RESEARCH PAPER

Cancer Biology & Therapy 9:10, 803-808; May 15, 2010; © 2010 Landes Bioscience

# MicroRNA-602 regulating tumor suppressive gene RASSF1A is overexpressed in hepatitis B virus-infected liver and hepatocellular carcinoma

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**Key words:** hepatocellular carcinoma, microRNA-602, chronic HBV hepatitis, RASSF1A, P73

**Abbreviations:** HCC, hepatocellular carcinoma; CH, chronic hepatitis; CL, cirrhotic liver; RASSF1A, Ras-associated domain family member 1A; MiRNA, microRNA

**Aims:** It is important to understand the role of microRNA in the transformation from chronic HBV hepatitis to hepatocellular carcinoma in hepatocarcinogenesis. Relationship of microRNA-602 with chronic HBV hepatitis, liver cirrhosis and HCC was investigated in this article.

**Results:** (1) 14 MicroRNAs were aberrantly expressed in HCC and CL compared with NL. Among these, microRNA-602 expression in CH, LC, NT and HCC was 2.939, 3.234, 2.439 and 4.134 times of that in NL respectively, which was significantly different ($p < 0.01$ for all vs. NL); RASSF1A expression in LC and HCC was lower than that in NL, while P73 protein expression in CL was higher than that in NL and HCC. (2) MicroRNA-602 expression in HepG2 2.2.15 and HepG2-HBX was 2.643 and 3.48 times of that in HepG2 ($p < 0.05$ for both). (3) MicroRNA-602 inhibition in HepG2 cells was associated with RASSF1A mRNA and protein expression increased to 4.37, 3.01 times respectively of those not, with cell apoptosis increased and cell proliferation rate decreased significantly, changes were similar in HepG2-HBX cells.

**Methods:** (1) MicroRNA expression was investigated in normal (NL), chronic HBV hepatitis (CH), HBV-positive cirrhotic (CL), HBV-positive HCC and corresponding normal para-tumorous livers (NT) and hepatoma cells was evaluated with microRNA microarray and verified by real-time PCR, and microRNA-602 was selected for further study. Expression of miR602-target genes RASSF1A and P73 were detected with RT-PCR and western blot. (2) MicroRNA-602 expression in HepG2 and HepG2-HBX was inhibited by miR-602 inhibitor transfection; RASSF1A and P73 expression was detected and cell apoptosis and proliferation were detected.

**Conclusions:** MicroRNA-602 plays a procarcinogenic role in HBV-related hepatocarcinogenesis by inhibiting RASSF1A. MicroRNA-602 might be an early diagnostic marker for HBV-mediated HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Chronic HBV hepatitis and HBV-positive liver cirrhosis are the most important causes and risk factors of HCC in East Asia and Africa. Understanding the pathogenesis of HBV-associated HCC is important in finding diagnostic markers and developing effective means of prevention and treatment of this highly malignant form of cancer.[1] MicroRNAs (miRNAs), a non-coding RNA family have been found to play pivotal roles in many pathophysiological processes including HCC development.[2-10] The presence of a molecular prognostic microRNA signature in primary HCC clinical specimens has also been confirmed by several recent studies.[5,7,8,11-13] Finding characteristic microRNA expression during the transformation from chronic hepatitis to HCC is very important in understanding the process of hepatocarcinogenesis and in developing potential diagnostic markers and treatment for this malignancy. In this article, microRNA expression in chronic HBV hepatitis (CH), HBV-positive cirrhotic liver (CL) and HBV-positive HCC were investigated. It was found that microRNA-602 was overexpressed and expression of its target tumor suppressive gene RASSF1A was decreased in CH, CL and HCC compared with normal liver (NL). Inhibiting microRNA-602 expression in hepatoma cell lines increased target gene RASSF1A expression, promoted hepatoma cell apoptosis and inhibited cell proliferation. Our results suggest that microRNA-602 is involved at an early stage of HBV-mediated hepatocarcinogenesis and may be a potential early marker for that condition.

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Submitted: 12/23/09; Revised: 01/28/10; Accepted: 02/08/10
Previously published online: www.landesbioscience.com/journals/cbt/article/11440

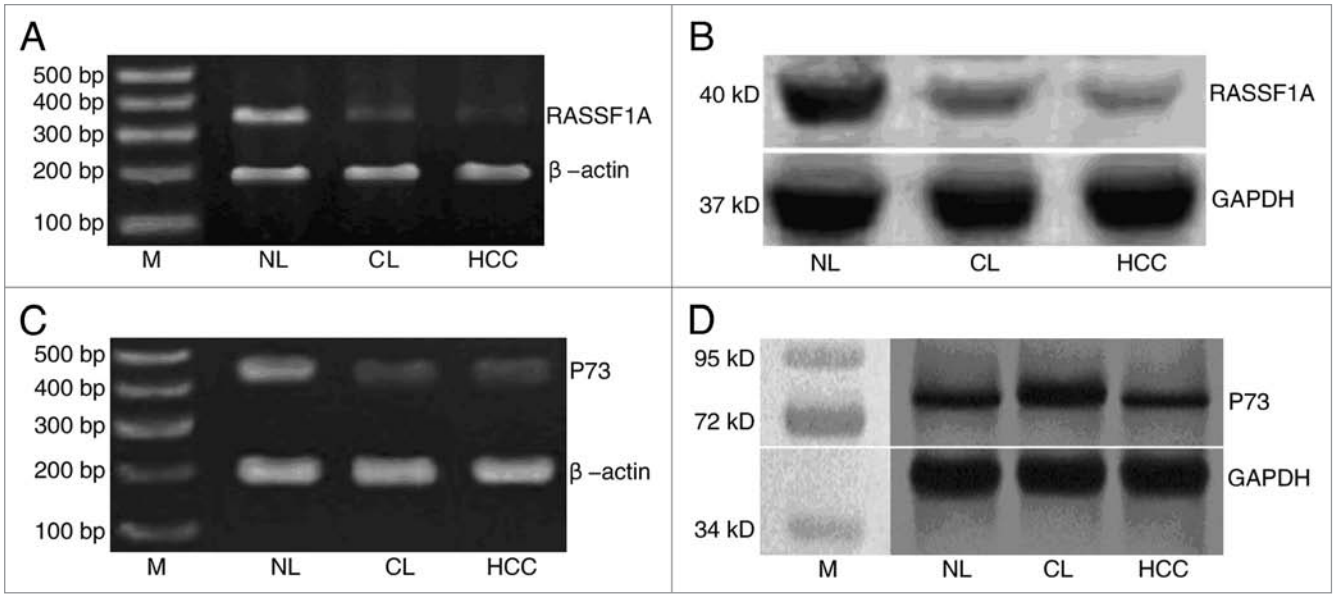


**Figure 1.** MicroRNA-602 target genes RASSF1A and P73 expression in different livers. mRNA and protein expression of microRNA-602 target genes RASSF1A and P73 in different livers assayed by RT-PCR and western blot. (A) mRNA levels of RASSF1A in NL, CL and HCC; (B) protein expression of RASSF1A in NL, CL and HCC; (C) mRNA levels of P73 in NL, CL and HCC; (D) Protein levels of P73 in NL, CL and HCC.

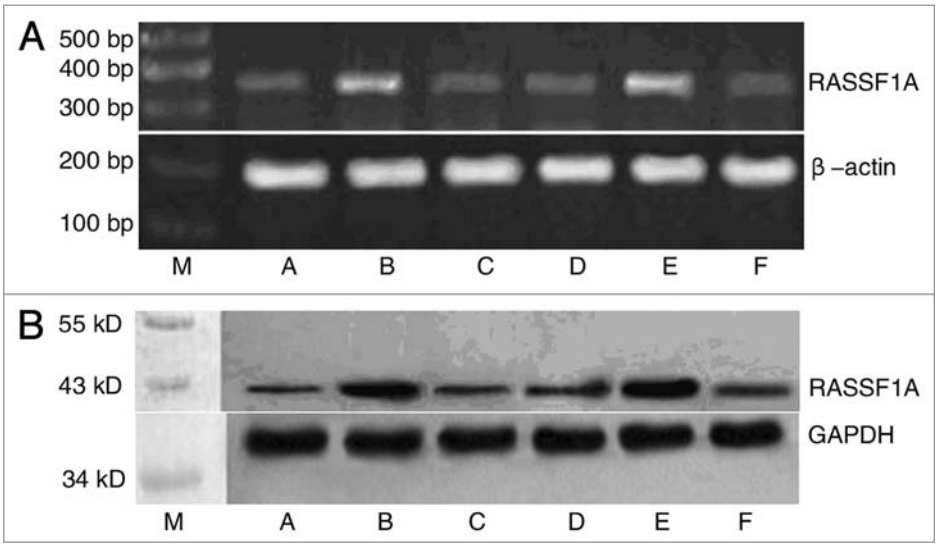


**Figure 2.** RASSF1A expression in hepatoma cells were regulated by microRNA-602. Change of RASSF1A mRNA and protein expression in HepG2 and HepG2-HBX before and after mir-602 inhibitor transfection were measured by RT-PCR and western blot, respectively. (A) RASSF1A mRNA after mir-602 inhibition. (B) RASSF1A protein after mir-602 inhibitor transfection. A: HepG2 control; B: mir-602 inhibitor transfection in HepG2; C: mock transfection in HepG2; D: HepG2-HBX control; E: mir-602 inhibitor transfection in HepG2-HBX; F: mock transfection in HepG2-HBX.

not group in hepatoma cells, which indicated that P73 was not regulated by microRNA-602 (data not shown).

**Cell apoptosis increased and cell proliferation decreased in HepG2 and HepG2-HBX after mir-602 inhibition.** In order to clarify whether mir-602 deregulation plays role in hepatocarcinogenesis, apoptosis and proliferation rates of HepG2 and HepG2-HBX cells were measured after mir-602 exposure. As determined by FACS, the apoptosis rate of HepG2 at 72 h after mir-602 inhibition was 28.35 ± 1.93%, which was significantly higher than apoptosis rate of HepG2 without mir-602 inhibition (5.35 ± 0.35%, $p < 0.01$). Apoptosis of HepG2-HBX after mir-602 inhibitor transfection was 22.57 ± 1.86 increased significantly compared to before, 4.42 ± 0.28%, $p < 0.05$. MTT assay indicated that at 48, 72 and 96 h after mir-602 exposure, the proliferation rate of HepG2 was inhibited to 48.83, 58.57 and 44.57% of control, respectively. The differences between the inhibition and control groups at 48, 72 and 96 h were significant, $p < 0.05$ for all points, while difference between mock and control group was not significant ($p > 0.05$). The changes in proliferation rate of HepG2-HBX

**Table 1.** Aberrant microRNA expression in NL, CH, CL, NT and HCC tissues tested with microarray

| MicroRNA | | Microarray | | | |
|---|---|---|---|---|---|
| | | CH/NL | CL/NL | NT/NL | HCC/NL |
| Overexpressed microRNAs | miR-602 | 2.939 | 3.234 | 2.439 | 4.134 |
| | miR-135a | 2.182 | 2.822 | 2.342 | 3.577 |
| | miR-210 | 4.331 | 5.159 | 2.194 | 4.274 |
| | miR-671-5p | 2.985 | 4.315 | 2.232 | 4.249 |
| | miR-30b* | 4.013 | 3.571 | 2.784 | 4.005 |
| | miR-222 | 2.669 | 4.073 | 2.188 | 3.472 |
| Under-expressed microRNAs | miR-143 | 0.189 | 0.273 | 0.401 | 0.173 |
| | miR-199a-5p | 0.332 | 0.404 | 0.439 | 0.195 |
| | miR-195 | 0.257 | 0.326 | 0.379 | 0.308 |
| | miR-27a | 0.271 | 0.456 | 0.401 | 0.318 |
| | miR-99a | 0.312 | 0.468 | 0.397 | 0.364 |
| | miR-519[e] | 0.348 | 0.377 | 0.434 | 0.374 |
| | miR-130a | 0.524 | 0.496 | 0.433 | 0.365 |
| | miR-597 | 0.395 | 0.483 | 0.491 | 0.424 |

MicroRNA expression in normal liver, chronic hepatitis, cirrhotic, HCC and corresponding nontumoral livers were evaluated with microRNA microarray. 14 microRNAs were aberrantly expressed in CH, CL, HCC and NT.

## Results



**MicroRNA-602 is overexpressed in chronic HBV hepatitis, cirrhotic, HCC and paratumoral tissue compared to normal liver.** MicroRNA expression profiles in NL, CH, CL, NT and HCC were detected by microRNA microarray. Fold-differences >2 between two groups were considered significant. As shown in **Table 1**, mean value of 6 microRNAs were higher in CH, CL, NT and HCC groups compared to NL, including microRNA-602, 135a, 210, 671-5p, 30b* and 222. Mean value of 8 microRNAs were lower in CH, CL, HCC, NT compared to NL, including microRNA-143, 199a-5p, 195, 27a, 99a, 519e, 130a and 597 (**Table 1**). Among these, microRNA-602 was selected for further study since it was predicted to target and inhibit two classical tumor suppressive genes RASSF1A and P73. The mean value of MicroRNA-602 expression in CH, CL, NT and HCC was 2.939, 3.234, 2.439 and 4.134 times of that in NL ($p < 0.01$ for all), while it was not different among CH, CL, NT and HCC groups. MicroRNA-602 expression was further evaluated by real-time PCR, which verified the expression difference of microRNA-602 in NL, CL and HCC (NL: LC: HCC 0.37 ± 0.14: 1.00 ± 0.36: 1.29 ± 0.27, $p < 0.01$ for all), while it was not statistically different between CH and HCC group.

**Expression of mir-602 target genes RASSF1A was lower in HBV-exposed liver compared to normal liver.** Tumor suppressive gene RASSF1A and P73 are considered to be mir-602 targets. Their aberrant expression and loss of tumor suppressive function have been shown to be involved in carcinogenesis including hepatocarcinogenesis.[30,41] RASSF1A and P73 mRNA and protein expression were detected in NL, LC and HCC by RT-PCR and western blot. As shown in **Figure 1A**, mean RASSF1A mRNA levels in NL, CL and HCC were 1.047 ± 0.100, 0.454 ± 0.083, 0.331 ± 0.069, respectively ($p < 0.01$ for CL or HCC compared to NL respectively). The mean RASSF1A protein levels in NL, CL, HCC were 0.878 ± 0.097, 0.395 ± 0.073, 0.229 ± 0.064 respectively ($p < 0.01$ for CL or HCC compared to NL respectively; **Fig. 1B**). The mean P73 mRNA levels in NL, CL, HCC were 1.232 ± 0.341, 0.267 ± 0.058, 0.311 ± 0.049 ($p < 0.01$ for CL or HCC compared to NL respectively; **Fig. 1C**). However, P73 protein expression in HCC was similar to NL, while significantly lower than in CL (NL: CL: HCC 0.063 ± 0.045: 0.098 ± 0.033: 0.045 ± 0.016, **Fig. 1D**). The difference in RASSF1A mRNA or protein levels between CL and HCC group were not significant, which was similar to the difference of P73 mRNA levels between CL and HCC group ($p > 0.05$, respectively). The expression of RASSF1A in these livers was consistent with changes in microRNA-602 levels since overexpressed microRNA-602 in cirrhotic and HCC livers inhibited RASSF1A. However, P73 protein levels in these tissues were not consistent with that of miRNA-602.

**MicroRNA-602 was overexpressed in HBV-related hepatoma cell.** To determine if microRNA-602 expression was different in hepatoma cell lines with different forms of HBV expression, microRNA-602 expression in HBV-negative HepG2, HBV-secreting HepG2 2.2.15 and HBX-transfected HepG2-HBX cells was also evaluated with real-time PCR. As the results showed, microRNA-602 expression was significantly higher in HepG2 2.2.15 and HepG2-HBX than in HepG2 with ratio of 2.67: 3.48: 1.00 respectively ($p < 0.01$ for HepG2 2.2.15 and HepG2-HBX compared to HepG2 respectively), which indicated that microRNA-602 expression levels may be associated with the presence of HBV. The mRNA and protein expression of RASSF1A in HepG2, HepG2-HBX and HepG2 2.2.15 was 0.121 ± 0.014, 0.104 ± 0.023, 0.097 ± 0.015 and 0.106 ± 0.033, 0.078 ± 0.019, 0.085 ± 0.017 respectively ($p < 0.05$ for HepG2-HBX vs. HepG2 or HepG2 2.2.15 vs. HepG2).

**Inhibition of mir-602 expression in hepatoma cells increases its target gene RASSF1A but not P73 expression.** To verify that mir-602 has a carcinogenic role in HCC development through RASSF1A, mir-602 expression was exposed to mir-602 inhibitor in HepG2 and HepG2-HBX cells and RASSF1A expression was measured. As the results showed, RASSF1A mRNA expression in HepG2 was much higher with mir-602 inhibitor exposure than without (**Fig. 2A**). RASSF1A level in HepG2 with exposure was 0.529 ± 0.032, and was 0.121 ± 0.014 in HepG2 without. Similarly, RASSF1A protein levels increased after microRNA-602 inhibition in HepG2, RASSF1A levels were 0.289 ± 0.054 in exposure group, and 0.106 ± 0.033 in the group without exposure ($p < 0.05$, for exposure group vs. without exposure group, **Fig. 2B**). Changes of RASSF1A HepG2-HBX and HepG2 2.2.15 after mir-602 inhibition were similar to those in HepG2. However, mRNA or protein expression of P73, another predicted target gene of microRNA-602, was not different among microRNA-602 inhibitor transfection group and

after mir-602 inhibition was similar to that of HepG2 (data not shown).

## Discussion

MicroRNAs negatively regulate its target gene expression either by translational inhibition or by mRNA degradation.[14-17] Aberrantly expressed microRNAs have been proven to modulate key cellular processes involved in carcinogenesis,[2-4,18] and to be related with metastasis, survival, differentiation, recurrence or chemotherapy response in malignancy.[5-8,10-13,19-21] Interestingly, altered expression of some miRNAs has been shown to be associated with distinctive risk factors such as hepatitis B viral hepatitis and alcohol use.[11-13,20] Studies have indicated that microRNA profile can be used for defining clinical phenotypes, as well as for molecular diagnostic markers for cancers including HCC.[4,7,11,13,18,22] However, the relationship between microRNA deregulation and the sequential hepatocarcinogenic transformation from chronic HBV hepatitis, liver cirrhosis to HCC is not yet clear. Among the aberrant expressed microNRAs in HCC and HBV-infected livers, the overexpressed microRNA-602 was selected for further study for it may target and inhibit two classical tumor suppressive genes (TSG) RASSF1A and P73.[23] The current results that mir-602 expression was significantly higher in chronic HBV hepatitis liver, CL and HBV-positive HCC than in normal liver, with lower expression of its target genes RASSF1A suggests that mir-602 is involved in HBV-positive hepatocarcinogenesis; Mir-602 may be involved at early stage of hepatocarcinogenesis since mir-602 is overexpressed through the hepatocarcinogenesis from chronic HBV infection, liver cirrhosis to HCC. The procarcinogenic role of mir-602 in HCC was also verified by the cell lines results in this article.

Although cell or animal models mimicking natural HBV infection are lacking, different expression of mir-602 in HepG2, HepG2-HBX and HepG2 2.2.15 in this article suggests that microRNA-602 expression is regulated by chronic HBV infection. However, the way in which HBV regulates mir-602 expression is not clear. DNA methylation, HBV chromosome integration and metabolism in addition to genome copy change have been suggested to regulate microRNA expressions.[24-29] Since microRNA-602 was found to be overexpressed in HBV-positive livers, DNA methylation which suppresses microRNA expression is not a likely means of HBV regulation of microRNA-602 expression. Specific unstable areas of the genome (fragile sites) in HCC undergoing either deletion or increased gene dosage have been shown to be related to HBV integration and HCC prognosis.[30-34] MicroRNAs are often located at fragile genomic sites in cancers and exhibit high frequency genomic alteration in cancers.[24,28] This indicates that HBV insertion at fragile sites may result in microRNA aberrant expression. The fact that microRNA-602 is located in chromosome 9q34.3, a common HBV integration site or cancer related gene region (CAGR)[32-34] suggests that HBV insertion at this site may upregulate mir-602 expression, although further investigation is needed to prove this hypothesis. HBX is regarded as one major molecules involved in HBV-related hepatocarcinogenesis. However, there has been debate about HBX expression in HepG2 2.2.15 cells. In this regard, HepG2-HBX stably transfected with HBX were further evaluated for effects of HBX on microRNA-602 expression in the current work. Differences in microRNA-602 levels in these cells suggest mir-602 overexpression is related with HBX transfection.

Bioinformatics analysis suggests that mir-602 targets and inhibits two tumor suppressive genes: Ras-associated domain family member 1A (RASSF1A) and P73. Our results confirmed that RASSF1A is target gene of microRNA-602. RASSF1A affects several cellular functions such as cell migration, proliferation and apoptosis, induces cell cycle arrest and senescence, promotes microstability and senescence in human cancer cells including HCC.[30,31,35,36] RASSF1A loss is an early event in HCC pathogenesis and can be found in premalignant liver tissues and blood 1–9 y before clinical HCC diagnosis.[37,38] The current results found early RASSF1A gene loss in hepatocarcinogenesis is consistent with the early aberrant microRNA-602 expression, which also confirms the important regulatory role of microRNA-602 in early stages of HBV-positive HCC development. This result also suggests that microRNA can target and inhibit RASSF1A transcription and/or translation, which coordinates with promoter hypermethylation[38,39] to inhibit tumor suppressive activity of RASSF1A and promotes hepatocarcinogenesis at a very early stage. Since mir-602 inhibition increased RASS1A mRNA and protein levels by 4.37- and 4.35-fold respectively, microRNA targeting may play more important role in RASSF1A inhibition during HBV-mediated hepatocarcinogenesis. P73 is a P53 superfamily member and shares substantial homology with p53, showing many of the p53-like properties.[40] Not similar to RASSF1A, P73 protein levels in HCC was not different from that in NL, while much lower than in CL, which does not support P73 is a target gene of mir-602.

In conclusion, the results in this article suggest that microRNA-602 plays an important regulatory role in HBV-mediated hepatocarcinogenesis by inhibiting the tumor suppressive function of RASSF1A, from very early stage of chronic HBV hepatitis and HBV-positive cirrhosis through HCC. MicroRNA-602 may be a potential diagnostic marker for HCC at very early premalignant stages of chronic HBV hepatitis and cirrhosis, and may serve as a therapeutic and preventive target for HBV-positive HCC.

## Material and Methods

Samples of 15 chronic HBV hepatitis livers and 6 HBV-positive cirrhotic livers were collected by fine-needle biopsy. Liver samples of eight normal individuals (three patients with gallbladder stones, two with liver trauma and two with idiopathic thrombocytopenic purura) were obtained by biopsy during surgery. 21 HBV-positive HCC and corresponding non-malignant paratumorous specimens were collected by radical hepatectomy. All tissues were obtained with informed consent from the patients and verified by biochemistry and pathological examination. The study was approved by the Institutional Review Board. HepG2-HBX cells stably expressing HBX, HBV-negative HepG2 and

HBV-secreting HepG2 2.2.15 were cultured in RMPI 1640 with 10% fetal bovine serum.

**MicroRNA arrays.** MicroRNA microarray were performed on five normal livers, five chronic hepatitis, six HBV-positive cirrhotic livers, eight HBV-positive HCC and corresponding nontumorous tissues. Briefly, 100 ng RNA of each specimen was harvested using TRIzol (Invitrogen) and RNeasy mini kit (QIAGEN) according to manufacturer's instructions. After RNA measurement on a Nanodrop instrument, samples were labeled using a miRCURY™ Hy3™/Hy5™ Power labeling kit, and hybridized on a miRCURY™ LNA Array (v.11.0). The samples were hybridized on a hybridization station. Scanning was performed with the Axon GenePix 4000B microarray scanner. Signal intensity was calculated after background subtraction and four replicated spots of each probe on the same slide and median values calculated. The differentially expressed miRNAs were identified through fold-change screening.

**Quantitative real-time PCR.** Quantitative real-time PCR was applied to verify microRNA-602 (mir-602) expression levels in eight NL, 15 CL, 21 HCC tissue samples including those evaluated with microRNA microarray and three hepatoma cell lines including HepG2, HBV-secreting HepG2 2.2.15 and HBX-transfected HepG2-HBX. Primers for mir-602 were F: 5'-GAC ACG GGC GAC AGC T-3'; R: 5'-TGC GTG TCG TGG AGT C-3', and for control U6: F: 5'-GCT TCG GCA GCA CAT ATA CTA AAA T-3', R: 5'-CGC TTC ACG AAT TTG CGT GTC AT-3', with expected products of 60 and 89 bp, respectively. Human TaqMan MiR assays (Applied Biosystems, CA) were according to the manufacturer's instructions. Cycle passing threshold (Ct) was recorded and normalized to RNU6B expression. Relative expression was calculated as 2Ct_miR-602-Ct_RNU6B. All qRT-PCR values were calculated as ratios to the U6-normalized qRT-PCR miR-602 value in the first normal specimen.

**Transfection of microRNA-602 inhibitor (mir-602i) into HepG2, HepG2 2.2.15 and HepG2-HBX cells.** Synthesized RNA duplexes of miR-602 inhibitor were purchased from Dharmacon (Lafayette, CA). After culture in RPMI 1640 plus 10% fetal bovine serum and passage for three generations, 30–50% confluent cells were transfected with 60 nM of miR-602 inhibitor, or inhibitor-negative control using DharmaFECT4 (Dharmacon). Cell RNA and proteins were harvested and tested at 72 h after transfection.

**RT-PCR.** Primers for RASSF1A, P73 and control β-actin were designed according to GeneBank. Primers for RASSF1A were: F: 5'-GCT CTG TGG CGA CTT CAT CT-3', R: 5'-TAG GAG GGT GGC TTC TTG C-3'; Primers for P73 were: F: 5'-ATC GGG AGG GAC TTC AAC G-3', R: 5'-TCC GCC GCT TCT TCA CA-3'; Primers for β-actin were: F: 5'-AGG GAA ATC GTG CGT GAC-3', R: 5'-ACC CAG GAA GGA AGG CT-3'. The expected products were 357 bp, 451 bp and 198 bp, respectively. cDNA was synthesized with Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, CA). PCR was conducted with following condition: 94°C x 30 s, 53°C x 45 s, 2°C x 45 s for 35 cycles, and 72°C x 5 min. PCR products were electrophoresed on 2% gels and stained with ethidium bromide. Integral optical density (IOD) of the band was analyzed with Gel Pro 4.0 software.

**Western blots.** Protein expression changes of RASSF1A and P73 in tissues and hepatoma cells were detected by western blots. Briefly, cells were lysed in Laemmli sample buffer (Bio-Rad) supplemented with a protease inhibitor (Roche, NJ). Protein concentrations were measured using a BCA Protein Assay kit (Pierce, IL). Cell lysates (50 μg) were electrophoresed on 10 to 20% polyacrylamide gels (Bio-Rad) and transferred to Immobilon-PSQ membranes (Millipore, MA). The membranes were blocked with TBS containing 5% skim milk and 0.1% Tween-20, then incubated with the primary antibody. Twenty μl of 1:300 anti-RASSF1A, anti-P73, and anti-GAPDH antibodies (Abcam for RASSF1A, CST for P73 and ProMab for GAPDH) were used according to the manufacturer's instructions. After washing, the membranes were incubated with the secondary antibody, horseradish peroxidase-conjugated IgG (Calbiochem, NJ) and analyzed using enhanced chemiluminescence-plus reagent (GE Healthcare, UK).



**Cell apoptosis and proliferation assay.** Apoptosis was detected by Annexin V-FITC/PI double staining. Briefly, 1 x $10^6$/mL cells 72 h after transfection were harvested, digested and stained with annexin V conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min and detected by FACScan. Data was analyzed with CELL Quest software. Cells at 0, 1, 2, 3, 4, 5, 6, 7 d after transfection were treated with methyl thiazolyl tetrazolium (MTT, Gibco) and cultured for 4 h. After 150 μl dimethyl sulfoxide (DMSO, Gibco) was added, the cells were detected for intensity of optical density (IOD) at 490 nm. Cell proliferation curves were obtained as IOD as a function of time.

**Statistics.** All experiments were repeated 3 times, and data were recorded as $\bar{x} \pm s$ and analyzed by Student's t-test and one-way ANOVA by SPSS13.0 software. Unless specially stated, data of CHL, LC, HCC or NT were compared with that of NL in each experiment and p value obtained. $p < 0.05$ was considered to be significantly different.

**Acknowledgements**

The work was supported by: National Natural Science Foundation grants of China (NSF No. 30672067, 30700190), and grant of New-Century Excellent Talent Program of Education Ministry of China (No. NCET-07-318).

## References


1. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002; 31:339-46.
2. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6:857-66.
3. Gregory RI, Shiekhattar R. MicroRNA biogenesis and cancer. Cancer Res 2005; 65:3509-12.
4. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005; 9:834-8.
5. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007; 133:647-58.
6. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, et al. Cyclin $G_1$ is a target of miR-122a, a microRNA frequently downregulated in human hepatocellular carcinoma. Cancer Res 2007; 67:6092-9.
7. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006; 25:2537-45.
8. Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008; 47:897-907.
9. Shah YM, Morimura K, Yang Q, Tanabe T, Takagi M, Gonzalez FJ. Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. Mol Cell Biol 2007; 27:4238-47.
10. Huang YS, Dai Y, Yu XF, Bao SY, Yin YB, Tang M, et al. Microarray analysis of microRNA expression in hepatocellular carcinoma and nontumorous tissues without viral hepatitis. J Gastroenterol Hepatol 2008; 23:87-94.
11. Jiang J, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis hepatitis, cirrhosis and patient survival. Clin Cancer Res 2008; 14:419-27.
12. Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP, et al. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology 2008; 47:1223-32.
13. Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated to clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008; 47:1807-9.
14. He L, Hannon GJ. MicroRNAs: Small RNAs with big role in gene regulation. Nat Rev Genet 2004; 5:522-31.
15. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 2005; 122:553-63.
16. Didiano D, Hobert O. Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat Struct Mol Biol 2006; 13:849-51.
17. Maroney PA, Yu Y, Fisher J, Nilsen TW. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol 2006; 13:1102-7.
18. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005; 353:1793-801.
19. Iizuka N, Oka M, Yamada-Okabe H, Nishida M, Maeda Y, Mori N, et al. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. Lancet 2003; 361:923-9.
20. Lee JS, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004; 40:667-76.
21. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006; 130:2113-29.
22. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lympho-cytic leukemias. Proc Natl Acad Sci USA 2004; 101:11755-60.
23. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007; 129:1401-14.
24. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 2004; 101:2999-3004.
25. Lujambio A, Calin GA, Villanueva A, Ropero S, Sánchez-Céspedes M, Blanco D, et al. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci USA 2008; 105:13556-61.
26. Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, Setién F, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 2007; 67:1424-9.
27. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 2007; 68:4123-32.
28. Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, et al. MicroRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci USA 2006; 103:9136-41.
29. Zhang L, Volinia S, Bonome T, Calin GA, Greshock J, Yang N, et al. Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer. Proc Natl Acad Sci USA 2008; 105:7004-9.
30. Luo JH, Ren B, Keryanov S, Tseng GC, Rao UN, Monga SP, et al. Transcriptomic and genomic analysis of human hepatocellular carcinomas and hepatoblastomas. Hepatology 2006; 44:1012-24.
31. Poon TC, Wong N, Lai PB, Rattray M, Johnson PJ, Sung JJ. A tumor progression model for hepatocellular carcinoma: bioinformatic analysis of genomic data. Gastroenterology 2006; 131:1262-70.
32. Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Bréchot C, et al. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. Gut 2005; 54:1162-8.
33. Pang E, Wong N, Lai PB, To KF, Lau JW, Johnson PJ. A comprehensive karyotypic analysis on a newly developed hepatocellular carcinoma cell line, HKCI-1, by spectral karyotyping and comparative genomic hybridization. Cancer Genet Cytogen 2000; 121:9-16.
34. Gao HF, Tu H. Identification of hepatitis B virus integration sites in hepatocellular carcinomas. Chin J Hepatol 2004; 12:567-8.
35. Thaler S, Hähnel PS, Schad A, Dammann R, Schuler M. RASSF1A mediates $p21^{Cip1/Waf1}$-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt. Cancer Res 2009; 69:1748-57.
36. van der Weyden L, Tachibana KK, Gonzalez MA, Adams DJ, Ng BL, Petty R, et al. The RASSF1A isoform of RASSF1 promotes microtubule stability and suppresses tumorigenesis. Mol Cell Biol 2005; 25:8356-67.
37. Chan KC, Lai PB, Mok TS, Chan HL, Ding C, Yeung SW, et al. Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. Clin Chem 2008; 54:1528-36.
38. Zhang YJ, Wu HC, Shen J, Ahsan H, Tsai WY, Yang HI, et al. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin Cancer Res 2007; 13:2378-84.
39. Yeo W, Wong N, Wong WL, Lai PB, Zhong S, Johnson PJ. High frequency of promoter hypermethylation of RASSF1A in tumor and plasma of patients with hepatocellular carcinoma. Liver Int 2005; 25:266-72.
40. Tannapfel A, John K, Mise N, Schmidt A, Buhlmann S, Ibrahim SM, et al. Autonomous growth and hepatocarcinogenesis in transgenic mice expressing the p53 family inhibitor DNp73. Carcinogenesis 2008; 29:211-8.