Cellular & Molecular Biology
Cell. Mol. Biol. 2015; 61 (3): 12-16
Published online June 8, 2015 (http://www.cellmolbiol.com)
Received on January 22, 2015, Accepted on June 2, 2015.
doi : 10.14715/cmb/2015.61.3.4

miR-145 regulates chemoresistance in hepatocellular carcinoma via epithelial mesenchymal transition

B-L. Ju[1], Y-B. Chen[1], W-Y. Zhang[1], C-H. Yu[1], D-Q. Zhu[2] and J. Jin[3]

[1] Department of Hepatobilliary Surgery, General Hospital of Beijing Command, PLA. Beijing 100700, China
[2] Department of Pharmacy, Shanghai Tongji Hospital, Tongji University, Shanghai, 200065, China
[3] Department of Pharmacy, Shanghai Ninth People's Hospital, Shanghai, 200011, China

Corresponding author: : De-qiu Zhu, Department of Pharmacy, Shanghai Tongji Hospital, Tongji University, Shanghai, 200065, China. E-mail: zdq_0726@163.com
Jian Jin, Department of Pharmacy, Shanghai Ninth People's Hospital, Shanghai, 200011, China. E-mail: jinjjij@126.com

Abstract

Resistance to chemotherapeutic drugs is a major obstacle in hepatocellular carcinoma (HCC) therapy. MicroRNA-145 (miR-145) has been shown to be down-regulated in several cancers and may be involved in the process of carcinogenesis. The present study aimed to evaluate the effects of miR-145 in adriamycin (ADM)-resistant human HCC cells. We found that miR-145 was significantly reduced in HepG2/ADM and HuH7/ADM cells compared with the chemosensitive parental cells. Up-regulation of miR-145 increased the ADM cytotoxicity in chemoresistant tumor cells. In addition, Smad3 was identified as the target of miR-145 and miR-145 overexpression inhibited Smad3 expression both at the mRNA and protein levels. The luciferase reporter assay confirmed that Smad3 was a direct target of miR-145. Moreover, up-regulation of miR-145 suppressed Smad3 related EMT features as shown by increased expression of E-cadherin and reduced vimentin level in HepG2/ADM and HuH7/ADM cells. Our study demonstrated that miR-145 modulated both chemoresistance and EMT in HCC cells, and up-regulation of miR-145 might be a potential therapeutic strategy for treatment of chemoresistant HCC.

Key words: Hepatocellular carcinoma, chemoresistance, miR-145, epithelial mesenchymal transition.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant tumors, ranking the third cause of cancer-related mortality in the world (1). Statistical studies have shown that there is estimated 350 000 new cases and nearly one million deaths annually (2, 3). Current treatments for HCC include chemotherapy, radiotherapy, and surgical operation. However, the development of acquired drug resistance to conventional chemotherapeutics has become a major obstacle in HCC treatment (4, 5). Such limitation highlights the imperative need for identifying novel treatment strategies which may help overcome drug resistance and enhance tumor cell response to anti-cancer drugs.

It has been acknowledged that the pathogenesis of liver cancer is a multistep process regulated by aberrantly protein expression and alterations of morphological and molecular features during malignant progression (6, 7). Epithelial-mesenchymal transition (EMT) is a complex, reversible process which induces epithelial cells to transform to mesenchymal phenotype (8). Although accumulating evidences suggest that EMT plays an important role in regulating the chemoresistance properties of liver cancer, but the molecular mechanism still remains elusive (9-11).

MicroRNAs (miRNAs) are a class of short non-coding RNA molecules (19–25 nucleotides in length) which repress the expression of target genes at the post-transcriptional level (12). Aberrant microRNA expression features significantly in many cancers and plays critical roles in tumor cell behavior, such as proliferation, differentiation and apoptosis (13-15). Recent studies have shown that miRNAs interact with EMT to develop chemoresistance. Several miRNAs has been identified to participate in the development of chemoresistance to anti-epidermal growth factor receptor in non small cell lung cancer cells (16). Among those miRNAs, miR-200 family is one of the most extensively studied miRNAs participated in both EMT and chemoresistance of cancer cells (17).

miR-145 has been found to be down-regulated in several cancers such as bladder cancer (18), liver cancer (19), and lung adenocarcinoma (20), suggesting reduced miR-145 expression may be involved in the general process of carcinogenesis. However, to date the role of miR-145 in EMT and chemoresistance of liver cancer cells has never been investigated. In the present study, we aimed to evaluate the relevance of miR-145 in chemoresistance of HCC cells using HepG2/adriamycin (ADM) and HuH7/ADM models.

Materials and methods

Cell culture

Human HCC cell lines HepG2 and HuH7 were purchased from the ATCC (Manassas, VA, USA). ADM was purchased from Sigma-Aldrich (St. Louis, MO, USA). Multidrug resistant human HCC cell lines, HepG2/adriamycin (ADM) and HuH7/ADM were established by treating HepG2/WT and HuH7/WT cells with stepwise increasing concentrations of ADM (21,22). All cells were maintained at 37°C in 5% CO_2 incubator and cultured in DMEM (Gibco, Carlsbad, CA, USA) supple-

mented with 10% FBS and 1% penicillin⁄streptomycin.

CCK-8 assay

HCC cells were seeded onto 96-well plates at 3.0 x 10^3 cells⁄well. The medium was replaced with the corresponding serum-free medium for 24 h to synchronize the cell cycle, then serum-free medium was replaced with complete medium containing the drugs at the indicated concentrations for 48 h. Then 10 μL⁄well CCK8 solution (Dojindo, Kumamoto, Japan) was added, the plates incubated for 3 h, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex, Chantilly , VA, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Cells were seeded on to 12-well plates and total RNAs were isolated by TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was reverse transcribed and amplified using the miRNA detection kit (Ambion, USA) according to the protocol. PCR reactions were performed on ABI 7500 Real-Time PCR System (Applied Biosystems) with the following conditions: 95˚C, 10 min for 1 cycle, then 95˚C, 15 sec, 60˚C, 1 min for 40 cycles. The U6 small nuclear RNA was used as a control. The mRNA expression of Smad3 was measured by real time PCR with GAPDH used as control. The primer sequences were 5'- CAGATGTGTGGTCCTTTG- 3' (forward); 5'- ATTCGGGTTGTAGGAGTCT- 3' (reverse).

Luciferase activity assay

Luciferase reporters were generated based on the firefly luciferase expressing vector pMIR-REPORT (Ambion, USA). Cells were seeded in 24-well plates at the density of 5x10^4 cells per well the day before transfection. Luciferase reporter (500 ng), 50 pmol (miRNA-145 mimic or control) and 40 ng of pRL-TK were added in each well. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, USA).

Western blot

Tumor cells were lysed in 50 μl cell lysis buffer (Cell Signaling, Danvers, MA , USA) containing protease inhibitors (Sigma, USA). Whole cell lysates were prepared and fractioned were separated by 10% SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with anti-Smad3 (Abcam, Cambridge, USA), anti-E-cadherin, or anti-vimentin (Cell Signaling Technology, USA) antibodies at 4°C overnight. The membranes were washed three times with TBST and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein expression was detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data were presented as means ± SD and treated for statistics analysis by SPSS program. Comparison between groups was made using ANOVA and statistically significant difference was defined as $P<0.05$.

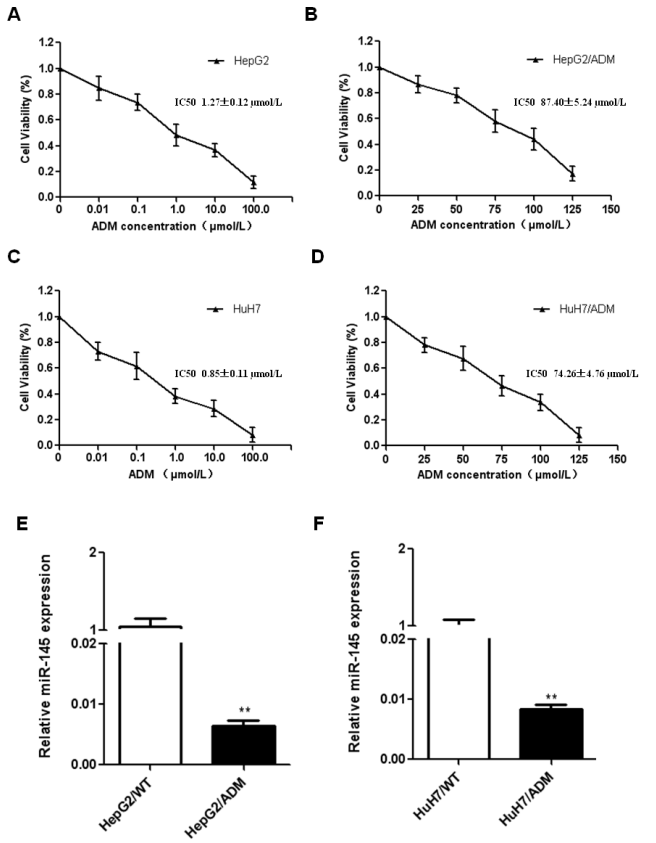

Figure 1. miR-145 was decreased in chemoresistant HCC cells. CCK-8 assay was used to measure the cytotoxicity of adriamycin on HepG2 (A), HepG2/ADM (B), HuH7 (C), and HuH7/ADM (D) cells at different concentrations. qRT-PCR data confirmed the down-regulation of miR-145 in HepG2/ADM (E) and HuH7/ADM (F) cells compared with their parental HepG2/WT and HuH7/WT cells. ** $P<0.01$.

Results

Down-regulation of miR-145 in chemoresistant HCC cells

Firstly, we incubated HepG2 and HuH7 cells respectively with ADM at a stepwise increasing concentration and selected the resistance cells by removing the non-resistant dead cells. We then tested the ADM sensitivity of each cell lines and showed that the lethal dose (IC_{50}) of ADM at 48 h was 1.27±0.12 μmol/L in HepG2 cells and 87.40±5.24 μmol/L in HepG2/ADM cells (Fig. 1A and B). Similarly, the ADM IC_{50} increased from 0.85±0.11 μmol/L in HuH7 cells to 74.26±4.76 μmol/L in HuH7/ADM cells (Fig. 1C and D). To understand the functional relevance of miR-145 in cancer cells chemoresistance, we evaluated the expression of miR-145 in HCC cells resistant to ADM by qRT-PCR. Results showed that miR-145 was significantly down-regulated in HepG2/ADM and HuH7/ADM cells compared to their parental cells (Fig. 1E and F). These results implied that miR-145 might be involved in the chemoresistance of HCC cells to ADM.

miR-145 regulated the chemoresistance of HCC cells

In order to confirm that miR-145 participated in ADM cytotoxicity, we enhanced miR-145 expression

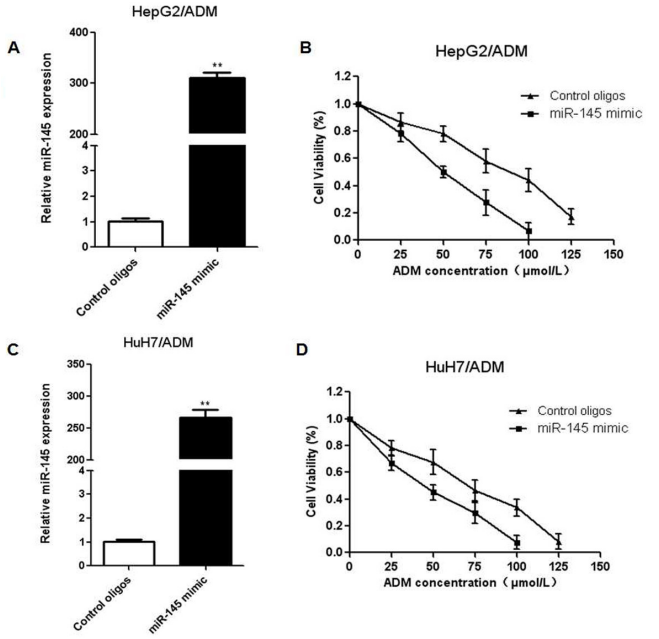

Figure 2. miR-145 was involved in chemoresistance of HCC cells. HepG2/ADMcells (A) were transfected with miR-145 mimic or control oligos, and cell viability was evaluated using CCK-8 assay (B). Similarly, HuH7/ADM cells (C) were transfected with miR-145 mimic or control oligos followed by CCK-8 assay for cell viability determination (D). ** P<0.01.

in HCC cells by transfection with miR-145 mimic. As a result, HepG2/ADM cells displayed a dramatic increase in miR-145 levels as shown by qRT-PCR (Fig. 2A). Interestingly, we found that up-regulation of miR-145 significantly augmented the ADM cytotoxicity in HepG2/ADM cells compared to those transfected with negative controls (Fig. 2B). In addition, similar results were also observed in HuH7/ADM cells (Fig. 2C and D). These results demonstrated that overexpression of miR-145 could enhance the chemotherapeutics sensitivity in HCC cells.

Smad3 was a target of miR-145

It is well known that miRNAs affect cellular processes by regulating genes expression at the post-translational level (14). We identified that Smad3 was a potential target of miR-145 by performing computational predictions. To determine whether miR-145 targeted Smad3 *in vitro*, we transfected HepG2/ADM cells with miR-145 mimic and detected Smad3 mRNA expression using qRT-PCR. We found that up-regulation of miR-145 resulted in a reduction of Smad3 mRNA in HepG2/ADM (Fig. 3A) and HuH7/ADM cells (Fig. 3B). Western blot analysis showed that overexpression of miR-145 remarkably inhibited Smad3 protein expression in HepG2/ADM (Fig. 3C) and HuH7/ADM cells (Fig. 3D). These results indicated that miR-145 regulated the expression of Smad3 at both the mRNA and protein levels. To determine if Smad3 was a direct target of miR-145, fluorescent reporter assays were performed. Smad3 3'-UTR with the predicted binding site of miR-145 was cloned into a fluorescent reporter vector. Up-regulation of miR-145 reduced the luciferase activity in HepG2/ADM and HuH7/ADM cells transfected with a vector containing Smad3 3' -UTR compared with controls (Fig. 3E and F). These results indicated that miR-145 targeted Smad3 3'-UTR region directly.

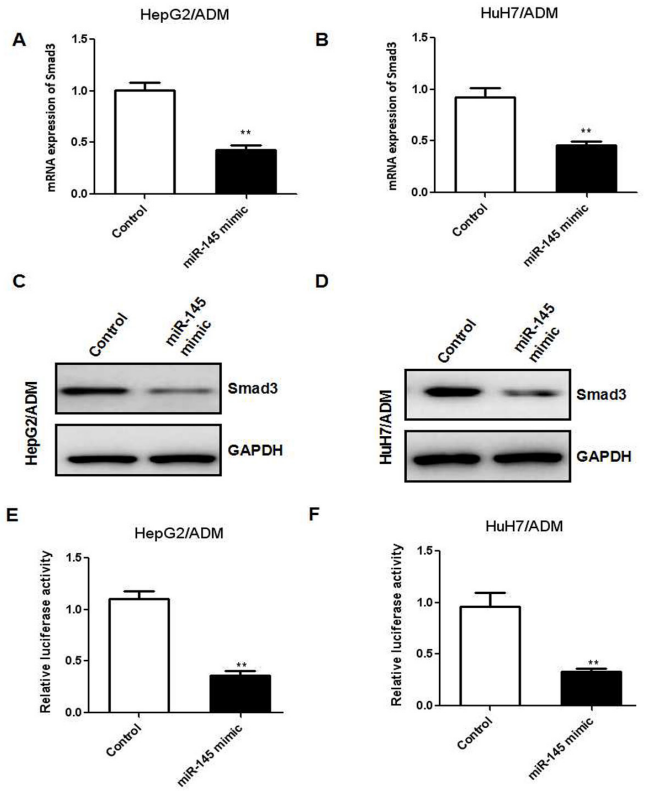

Figure 3. Smad3 was a target of miR-145. Chemoresistant tumor cells were transfected with miR-145 mimic or control oligos. After transfection, the mRNA (A and B) and protein (C and D) levels of Smad3 in HepG2/ADM and HuH7/ADM cells was determined by qRT-PCR and western blot, respectively. Luciferase reporter assay validated that the 3'UTR of Smad3 mRNA was targeted by miR-145 (E and F). ** P<0.01.

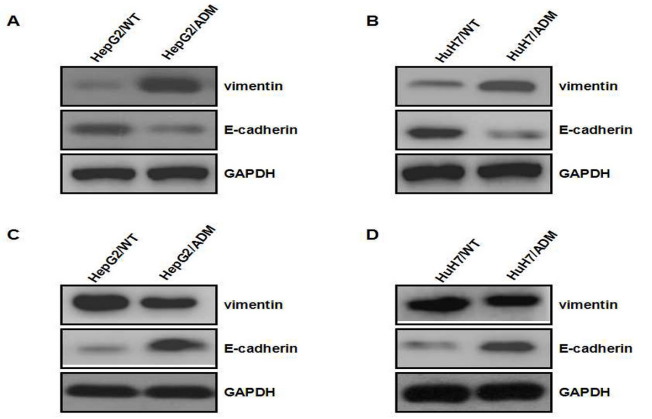

Figure 4. miR-145 regulated EMT in HCC cells. The expression of EMT-related biomarkers including vimentin and E-cadherin in HepG2/ADM (A) and HuH7/ADM (B) cells were measured by western blot. The protein levels of vimentin and E-cadherin were detected by western blot in HepG2/ADM (C) and HuH7/ADM (D) cells transfected with miR-145 mimic or control oligos.

miR-145 regulated EMT in HCC cells

It has been reported that Smad3 plays a critical role in EMT. Thus we next investigated whether miR-145 was involved in regulation of EMT. We found that HepG2/ADM and HuH7/ADM cells displayed mesenchymal-like markers, such as the high expression of vimentin and decreased expression of E-cadherin (Fig. 4A and B). Then, we transfected miR-145 mimic into HepG2/ADM and HuH7/ADM cells and found that up-regulation of miR-145 enhanced the expression of E-cadherin and reduced vimentin expression (Fig. 4C and D). These results demonstrated that up-regulation of miR-

145 reversed EMT in drug-resistant cancer cells.

Discussion

The acquired drug resistance to traditional chemotherapeutics has become a major obstacle to the triumph of chemotherapy (23). Accumulating evidences have shown that abnormal expression of miRNAs regulates oncogenic factors or tumor suppressors, leading to onset/offset of growth, angiogenesis, and metastasis of human cancers (13-15). It was further noticed that miRNAs play critical roles in regulating tumor cell response to chemotherapeutic agents (24). miR-145 has been reported to be frequently down-regulated in a number of cancers (18-22). Recent studies suggested that miR-145 suppressed cancer cell proliferation and potentially functioned as a tumor suppressor (21). Moreover, decreased expression of miR-145 was correlated with poor histological grade and prognosis in patients with liver cancer (25). The anthracycline drug, adriamycin, is one of the most important anti-cancer chemotherapeutic drugs. However, adriamycin resistance has been widely reported in the analysis of cancer research and various factors have been shown to contribute to this chemoresistance, such as increased GSH transferases, enhanced efflux through P-glycoprotein and multidrug resistance-associated protein drug pumps, and altered topoisomerase activity (26). In the present study, we firstly demonstrated that miR-145 was significantly reduced in HepG2/ADM and HuH7/ADM cells, and its overexpression enhanced the cytotoxicity of anti-cancer drugs, suggesting a role of miR-145 in chemoresistance.

miRNAs control the cells biological function by targeting genes expression, therefore it is critical to elucidate the functional targeted genes (12). Smad3 is involved in many cellular processes such as cell growth, apoptosis, cell cycle arrest and differentiation (27). Down-regulation of Smad3 is observed in diverse types of malignancies, including gastric cancers (28), breast cancers (29), and acute lymphoblastic leukemia (30). In the present study, we identified Smad3 as a candidate gene of miR-145; up-regulation of miR-145 led to high expression of Smad3 in HepG2/ADM and HuH7/ADM cells. In addition, miR-145 overexpression inhibited the luciferase activity in HepG2/ADM and HuH7/ADM cells transfected with a vector containing Smad3 3' -UTR compared with controls, demonstrating that miR-145 directly targeted Smad3.

EMT is involved in wound healing, stem cell behaviour, development, and contributes to cancer progression (9, 10). It plays crucial roles in the acquired chemoresistance in many kinds of cancer, including HCC (31). Smad3 was found to be critical for TGF-β-induced EMT; by forming a complex with Snail1, Smad3 plays as a pivotal role in EMT signaling and directly targets the E-cadherin expression (32, 33). Recent study showed that upregulated Smad3 promoted EMT and predicted poor prognosis in pancreatic ductal adenocarcinoma (34). Furthermore, inhibition of Smad3 activation by HSP70 could attenuate EMT of peritoneal mesothelial cells (35). Thus, we hypothesize that Smad3 inhibition by miR-145 could reverse EMT and sensitize tumor cells to chemotherapeutics. Indeed, our study show that HepG2/ADM and HuH7/ADM cells exhibited enhanced expression of vimentin and reduced E-cadherin level compared with their parental cells. However, such EMT features were reversed by overexpression of miR-145, suggesting that miR-145 may modulate EMT via regulation of Smad3 in HCC cells.

In conclusion, our study for the first time demonstrated that miR-145 was a potential modulator of both chemoresistance and EMT in HCC cells. In addition, up-regulation of miR-145 or inhibition of Smad3 might be potential therapeutic strategies for the treatment of chemoresistant liver cancer.

References

1. Forner, A. and Bruix, J., Biomarkers for early diagnosis of hepatocellular carcinoma. *Lancet Oncol*. 2012, **13:** 750-751. doi: 10.1016/S1470-2045(12)70271-1

2. Siegel, R., Ma, J., Zou, Z. and Jemal, A., Cancer statistics, 2014. *CA Cancer J Clin.* 2014, **64:** 9-29. doi: 10.3322/caac.21208.

3. El-Serag, H. B. and Rudolph, K. L., Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*. 2007, **132:** 2557-2576. doi: 10.1053/j.gastro.2007.04.061.

4. Sui, H., Zhu, L., Deng, W. and Li, Q., Epithelial-mesenchymal transition and drug resistance: role, molecular mechanisms, and therapeutic strategies. *Oncol Res Treat*. 2014, **37:** 584-589. doi: 10.1159/000367802.

5. Longley, D. B. and Johnston, P. G., Molecular mechanisms of drug resistance. *J Pathol.* 2005, **205:** 275-292. doi: 10.1002/path.1706.

6. Dufour, J. F. and Johnson, P., Liver cancer: from molecular pathogenesis to new therapies: summary of the EASL single topic conference. *J Hepatol.* 2010, **52:** 296-304. doi: 10.1016/j.jhep.2009.11.010.

7. Holczbauer, A., Factor, V. M., Andersen, J. B., Marquardt, J. U., Kleiner, D. E., Raggi, C., Kitade, M., Seo, D., Akita, H., Durkin, M. E. and Thorgeirsson, S. S., Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. *Gastroenterology.* 2013, **145:** 221-231. doi: 10.1053/j.gastro.2013.03.013.

8. Gujral, T. S., Chan, M., Peshkin, L., Sorger, P. K., Kirschner, M. W. and MacBeath, G., A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. *Cell.* 2014, **159:** 844-856. doi: 10.1016/j.cell.2014.10.032.

9. Steinway, S. N., Zanudo, J. G., Ding, W., Rountree, C. B., Feith, D. J., Loughran, T. J. and Albert, R., Network modeling of TGFbeta signaling in hepatocellular carcinoma epithelial-to-mesenchymal transition reveals joint sonic hedgehog and Wnt pathway activation. *Cancer Res.* 2014, **74:** 5963-5977. doi: 10.1158/0008-5472.CAN-14-0225.

10. Yang, Y. M., Lee, W. H., Lee, C. G., An, J., Kim, E. S., Kim, S. H., Lee, S. K., Lee, C. H., Dhanasekaran, D. N., Moon, A., Hwang, S., Lee, S. J., Park, J. W., Kim, K. M. and Kim, S. G., Galpha gep oncogene deregulation of p53-responsive microRNAs promotes epithelial-mesenchymal transition of hepatocellular carcinoma. *Oncogene. 0*, 2014. doi: 10.1038/onc.2014.218.

11. Cheng, J., Li, M. and Lv, Y., Sublethal heat treatment promotes epithelial-mesenchymal transition and enhances the malignant potential of hepatocellular carcinoma. *Hepatology.* 2014, **59:** 1650. doi: 10.1002/hep.26630.

12. Pritchard, C. C., Cheng, H. H. and Tewari, M., MicroRNA profiling: approaches and considerations. *Nat Rev Genet.* 2012, **13:** 358-369. doi: 10.1038/nrg3198.

13. Rachagani, S., Kumar, S. and Batra, S. K., MicroRNA in pancreatic cancer: pathological, diagnostic and therapeutic implications. *Cancer Lett.* 2010, **292:** 8-16. doi: 10.1016/j.canlet.2009.11.010.

14. Lin, L., Liang, H., Wang, Y., Yin, X., Hu, Y., Huang, J., Ren, T., Xu, H., Zheng, L. and Chen, X., microRNA-141 inhibits cell proliferation and invasion and promotes apoptosis by targeting hepatocyte nuclear factor-3beta in hepatocellular carcinoma cells. *BMC Cancer.* 2014, **14:** 879. doi: 10.1186/1471-2407-14-879.
15. Cheng, C. J., Bahal, R., Babar, I. A., Pincus, Z., Barrera, F., Liu, C., Svoronos, A., Braddock, D. T., Glazer, P. M., Engelman, D. M., Saltzman, W. M. and Slack, F. J., MicroRNA silencing for cancer therapy targeted to the tumour microenvironment. *Nature.* 2014. doi: 10.1038/nature13905.
16. Bryant, J. L., Britson, J., Balko, J. M., Willian, M., Timmons, R., Frolov, A. and Black, E. P., A microRNA gene expression signature predicts response to erlotinib in epithelial cancer cell lines and targets EMT. *Br J Cancer.* 2012, **106:** 148-156. doi: 10.1038/bjc.2011.465.
17. Feng, X., Wang, Z., Fillmore, R. and Xi, Y., MiR-200, a new star miRNA in human cancer. *Cancer Lett.* 2014, **344:** 166-173. doi: 10.1016/j.canlet.2013.11.004.
18. Chiyomaru, T., Enokida, H., Tatarano, S., Kawahara, K., Uchida, Y., Nishiyama, K., Fujimura, L., Kikkawa, N., Seki, N. and Nakagawa, M., miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. *Br J Cancer.* 2010, **102:** 883-891. doi: 10.1038/sj.bjc.6605570.
19. Wang, Z., Zhang, X., Yang, Z., Du H, Wu, Z., Gong, J., Yan, J. and Zheng, Q., MiR-145 regulates PAK4 via the MAPK pathway and exhibits an antitumor effect in human colon cells. *Biochem Biophys Res Commun.* 2012, **427:** 444-449. doi: 10.1016/j.bbrc.2012.06.123.
20. Vaksman, O., Stavnes, H. T., Kaern, J., Trope, C. G., Davidson, B. and Reich, R., miRNA profiling along tumour progression in ovarian carcinoma. *J Cell Mol Med.* 2011, **15:** 1593-1602. doi: 10.1111/j.1582-4934.2010.01148.x.
21. Noh, J. H., Chang, Y. G., Kim, M. G., Jung, K. H., Kim, J. K., Bae, H. J., Eun, J. W., Shen, Q., Kim, S. J., Kwon, S. H., Park, W. S., Lee, J. Y. and Nam, S. W., MiR-145 functions as a tumor suppressor by directly targeting histone deacetylase 2 in liver cancer. *Cancer Lett.* 2012, **335:** 455-462. doi: 10.1016/j.canlet.2013.03.003.
22. Cho, W. C., Chow, A. S. and Au, J. S., Restoration of tumour suppressor hsa-miR-145 inhibits cancer cell growth in lung adenocarcinoma patients with epidermal growth factor receptor mutation. *Eur J Cancer.* 2009, **45:** 2197-2206. doi: 10.1016/j.ejca.2009.04.039.
23. Brown, R., Curry, E., Magnani, L., Wilhelm-Benartzi, C. S. and Borley, J., Poised epigenetic states and acquired drug resistance in cancer. Nat Rev Cancer. 2014, **14:** 747-753. doi: 10.1038/nrc3819.
24. Sarkar, F. H., Li, Y., Wang, Z., Kong, D. and Ali, S., Implication of microRNAs in drug resistance for designing novel cancer therapy. *Drug Resist Updat.* 2010, **13:** 57-66. doi: 10.1016/j.drup.2010.02.001.
25. Varnholt, H., Drebber, U., Schulze, F., Wedemeyer, I., Schirmacher, P., Dienes, H. P. and Odenthal, M., MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. *Hepatology.* 2008, **47:** 1223-1232. doi: 10.1002/hep.22158.
26. Kim, H. J., Kim, M. Y., Jin, H., Kim, H. J., Kang, S. S., Kim, H. J., Lee, J. H., Chang, K. C., Hwang, J. Y., Yabe-Nishimura, C., Kim, J. H. and Seo, H. G., Peroxisome proliferator-activated receptor {delta} regulates extracellular matrix and apoptosis of vascular smooth muscle cells through the activation of transforming growth factor-{beta}1/Smad3. *Circ Res.* 2009, **105:** 16-24. doi: 10.1161/CIRCRESAHA.108.189159.
27. Islam, S. S., Mokhtari, R. B., El, H. Y., Azadi, M. A., Alauddin, M., Yeger, H. and Farhat, W. A., TGF-beta1 induces EMT reprogramming of porcine bladder urothelial cells into collagen producing fibroblasts-like cells in a Smad2/Smad3-dependent manner. *J Cell Commun Signal.* 2014, **8:** 39-58. doi: 10.1007/s12079-013-0216-4.
28. Han, S. U., Kim, H. T., Seong, D. H., Kim, Y. S., Park, Y. S., Bang, Y. J., Yang, H. K. and Kim, S. J., Loss of the Smad3 expression increases susceptibility to tumorigenicity in human gastric cancer. *Oncogene.* 2004, **23:** 1333-1341. doi: 10.1038/sj.onc.1207259.
29. Jeruss, J. S., Sturgis, C. D., Rademaker, A. W. and Woodruff, T. K., Down-regulation of activin, activin receptors, and Smads in high-grade breast cancer. *Cancer Res.* 2003, **63:** 3783-3790.
30. Wolfraim, L. A., Fernandez, T. M., Mamura, M., Fuller, W. L., Kumar, R., Cole, D. E., Byfield, S., Felici, A., Flanders, K. C., Walz, T. M., Roberts, A. B., Aplan, P. D., Balis, F. M. and Letterio, J. J., Loss of Smad3 in acute T-cell lymphoblastic leukemia. *N Engl J Med.* 2004, **351:** 552-559. doi: 10.1056/NEJMoa031197.
31. Zhou, Y., Li, Y., Ye, J., Jiang, R., Yan, H., Yang, X., Liu, Q. and Zhang, J., MicroRNA-491 is involved in metastasis of hepatocellular carcinoma by inhibitions of matrix metalloproteinase and epithelial to mesenchymal transition. *Liver Int.* 2013, **33:** 1271-1280. doi: 10.1111/liv.12190.
32. Vincent, T., Neve, E. P., Johnson, J. R., Kukalev, A., Rojo, F., Albanell, J., Pietras, K., Virtanen, I., Philipson, L., Leopold, P. L., Crystal, R. G., de Herreros, A. G., Moustakas, A., Pettersson, R. F. and Fuxe, J., A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nat Cell Biol.* 2009, **11:** 943-950. doi: 10.1038/ncb1905.
33. Dang, H., Ding, W., Emerson, D. and Rountree, C. B., Snail1 induces epithelial-to-mesenchymal transition and tumor initiating stem cell characteristics. *BMC Cancer.* 2011, **11:** 396. doi: 10.1186/1471-2407-11-396.
34. Yamazaki, K., Masugi, Y., Effendi, K., Tsujikawa, H., Hiraoka, N., Kitago, M., Shinoda, M., Itano, O., Tanabe, M., Kitagawa, Y. and Sakamoto, M., Upregulated SMAD3 promotes epithelial-mesenchymal transition and predicts poor prognosis in pancreatic ductal adenocarcinoma. *Lab Invest.* 2014, **94:** 683-691. doi: 10.1038/labinvest.2014.53.
35. Liu, J., Bao, J., Hao, J., Peng, Y. and Hong, F., HSP70 inhibits high glucose-induced Smad3 activation and attenuates epithelial-to-mesenchymal transition of peritoneal mesothelial cells. *Mol Med Rep.* 2014, **10:** 1089-1095. doi: 10.3892/mmr.2014.2279.