Hepatitis C Virus Infection Induced Liver Tumors in a Humanized Mouse Model

By Ningbin Wu

B.S. in Pharmaceutical Sciences, June 2010, Sun Yat-sen University, China

Thesis submitted to

The Faculty of
Columbian College of Arts and Sciences
The George Washington University
in partial fulfillment of the requirement
of Master of Science

August 31, 2012

Thesis directed by

Ajit Kumar,
Professor of Biochemistry and Molecular Biology

© Copyright 2012 by Ningbin Wu
All rights reserved

Acknowledgments

I would like to thank Dr. Ajit Kumar for his inspiration and kind guidance throughout my thesis research. Also I thank Dr. Steve Feinstone (Adjunct Professor of Biochemistry and Molecular Biology, GWU) and his lab members, Ms. Abeba Tesfaye and Mr. Qingwen Cui, for the mouse tumor tissue samples. I wish to express my gratitude to Dr. Krishna Banaudha and Mr. Wenjie Bao for their technical support.

Abstract of Thesis

Hepatitis C Virus Infection Induced Liver Tumors in a Humanized Mouse Model

Hepatitis C Virus is a major cause for liver fibrosis, cirrhosis and hepatocellular carcinoma. Around 300 million people worldwide are infected with HCV. A narrow host range of HCV infection has hampered the availability of small animal model, to study the development of novel therapy. The MUP-uPA mice are crossed with SICD/bg mice to obtain triply homozygous mice expressing urokinase plasminogen activator gene under the control of mouse urinary promoter. These immunodeficient mice survive on xenografted human hepatocytes as their hepatocyte cells dead early in the life. After engraftment of fresh or frozen human hepatocytes, these mice can be infected with most HCV genotypes and develop liver tumors within six weeks post infection. Earlier research has shown that PTEN tumor suppressor is down regulated in diverse types of tumors. Also, c-MYC protooncogene is commonly increased as a tumor marker in most tumors. We observed that PTEN is decreased and c-MYC is up regulated in humanized mouse model of HCV infection induced tumors. However, Caspase 3, an important effector in apoptosis, is hardly changed. We also detected the expression levels of miRNAs (that were reported as liver cancer markers in other studies) to further confirm the HCV infection induced tumors in MUP-uPA mice. The results show that miRNA-221, 21 and 141 are induced and miRNA-122, 181 23a and 26a are markedly reduced in HCV infected liver tumors.

Table of Contents

Acknowledgments ……iii

Abstract……iv

List of Figures……vi

List of Abbreviations……vii

Chapter 1: Introduction and hypothesis……1

Chapter 2: Materials and methods……5

Chapter 3: Results……8

Chapter 4: Discussion……15

Chapter 5: Conclusion……18

References……19

List of Figures

Figure 1……9

Figure 2……12

List of Abbreviation

HCV	Hepatitis C Virus
PTEN	Phosphatase and Tensin Homolog located on chromosome Ten
miRNA	MicroRNA
ncRNA	Non-coding RNA
PI3K	Phosphatidylinositol-3-kinase
HCC	Hepatocellular Carcinoma
RT-PCR	Reverse Transcription Polymerase Chain Reaction

Chapter 1: Introduction and Hypothesis

Introduction

HCV is a small, enveloped, single-stranded, positive-sense RNA virus of the family *Flavivridae*. The genome consists of a single open reading frame of 9.6kb long and the 5' UTR contains internal ribosome entry site (IRES) that initiate the translation of a ~3000 amino acids ployprotein. The viral and cellular proteases catalyze this large protein into 10 smaller functional proteins (Moradpour et al., 2007). Virus particles consist of core, envelope protein 1 and envelope protein 2. The other 7 proteins, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, are non-structural proteins that are required for complete virus life cycle. In most cases of HCV infection, it takes years to develop chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002) .

Lack of small animal models has impeded the progress of finding efficient therapies and developing of a preventive vaccine (Lauer and Walker, 2001). There are mainly three reported *in vivo* experimental models. Chimpanzees, which are the only immunocompetent animal model, can be used to test the efficacy of vaccine

candidates. Infection with a single strand of HCV made it possible to analyze the biological difference between diverse types of HCV (Bukh, 2004). However, it is preferable to develop a small animal model because of the limitations on working with the chimpanzee. Interestingly, tree shrew (*Tupaia belangeri*) is reported to be another natural host of HCV, and recent research suggests that tree shrew is a practical animal model to study HCV infection, which is a time-dependent chronic process (Amako et al., 2010; Xie et al., 1998; Xu et al., 2007). But lack of sufficient genetic information of tree shrew hampers its usage. To overcome these limitations, humanized mouse models are introduced. The first chimeric mice were reported by Mercer et al (Mercer et al., 2001). These mice are immunodeficient and carry a urokinase plasminogen activator transgene (uPA). SCID-beige mice are commonly used as xenograft recipients due to its lack of functional lymphoid and decreased natural killer cell. The Alb-uPA mice carry a tandom array of four murine urokinase plasminogen activator genes controlled by an albumin promoter. Plasminogen activators catalyze the reaction of plasminogen to plasmin, which functionally degrades fibrin clots (Sandgren et al., 1991), expression of uPA causes destruction of liver in early stage of life (Bissig et al., 2010; Mercer et al., 2001). Unfortunately, only mice dizygous for the uPA transgene are highly capable for engraftment with human liver. But the dilemma is that dizygous mice usually die soon after birth. In order to overcome these problems, Dr. Steve Feinstone put uPA genes under the control of the mouse major urinary protein (MUP) (Braun and Sandgren, 2000) promoter which is not expressed until after 3 weeks of life. This allows the mice to grow relatively robustly in the early stage of life. Dr. Feinstone's lab crossed the MUP-uPA

mice with SICD/bg mice until they had mice that were triply homozygous (for uPA). These mice have a high infant survival rate and are healthy enough to be engrafted with human hepatocytes. The engrafted mice can be infected with HCV to study the effects infected human hepatocyte xenografts on tumor development.

Inactivation of tumor suppressor genes has been suggested to play a critical role in genesis and progression of tumor. Tumor suppressor genes protect cells from forming tumors through repression of genes that are essential for the cell cycle progression, initiation of apoptosis, repair of DNA or inhibition of metastasis (Sherr, 2004). PTEN (phosphatase and tensin homolog located on chromosome 10) is one of the most frequently affected tumor suppressor genes in different kinds of tumor (Carracedo et al., 2011). PTEN functions as a negative regulator of phosphoinositide 3,4,5-triphosphate (PIP3), which is an activator in PI3K/AKT pathway. This pathway is considered as one target of cancer therapy (Wong et al., 2010). However, recent studies emphasize the importance of nuclear PTEN protein in its tumor suppressor function. Nuclear PTEN is involved in the regulation of genomic stability and cell cycle progression (Baker, 2007; Shen et al., 2007; Trotman et al., 2007). In addition, our study of *in vitro* human hepatocyte cell culture system shows that HCV replication requires down regulation of nuclear PTEN (Wenjie Bao's results, not published).

MicroRNAs are small (about 22 nucleotides) noncoding RNAs that can target mRNAs and reduce the translation products of mRNAs. Aberrant expression of microRNAs has been reported in variety of cancers. MicroRNAs referred to as "oncomirs" are differentially expressed in cancer, and are able to affect cell

transformation and carcinogenesis by functioning as oncogenes or tumor suppressors. MiRNA profiles have been studied as potential biomarker for different types of tumors (Tricoli and Jacobson, 2007). A number of investigators have shown that miRNAs activities are involved in liver tumorigenesis (Banaudha et al., 2011; Ji et al., 2009; Pineau et al., 2010; Wang et al., 2012). Therefore, we wished to establish if altered expression of miRNAs can serve as reliable markers HCV-infection induced liver cancer in MUP-uPA mouse model.

Hypothesis

Down regulation of PTEN is essential for HCV-infection induced liver tumor in human hepatocyte engrafted MUP-uPA mice. This HCV-infection induced humanized mouse can serve as a reliable model for validation of altered expression of miRNAs in liver tumorigenesis.

Chaper 2: Materials and Methods

HCV-induced Humanized Mouse Liver Tumor Samples: samples were obtained from Dr. Steve Feinstone's lab. They developed their colony of the MUP-uPA/SCID/bg mice and then began a program to engraft these mice between four and 12 mouths of age with fresh and frozen human hepatocytes. Immuno-staining of mouse liver tissue for human albumin (brown/black staining) was applied to confirm successful engrafment. These mice revealed large areas of mouse liver replaced by human hepatocytes, which mean they are potential host for HCV. Infection of the human hepatocyte engrafted mice with different HCV genotypes was initiated and mouse tumors were detected within six weeks of HCV infection. 7 non-infection samples and 8 infection samples (infected with HCV 1a strain) were applied in our experiment.

Protein Extraction, Subcellular Protein Fractionation and Western Blot: Tissue samples were quickly cut into small pieces after being removed from -80^{0}C and then rinsed in RIPA buffer supplemented with 1 X protease inhibitor cocktail (Roche) for total protein extraction. Homogenize liver pieces using Homogenization

Protocol for Liver Tissue (Next Advance). Briefly, tightly screw the centrifuge tubes closed and place them into the Bullet Blender®. Set controls for SPEED 8 and TIME 3 minutes. Visually inspect samples to confirm samples are totally homogenized. For nuclear and cytoplasmic extraction, NE-PER nuclear and cytoplasmic extraction reagents (Thermo Pierce) was utilized. 200 µL of CER I (Cytoplasmic Extraction Reagent I) was combined with the homogenization protocol. 11 µL of CER II (Cytoplasmic Extraction Reagent II) was added to precipitate nuclear part. Then 100 µL of NER (Nuclear Extraction Reagent) was used to extract nuclear fraction. The protein samples were stored in -80^{0}C until use. Before processing for immunoblotting, protein concentration was determined by BCA Protein Assay Kit (Thermo Pierce). Equal amounts of protein were loaded on 10% precast Mini-PROTEIN gel (BIO-RAD). The gel was transferred to nitrocellulose membrane. Membrane was blocked with 5% non-fat dry milk for 1 hour prior to incubation with primary antibodies (1 hour at room temperature or overnight at -4^{0}C). The list of primary antibodies and secondary antibodies is as follows: primary antibodies against PTEN (ab#79156, 1:1000, Abcom), GAPDH (ab8245, 1:2000, Abcom), Lamin-B1 (ab#16048, 1:1000, Abcom), p-AKT (#4060, 1:500, Cell Signaling), pan-AKT (#2920, 1:1000, Cell Signaling), DLC-1 (ab109991, 1:1000, Abcom), Cyclin D1 (ab6152, 1:500, Abcom), Caspase 3 (ab90437, 1:500, Abcom) and HRP conjugated anti-rabbit, anti-mouse, anti-goat antibodies (1:20000, Abcam). SuperSignal West Dura Chemiluminescent Substrate was used and the signal was visualized under BIO-RAD ChemiDoc™ XRS+ System. The images were analyzed using Image Lab™ Software developed by BIO-RAD.

RNA Extraction and Quantization: TRIzol® Reagent (Ambion) was applied to total RNA isolation according to the optimized protocol. Briefly, tissue samples were quickly cut into small pieces after removed from -80^{0}C and rinsed in 1 mL of TRIzol® Reagent. Homogenize liver pieces using Homogenization Protocol for Liver Tissue (Next Advance). Total RNA was separated by 0.2 mL of chloroform and precipitated overnight by 0.5 mL of isopropanol. Total RNA fraction was washed by 75% ethanol. RNA concentration was measured using the NanoDrop 1000 Spectrophotometer. RNA quality was checked by the integrity of 18S and 28S.

Real time Quantitative PCR of small RNA: 1ug of total RNA was added to QuantiMir RT Kit Small RNA Quantification System (System Biosciences) following the manufacture's protocol. The product of reverse transcription was then diluted (40 fold). Real time Quantitative PCR was performed using iTaq SYBR Green Supermix with Rox (Bio-Rad) on ABI 7300 Real Time PCR system. Q-RT-PRC forward primers of hsa-miR-141, hsa-miR-23a, hsa-miR-122, hsa-miR-181, hsa-miR-21, hsa-miR-221, hsa-miR-26a and hsa-miR-16 are 5'-TAACA CTGTC TGGTA AAGAT GG-3', 5'-ATCAC ATTGC CAGGG ATTTC C-3', 5'-TGGAG TGTGA CAATG GTGTT TG-3', 5'-AACAT TCAAC GCTGT CGGTG AGT-3', 5'-TAGCT TATCA GACTG ATGTT GA-3', 5'-AGCTA CATTG TCTGC TGGGT TTC-3', 5'-TTCAA GTAAT CCAGG ATAGG CT-3' and 5'-TAGCA GCACG TAAAT ATTGG CG-3'

Statistical analysis: All Quantitative Real time PCR and densitometry data and was analyzed using Microsoft Excel 2010.

Chaper 3: Results

PTEN is down regulated in HCV-infection induced liver tumors.

PTEN expression is commonly deregulated in human cancer (Carracedo et al., 2011). Nuclear localization of PTEN is critical for this tumor suppressor (Baker, 2007). From our previous experiment of *in vitro* model of HCV-infected human primary hepatocytes, PTEN was significantly down regulated in the nuclear and hardly changed in the cytoplasmic. So it is reasonable to detect PTEN expression level in HCV-infected MUP-uPA mouse tumor. As expected, we observed a marked decline of PTEN in the nuclear in infected mouse tissue compared to uninfected group (Fig 1A). Most phenotypes of PTEN down regulation can be attributed to the negative regulator of PI3 signaling cascade. PTEN can dephosphorylate PI3P, which a second messenger in PI3K/AKT pathway. In most cases, the activation of the PI3K/AKT pathway is used as a functional validation of PTEN depletion (Blanco-Aparicio et al., 2007; Salmena et al., 2008). We detected increased expression level of phosphorylation of nuclear AKT (Fig 1A). These results are consistent with our previous observation in HCV-infected primary human hepatocytes. Moreover, we

also noted decreased cytoplasmic PTEN and increased cytoplasmic p-AKT (Fig 1B), which was different from the results of *in vitro* system.

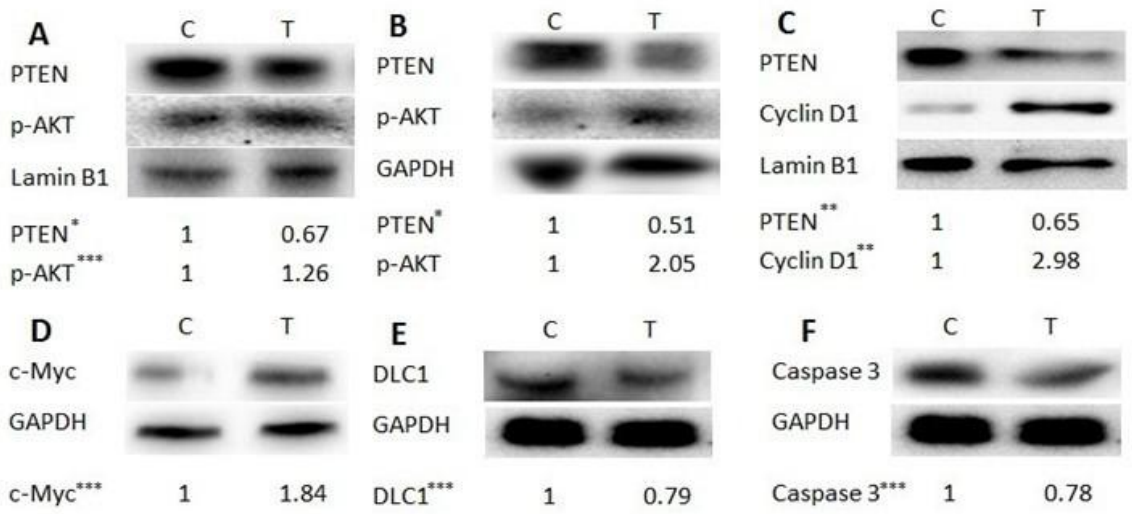

Figure 1: *Western Blot analysis in control liver tissues and HCV-infected tumor*. C, T respectively represent two uninfected and four HCV-infected livers. Uninfected tissues were harvested from mice engrafted but not inoculated with HCV. HCV-infected liver were engrafted and inoculated with HCV 1a stain. (*)$p<0.003$, (**)$p<0.01$, (***)$p<0.04$.

Decline of nuclear PTEN is involved in Cyclin D1 checkpoint.

Nuclear PTEN has been suggested as an important factor in cell cycle arrest. Normal levels of nuclear PTEN suppress functional Cyclin D1 levels, which are involved in G1 cell cycle arrest (Weng et al., 2001). Cyclin D1 is assembled with

cyclin-dependent kinases (CDKs); in this case they are CDK4 and CDK6. These complexes trigger progression into the S phase by phosphorylation and release the retinoblastoma (Rb) protein (Radu et al., 2003). In addition, CDK-independent activities of Cyclin D1 are also suggested to contribute to tumorigenesis (Ewen and Lamb, 2004). Our results showed increasing nuclear Cyclin D1 levels in HCV-infected liver tumors (Fig 1C). The normal function of PTEN is to stabilize cell cycle checkpoint (Chang et al., 2008; Wang and Jiang, 2008). It suggests that depletion of nuclear PTEN regulates the depression of Cyclin D1 mediated cell cycle checkpoint and increased cell proliferation.

C-Myc proto-oncogene expression is up regulated in HCV-infected liver.

c-Myc is a strong proto-oncogene and it is often found to be up regulated in many types of cancers (Teufel et al., 2007). The helix-loop-helix leucine zipper of c-Myc is essential as a transcription factor and its expression is necessary for cell growth and proliferation (Soucek et al., 2008). Artificially increasing c-Myc expression levels leads to tumor formation (Zender et al., 2008). We therefore reasoned that increased c-Myc level can be a marker to confirm our HCV-infection induced liver tumor mouse model. We noted increased amount of c-Myc in HCV-infected liver tumor (Fig 1D).

DLC1 is inhibited in HCV-infected tumors.

DLC1 is frequently deleted in HCC and has been reported that DLC1 is targeted by miR-141 in HCV-infected primary human hepatocytes (Banaudha et al., 2011). We also observed that DLC1 protein is inhibited in HCV-infection induced liver tumors (Fig 1E). Overall, expression of the tumorigenic markers attests to a novel *in*

vivo model of HCV-infection induced liver tumor in humanized mice; tumor progression depends upon suppression of apoptosis (Fig 1F).

MicroRNAs markers are analyzed in HCV-infected humanized mice.

miRNRAs are small non-coding RNAs that are highly conserved, tissue specific gene regulatory molecules. MicroRNAs have been suggested as biomarkers for diverse human tumors (reviewed in (Croce, 2009; Krutovskikh and Herceg, 2010; Lu et al., 2008)). We observed altered expression of microRNAs, some of which have been reported in the literature as markers for liver tumor. However, the reported studies have not clearly defined miRNA markers of HCV-infection associated liver tumors.

We reasoned that MUP-uPA mice could be a useful model to validate miRNAs as specific markers of HCV-infection induced liver tumor.

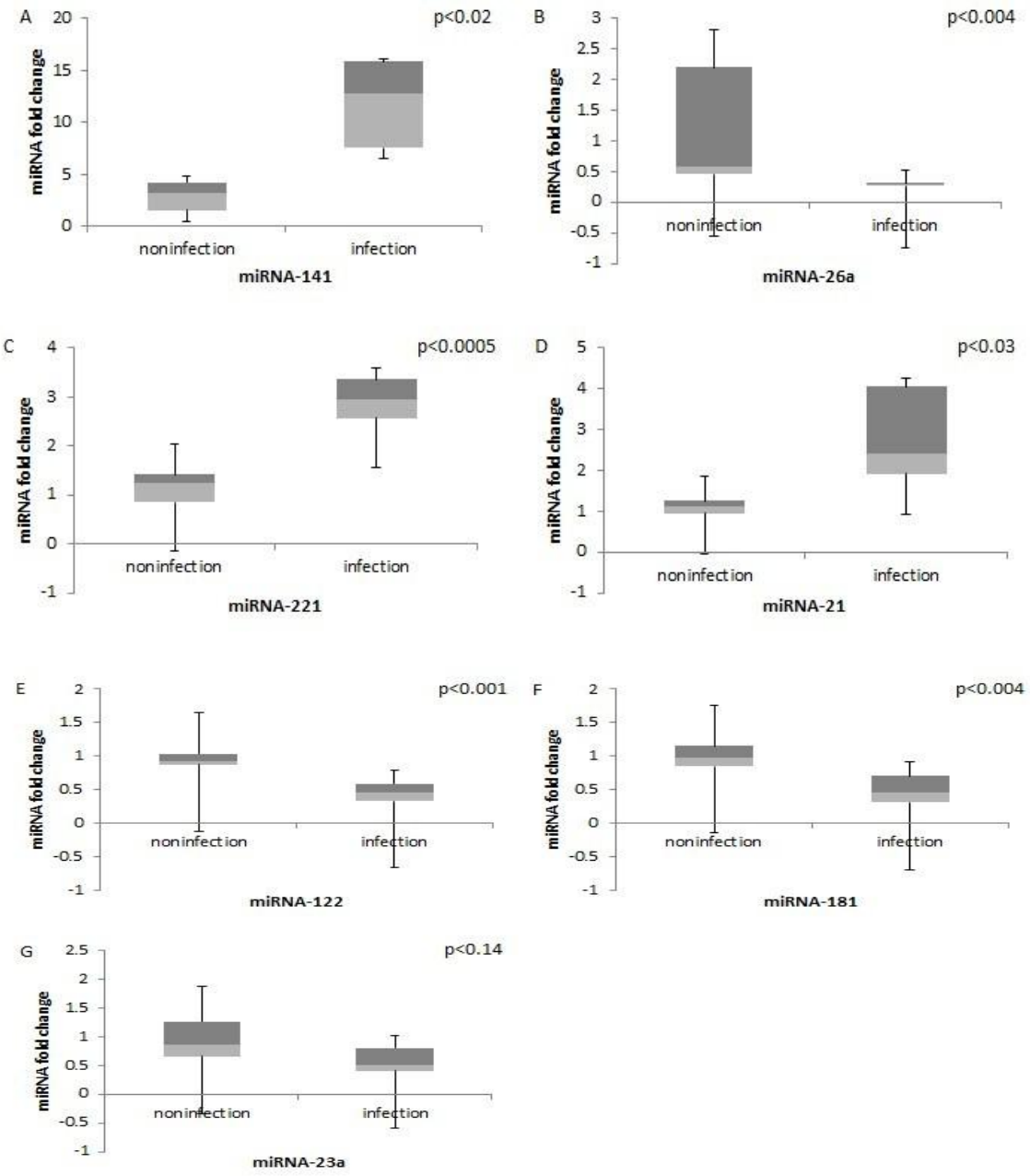

Figure 2: *QRT-PCR analysis of microRNA from control liver tissues and HCV-infected tumor*. A. the expression of miRNA-141 was determined by real-time PCR in both non-infected liver and HCV-induced liver tumor (*seven* uninfected and *eight* HCV infected). The y-axis refers to the relative miRNA expression fold (each non-infection or infection case versus the average of 7 non-infection cases). MiRNA

abundance was normalized to human U6 snRNA. P value was calculated by two-tailed t test. B, C, D, E, F and G show the expression level of miRNA-26a, miRNA-211, miRNA-21, miRNA-122, miRNA-181 and miRNA-23a, respectively.

MiR-141 targets DLC1 tumor suppressor in HCV-infected human hepatocytes (Banaudha et al., 2011). Our results showed nearly three folds induction of miR-141 in HCV infection induced liver tumors (Fig 2A). The results are consistent with reduced level of DLC1 protein in tumor tissue (Fig 1E). Reduced expression of miR-26a has been reported in hepatocellular carcinoma (HCC) cells; miR-26a targets Cyclin D2 and Cyclin E2 both involved in cell-cycle regulation (Kota et al., 2009). We found that miR-26a is down regulated in HCV-infected humanized mouse liver (Fig 2B). Overexpression of miR-21 or miR-221 has been reported to target cell cycle inhitors, which plays an important role in cancer (Medina et al., 2010; Pineau et al., 2010). MiR-21 expression was up regulated (2-4 folds) in HCV-infected mouse model (Fig 2D). The expression of miR-221 was also increased ~3 times (Fig 2C) in the HCV-infection induced liver tumors. MiR-122 is known to be abundantly expressed in the human liver. Recent studies have suggested that replication of HCV subgenomic RNA replication is dependent on miR-122 levels (Jopling et al., 2005). The expression level of miR-122 liver tumors is controversial. In our HCV-infected liver tumor system, we noted a down regulation of miR-122 expression (Fig 2E). Moreover we observed that the altered expression of miR-181 and miR-23a were less specific markers of liver tumors. MiR181, which was up-regulated in some types

of HCCs (Ji et al., 2009), was decreased in our HCV-infection induced liver tumor (Fig 2F). The expression of miR-23a was positively regulated to decrease glucose production through targeting PGC-1 alpha and G6PC(Wang et al., 2012) . We noted a decline of miR-23a in HCV-infected humanized mouse liver. Thus, our HCV-infection induced liver tumor model clearly defines microRNAs that are markers of HCV-induced liver tumor as compared to liver tumors induced by causes unrelated to virus infection.

Chapter 4: Discussion

In this study, we demonstrated that both nuclear and cytoplasmic PTEN were down regulated in HCV-infection induced liver tumor. Our previous results showed that inhibition of nuclear PTEN is directed by a virus-derived small RNA (vmr-11) in human primary hepatocytes (Wenjie Bao's result, not published). Decreasing the expression level of nuclear PTEN is related to impaired DNA double-strand breaks (DSBs) and genomic instability. However, we were unable to detect a specific vmr-11 signal in HCV-infection induced liver tumor (data not shown), suggestion that virus-derived mircoRNA (vmiR11) inhibition of nuclear PTEN expression may be an early event in HCV-infection associated hepatocarcinogenesis. We observed that the suppression of PTEN in the nuclear not only effect the PI3K signaling pathway but also Cyclin D1 pathway. Radu A. (Radu et al., 2003) introduced an oncogenesis model in which PTEN decreases nuclear cyclin D1 level through AKT/PKB pathway. Therefore, nuclear PTEN presents a cell-cycle-arrest dependent tumor suppress function in HCV-induced tumorigenesis. On the other hand, decline of cytoplasmic PTEN level is inconsist with the previous observation. Cytoplasmic PTEN level was

hardly changed in HCV-infected primary human hepatocytes, which is in early stage of HCV infection. The difference between the two research systems indicates that different stages of tumorigenesis require altered PTEN level. Overall, the results suggest an important role of PTEN in HCV-infected hepatocarcinogenesis.

Efficient HCV replication is correlated with miR-141 mediated reduction of DLC1 protein in virus-infected cell (Banaudha et al., 2011). Although, the regulation mechanism of DLC1 is not clear, we observed that miR-141 or DLC1 is involved in HCV-infected humanized mouse liver. However, DLC1 is not specific regulator for HCV-induced tumorigenesis; it is also found as tumor suppressor in human hepatocellular carcinoma cells or other types of epithelial tumors (Liao and Lo, 2008; Zhou et al., 2004).

C-Myc may be essential for keeping cell under the appropriate tumorigenesis environment (Soucek et al., 2008). As a common tumor marker, it also up regulated in HCV-infected liver tumor, although c-Myc has no clear regulating pathway. Nevertheless, c-Myc offers both an available marker of liver tumor and therapy target.

MiRNAs mainly bind to the 3' untranslated region of target mRNAs. The binding can either inhibit translation or degrade mRNA. Some oncogenic miRNAs have been reported such as miR-21 that overexpressed in most cancers (reviewed by (Krutovskikh and Herceg, 2010)). According to literature report, we selected a number of liver-cancer-related miRNAs to test whether our HCV-infected mouse can serve as a capable model to validate tumor biomarkers. Although expression levels of some miRNAs do not consist with others' reports, phenotypes of most miRNAs

are same to previous research. It is understandable because miRNA profiling changes in most human cancers (Calin and Croce, 2006).

Chapter 5: Conclusion

We present evidence that down regulation of PTEN is essential for HCV-infection induced liver tumor in human hepatocyte engrafted MUP-uPA mice. Declined DLC1 and increased c-Myc are found as tumor markers in HCV-infection induced liver tumor. We also confirm that this HCV-infection induced humanized mouse can serve as a reliable model for validation of altered expression of miRNAs in liver tumorigenesis; miRNA-221, 21 and 141 are induced and miRNA-122, 181 23a and 26a are markedly reduced in HCV infected liver tumors.

Chapter 6: Reference:

Amako, Y., Tsukiyama-Kohara, K., Katsume, A., Hirata, Y., Sekiguchi, S., Tobita, Y., Hayashi, Y., Hishima, T., Funata, N., Yonekawa, H.*, et al.* (2010). Pathogenesis of hepatitis C virus infection in Tupaia belangeri. Journal of virology *84*, 303-311.

Baker, S.J. (2007). PTEN enters the nuclear age. Cell *128*, 25-28.

Banaudha, K., Kaliszewski, M., Korolnek, T., Florea, L., Yeung, M.L., Jeang, K.T., and Kumar, A. (2011). MicroRNA silencing of tumor suppressor DLC-1 promotes efficient hepatitis C virus replication in primary human hepatocytes. Hepatology *53*, 53-61.

Bissig, K.D., Wieland, S.F., Tran, P., Isogawa, M., Le, T.T., Chisari, F.V., and Verma, I.M. (2010). Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. The Journal of clinical investigation *120*, 924-930.

Blanco-Aparicio, C., Renner, O., Leal, J.F., and Carnero, A. (2007). PTEN, more than the AKT pathway. Carcinogenesis *28*, 1379-1386.

Braun, K.M., and Sandgren, E.P. (2000). Cellular origin of regenerating parenchyma in a mouse model of severe hepatic injury. The American journal of pathology *157*, 561-569.

Bukh, J. (2004). A critical role for the chimpanzee model in the study of hepatitis C. Hepatology *39*, 1469-1475.

Calin, G.A., and Croce, C.M. (2006). MicroRNA-cancer connection: the beginning of a new tale. Cancer research *66*, 7390-7394.

Carracedo, A., Alimonti, A., and Pandolfi, P.P. (2011). PTEN level in tumor suppression: how much is too little? Cancer research *71*, 629-633.

Chang, C.J., Mulholland, D.J., Valamehr, B., Mosessian, S., Sellers, W.R., and Wu, H. (2008). PTEN nuclear localization is regulated by oxidative stress and mediates p53-dependent tumor suppression. Molecular and cellular biology *28*, 3281-3289.

Croce, C.M. (2009). Causes and consequences of microRNA dysregulation in cancer. Nature reviews Genetics *10*, 704-714.

Ewen, M.E., and Lamb, J. (2004). The activities of cyclin D1 that drive tumorigenesis. Trends in molecular medicine *10*, 158-162.

Hoofnagle, J.H. (2002). Course and outcome of hepatitis C. Hepatology *36*, S21-29.
Ji, J., Yamashita, T., Budhu, A., Forgues, M., Jia, H.L., Li, C., Deng, C., Wauthier, E., Reid, L.M., Ye, Q.H., *et al.* (2009). Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology *50*, 472-480.
Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M., and Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science *309*, 1577-1581.
Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R., *et al.* (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell *137*, 1005-1017.
Krutovskikh, V.A., and Herceg, Z. (2010). Oncogenic microRNAs (OncomiRs) as a new class of cancer biomarkers. BioEssays : news and reviews in molecular, cellular and developmental biology *32*, 894-904.
Lauer, G.M., and Walker, B.D. (2001). Hepatitis C virus infection. The New England journal of medicine *345*, 41-52.
Liao, Y.C., and Lo, S.H. (2008). Deleted in liver cancer-1 (DLC-1): a tumor suppressor not just for liver. The international journal of biochemistry & cell biology *40*, 843-847.
Lu, M., Zhang, Q., Deng, M., Miao, J., Guo, Y., Gao, W., and Cui, Q. (2008). An analysis of human microRNA and disease associations. PloS one *3*, e3420.
Medina, P.P., Nolde, M., and Slack, F.J. (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature *467*, 86-90.
Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., *et al.* (2001). Hepatitis C virus replication in mice with chimeric human livers. Nature medicine *7*, 927-933.
Moradpour, D., Penin, F., and Rice, C.M. (2007). Replication of hepatitis C virus. Nature reviews Microbiology *5*, 453-463.
Pineau, P., Volinia, S., McJunkin, K., Marchio, A., Battiston, C., Terris, B., Mazzaferro, V., Lowe, S.W., Croce, C.M., and Dejean, A. (2010). miR-221 overexpression contributes to liver tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 264-269.
Radu, A., Neubauer, V., Akagi, T., Hanafusa, H., and Georgescu, M.M. (2003). PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. Molecular and cellular biology *23*, 6139-6149.
Salmena, L., Carracedo, A., and Pandolfi, P.P. (2008). Tenets of PTEN tumor suppression. Cell *133*, 403-414.
Sandgren, E.P., Palmiter, R.D., Heckel, J.L., Daugherty, C.C., Brinster, R.L., and Degen, J.L. (1991). Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. Cell *66*, 245-256.
Shen, W.H., Balajee, A.S., Wang, J., Wu, H., Eng, C., Pandolfi, P.P., and Yin, Y. (2007). Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell *128*, 157-170.
Sherr, C.J. (2004). Principles of tumor suppression. Cell *116*, 235-246.

Soucek, L., Whitfield, J., Martins, C.P., Finch, A.J., Murphy, D.J., Sodir, N.M., Karnezis, A.N., Swigart, L.B., Nasi, S., and Evan, G.I. (2008). Modelling Myc inhibition as a cancer therapy. Nature *455*, 679-683.

Teufel, A., Staib, F., Kanzler, S., Weinmann, A., Schulze-Bergkamen, H., and Galle, P.R. (2007). Genetics of hepatocellular carcinoma. World journal of gastroenterology : WJG *13*, 2271-2282.

Tricoli, J.V., and Jacobson, J.W. (2007). MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis. Cancer research *67*, 4553-4555.

Trotman, L.C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N.P., Carver, B.S., Cordon-Cardo, C., Erdjument-Bromage, H.*, et al.* (2007). Ubiquitination regulates PTEN nuclear import and tumor suppression. Cell *128*, 141-156.

Wang, B., Hsu, S.H., Frankel, W., Ghoshal, K., and Jacob, S.T. (2012). Stat3-mediated activation of microRNA-23a suppresses gluconeogenesis in hepatocellular carcinoma by down-regulating Glucose-6-phosphatase and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha. Hepatology *56*, 186-197.

Wang, X., and Jiang, X. (2008). Post-translational regulation of PTEN. Oncogene *27*, 5454-5463.

Weng, L.P., Brown, J.L., and Eng, C. (2001). PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Human molecular genetics *10*, 599-604.

Wong, K.K., Engelman, J.A., and Cantley, L.C. (2010). Targeting the PI3K signaling pathway in cancer. Current opinion in genetics & development *20*, 87-90.

Xie, Z.C., Riezu-Boj, J.I., Lasarte, J.J., Guillen, J., Su, J.H., Civeira, M.P., and Prieto, J. (1998). Transmission of hepatitis C virus infection to tree shrews. Virology *244*, 513-520.

Xu, X., Chen, H., Cao, X., and Ben, K. (2007). Efficient infection of tree shrew (Tupaia belangeri) with hepatitis C virus grown in cell culture or from patient plasma. The Journal of general virology *88*, 2504-2512.

Zender, L., Xue, W., Zuber, J., Semighini, C.P., Krasnitz, A., Ma, B., Zender, P., Kubicka, S., Luk, J.M., Schirmacher, P.*, et al.* (2008). An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. Cell *135*, 852-864.

Zhou, X., Thorgeirsson, S.S., and Popescu, N.C. (2004). Restoration of DLC-1 gene expression induces apoptosis and inhibits both cell growth and tumorigenicity in human hepatocellular carcinoma cells. Oncogene *23*, 1308-1313.