistance and β-Catenin Activation in NAFLD-Associated Hepatocellular Carcinoma. *Cancer Res* 2015; **75**: 4803-4816 [PMID: 26383163 DOI: 10.1158/0008-5472.CAN-14-3786]