HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

Hepatology. 2015 September ; 62(3): 829–840. doi:10.1002/hep.27886.

Identification of MicroRNAs Specific for EpCAM+ Tumor Cells in Hepatocellular Carcinoma

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Abstract

Therapies that target cancer stem cells (CSCs) hold promise in eliminating cancer burden. However, normal stem cells are likely to be targeted due to their similarities to CSCs. It is established that EpCAM is a biomarker for normal hepatic stem cells and $EpCAM^+AFP^+$ hepatocellular carcinoma (HCC) cells have enriched hepatic CSCs. We sought to determine if specific miRNAs exist in hepatic CSCs that are not expressed in normal hepatic stem cells. We performed a pair-wised comparison of the miRNA transcriptome of $EpCAM^+$ and corresponding $EpCAM^-$ cells isolated from two primary HCC specimens, as well as from two fetal livers and three healthy adult liver donors via small RNA deep sequencing. We found that miR-150, miR-155 and miR-223 were preferentially highly expressed in $EpCAM^+$ HCC cells, which was further validated. Their gene surrogates, identified using miRNA and mRNA profiling in a cohort of 292 HCC patients, were associated with patient prognosis. We further demonstrated that miR-155 was highly expressed in $EpCAM^+$ HCC cells compared to corresponding $EpCAM^-$ HCC cells, fetal livers with enriched normal hepatic progenitors, and normal adult livers with enriched mature hepatocytes. Suppressing miR-155 resulted in a decreased $EpCAM^+$ fraction in HCC cells and reduced HCC cell colony formation, migration and invasion *in vitro*. The reduced levels of

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identified miR-155 targets predicted the shortened overall survival and time to recurrence of HCC patients. Conclusion: MiR-155 was highly elevated in EpCAM$^+$ HCC cells and might serve as a molecular target to eradicate the EpCAM$^+$ CSC population in human HCCs.

Keywords

hepatocellular carcinoma; EpCAM; miR-155; hepatic cancer stem cells

Introduction

Cancer stem cells (CSCs) are defined by their abilities to give rise to a new tumor possessing all cell types in the original cancer. They are thought to be responsible for cancer metastasis and tumor relapse (1, 2). Eradicating CSCs may be a critical step to achieve stable tumor remission, or even a cure, of aggressive malignances. However, CSCs and normal stem cells share many common cellular properties (e.g., self-renewal, differentiation) and molecular signaling pathways (e.g., Wnt/β-catenin, TGF-beta, Notch) (1, 3–11), which precludes the development of therapeutics that can specifically target CSCs. Therefore, one of the major hurdles in CSC eradiation is our poor understanding of molecular changes specific to CSCs but not to normal stem/progenitor cells.

Hepatocellular carcinoma (HCC), a major type of primary liver cancer, is the second most common cause of cancer-related mortality worldwide in men (12, 13)(Globocan2012). Studies have indicated that epithelial cell adhesion molecule (EpCAM) is a normal human hepatic stem cell (HpSC) marker, and that EpCAM$^+$ cells isolated from AFP$^+$ HCC clinical specimens or cell lines are hepatic CSCs (4, 5, 14, 15). Several technologies including transcriptomic and metabolomic profiling have been used to characterize HCC specimens with high level of EpCAM and AFP (EpCAM$^+$AFP$^+$ HCC) (3, 5, 16–18). However, molecular features associated with EpCAM$^+$AFP$^+$ HCCs are commonly found in EpCAM$^+$ normal HpSCs, such as the activation of Wnt/beta-catenin pathway and the up-regulation of microRNA-181s (1, 3, 19). Little is known about the global molecular alterations specific to hepatic CSCs. To search for CSC-specific molecular traits, one strategy is to perform a pair wise comparison of molecular profiles between EpCAM$^+$ HCC cells and EpCAM$^-$ HCC cells isolated from the same AFP$^+$ HCC patients and then to normal EpCAM$^+$ hepatic stem/progenitor cells.

MicroRNAs (miRNAs) are a class of ~22-nt non-coding RNA molecules that repress gene expression at the post-transcriptional level under normal and pathological conditions. They are functionally linked to normal stem cells and CSCs, are relevant to cancer therapy, and are expressed in a tissue/cell-specific manner (20–24). High-throughput next-generation sequencing has become the technology of choice for analyzing miRNA expression with an increased sensitivity and accuracy. This technology is able to detect a full-length miRNA within a single read, and can distinguish miRNAs that are very similar in sequence, thereby producing a precise count of each type of miRNA (25, 26). Thus, this technology, in principle, may provide sufficient resolutions to detect molecular changes specific to hepatic CSCs.

In this vein, we used a small RNA deep sequencing approach to profile the miRNA transcriptome of $EpCAM^+$ cells and corresponding $EpCAM^-$ cells from primary HCC clinical specimens, HCC cell lines, as well as normal livers. We identified several miRNAs including miR-150, miR-155, miR-223 that were specific to $EpCAM^+$ HCC cells. We further demonstrated that miR-155 was highly elevated in $EpCAM^+$ HCC cells compared to the rest groups of cells, and that blockage of miR-155 resulted in a decreased $EpCAM^+$ HCC cell proportion and the reduced HCC spheroid formation, colony formation, cellular migration and invasion.

Materials and Methods

Cell sorting from fresh HCC samples and HCC cells, HpSC and HB cell isolation, primary human hepatocytes isolation, hESC cell culture

Cell sorting from fresh HCC samples and cell lines was done as we did previously (3, 5, 16). Cell sorting for $EpCAM^+$ cells from primary HCC tumor was done using magnetic-activated cell sorting (MACS) according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). EpCAM microBeads (Miltenyi Biotec, CA) were used. $EpCAM^-$ cells were mainly HCC cells after depletion of $EpCAM^+$ HCC cells. The purity of sorted cells was evaluated by FACS. The research protocol was reviewed and approved by the local Institutional Review Board. Cell sorting for $EpCAM^+$ cells from cultured HCC cells were done via a BD FACSAria cell sorting system (BD Biosciences). FITC-conjugated anti-EpCAM monoclonal antibody Clone Ber-EP4 (DAKO, Carpinteria, CA) was used.

Human HpSCs and HBs were isolated from fetal livers and primary human hepatocytes were isolated from healthy adult liver donors using protocols published previously (15, 27, 28). Detailed information was in Supplementary Supporting text. Human ESCs, H9 and H1 cells (WiCell Institute, Madison, WI, USA) were cultured as described in the standard protocols http://www.wicell.org. The inactivated MEF cells (Applied StemCell Inc, CA, USA) were used as the feeder layer. Cells were incubated at 37°C in 5% of CO2 in air.

Other Materials and Methodologies are described in detail in the Supplementary Supporting text.

Results

Small RNA deep sequencing of $EpCAM^+$ and $EpCAM^-$ cells from HCC and normal livers

To globally identify miRNA(s) specifically altered in hepatic CSCs but not in normal hepatic stem cells nor in differentiated HCC cells, we performed small RNA deep sequencing using the samples listed in Table 1. 1) $EpCAM^+$ HCC cells and corresponding $EpCAM^-$ HCC cells isolated from two human primary AFP^+ HCC specimens and two HCC cell lines; 2) normal HpSCs and their descendants, hepatoblasts (HB) isolated from two human fetal livers (14, 15); 3) primary hepatocytes isolated from three healthy liver donors. In addition, we included pooled H1 and H9 cell lines as human embryonic stem cell (hESC) positive controls to monitor the validity of our small RNA deep sequencing and downstream bioinformatics pipelines procedure since sequencing data and miRNA expression data are available for hESCs (29, 30). To monitor the validity of isolated primary $EpCAM^+$ and

EpCAM$^-$ HCC cells, a group of stem cell-related genes and mature hepatocytes-related genes were examined. Supplementary Fig 1 showed that, in primary EpCAM$^+$ HCC cells, four pluripotent stem cell markers (Lin28B, SOX2, NANOG, POU5F1) and two endodermal stem cells markers (PDX1, LGR5) expressed at higher levels, while mature hepatocyte-related genes (CYP3A4 and GSTA1) expressed at lower levels compared to those in EpCAM$^-$ cells.

Sequencing analysis resulted in a total of 290.9 million reads. We found that 92.5 million reads (about 1/3) were aligned to human miRBase with the rest of 71.7 million reads to the human genome (Fig 1A, Supplementary Table 1). For the reads aligned to human miRBase, their size distribution was enriched in 21–24nt (Fig 1B), which was consistent with the size of mature miRNAs. In contrast, the size for reads aligned to human genome was about evenly distributed. Using our in-house bioinformatics analysis pipeline (Supplementary Fig 2), we identified a total of 1231 known mature miRNAs, accounting for a total of 67.9 million reads (Fig 1C, Supplementary Table 1). We have also identified 175 potential novel mature miRNAs from the mapped reads. However, for these miRNAs, the abundance was relative low (Supplementary Table 1) and only a few of them with the median read number was over 100 reads among 16 samples. Therefore, only identified known miRNAs with a median of reads higher than three, 600 miRNAs, were used for further analysis. The total reads of 600 miRNAs counted for 99.9% of total reads (67.88 vs. 67.93 million reads).

Among these miRNAs, members of the miR-302 family were the most abundant miRNAs in hESCs, accounted for more than 21.4% of total reads (1.3 million/6.0 million) (Supplementary Fig 3A), while miR-122 was the most abundant miRNA in differentiated hepatocytes, accounted for more than 24.4% of total reads (0.6 million/ 2.4 million) (Supplementary Fig 3B). These results were consistent with the reported data (29–32), confirming the validity of our small RNA sequencing and the downstream bioinformatics pipelines.

Comparison of whole miRNA transcriptome between EpCAM$^+$ and EpCAM$^-$ cells from HCC and normal livers

To compare the difference of the miRNA transcriptome among EpCAM$^+$ and EpCAM$^-$ cells from HCC and normal livers, we first normalized the expression data of 600 miRNAs based on the total mapped reads in each corresponding samples (Supplementary Fig 4) and then performed an unsupervised hierarchical clustering. Unsupervised clustering analysis revealed 2 distinct groups. One contained cultured HCC cell lines while the other contained primary cells (Fig 2A). Because cultured HCC cell lines have very distinct small RNA transcriptome profiles compared to those of primary cells, we only included data from primary cells for the initial data analysis.

We found that 99 out of 600 miRNAs were differentially expressed between EpCAM$^+$ and EpCAM$^-$ HCC cells from two primary HCC cases with an average fold change of more than 2. Meanwhile, 301 of 600 miRNAs were differentially expressed between HpSC (and HB) and hepatocytes (≥2 folds). Among them, 22 miRNAs had the consistent alteration from the comparison of EpCAM$^+$ and EpCAM$^-$ cells (21 up and 1 down) in both tumor and normal cells (Fig 2B, left panel). MiR-181c was one of 21 up-regulated miRNAs in both EpCAM$^+$

HCC cells and normal hepatic progenitors (Supplementary Fig 5), which was consistent with our previous findings (3, 19).

Identification of miRNAs specific to EpCAM$^+$ HCC cells

We utilized a stringent criterion to further define miRNAs specific to EpCAM$^+$ HCC cells due to the limited sample size for sequencing. We first identified 28 miRNAs with > 5-folds between EpCAM$^+$ and EpCAM$^-$ HCC cells (Fig 2B, right panel; 21 upregulated and 7 downregulated miRNAs in EpCAM$^+$ HCC cells). Our candidate miRNAs were then restricted to those with less than 2-fold changes in normal liver cell comparison. As shown in Fig 2C, we identified 10 miRNAs as candidates specific to EpCAM$^+$ HCC cells.

We performed qRT-PCR to validate the sequencing results. Seven miRNAs were included because of the availability of probes for qRT-PCR. As shown in Fig 2D, there was a significant correlation between the qRT-PCR data and sequencing data.

EpCAM$^+$AFP$^+$ HCC cases had metastatic feature and short survival, and activated CSC gene signaling signatures, while EpCAM$^-$AFP$^-$ HCC cases had good prognosis and activated mature hepatocyte gene signatures (3, 5, 33). We therefore examined if a differential expression of candidate miRNAs can be found among EpCAM$^+$AFP$^+$ and EpCAM$^-$AFP$^-$ tumor samples. We examined HCC cases with available miRNA expression data (GSE6857; Cohort 1) (3, 34, 35), especially those with extreme expression spectrums on EpCAM and AFP. Among ten candidate miRNAs (listed in Fig 2C), three miRNAs (miR-150, miR-155, and miR-223) were included in this miRNA array dataset. Consistently, all three miRNAs were significantly elevated in extreme EpCAM$^+$AFP$^+$ HCCs compared to extreme EpCAM$^-$AFP$^-$ HCC cases (Fig 2E).

We further searched mRNAs as functional surrogates that were significantly correlated with miR-150, miR-155, and miR-223. In cohort 1, 196 HCC samples had both miRNA and mRNA expression data (GSE14520) (34, 36) (Fig 3A). We found that 511 genes were significantly correlated with all three miRNAs (Fig 3B). Hierarchical clustering of these 511 genes revealed that they can significantly discriminate EpCAM$^+$AFP$^+$ cases from EpCAM$^-$AFP$^-$ HCC cases (Fig 3C). Furthermore, these gene surrogates could predict overall survival and time to recurrence (Fig 3D). These results were validated in another independent HCC cohort with 139 HCC cases (GSE1898 and GSE4024; Cohort 2)(37, 38) (Fig 3E). Cytokeratin 19 (CK19) has been reported as a predictive marker for poor prognosis of HCC patients and related to hepatic CSCs (39, 40). We thus compared the expression of CK19 in the two clusters predicted by gene surrogates of EpCAM$^+$ HCC cells-related miRNAs. Using the available mRNA microarray data in two HCC cohorts, we found that CK19 expression level was significantly higher in cluster 2 patients with poor prognosis compared to that in cluster 1 patients with good prognosis in both cohorts ($p<0.001$, Supplementary Fig 6). Thus, gene surrogates of EpCAM$^+$ HCC cells-related miRNAs were associated with metastatic features and patient prognosis.

MiR-155 overexpression in EpCAM$^+$ HCC cells

MiR-155 was chosen for further analysis because of its high abundance in EpCAM$^+$ primary HCC cells compared to EpCAM$^-$ HCC cells, normal hepatic progenitor cells and

hepatocytes (Fig 4A; ≥5-fold). Since basal levels of miR-155 was very low in cultured HuH7 and HuH1 cells from either sequencing and qRT-PCR analyses, we first enriched $EpCAM^+$ and $EpCAM^-$ HCC cells (Fig 4B, top panel) using the KnockOut medium/Serum Replacement culture and EpCAM siRNA methodologies described previously (3, 5, 33). Under these conditions, miR-155 was more abundantly expressed in $EpCAM^+$ cells than in $EpCAM^-$ cells from HuH7 HCC cells (Fig 4B, bottom panel). We also found that hypoxia could enrich $EpCAM^+$ CSC populations as previous reports (8, 41) and induced miR-155 expression (Fig 4C-HuH7, Supplementary Fig 7-HuH1).

In contrast, miR-155 level in human adult livers and fetal livers was lower than that in enriched $EpCAM^+$ HCC cells obtained from KnockOut medium culture methods (Fig 4D). We also examined miR-155 levels in different stages of mouse fetal livers and found the miR-155 level was low, with no change during mouse embryonic liver development (Fig. 4E). As a control, miR-181d showed a gradual decline in its expression following the liver development from 11.5 to 15.5 days (fetal liver) and from 1 week to 15 weeks after birth (Fig. 4E). Collectively, we concluded that miR-155 was specifically highly expressed in $EpCAM^+$ HCC cells, but not in $EpCAM^-$ HCC cells, nor in normal livers including fetal livers with more hepatic progenitors and adult livers with more differentiated hepatocytes.

HCC cells with suppressed miR-155 had reduced malignancy features

We hypothesized that silencing miR-155 could suppress HCC malignant features. MiRZip-155, a lentivirus vector encoding an anti-miR155 cassette, was used to suppress miR-155 functions via blocking the binding of miR-155 to its target genes. We found that cells with transfection of miRZip-155 had a reduced miR-155 activity determined by a luciferase reporter containing a miR-155 sequence at the 3'UTR (Fig 5A). We then examined whether MiRZip-155 can inhibit the malignant features of HCC cells. FACS analysis revealed that suppressing miR-155 by miRZip-155 led to a reduced $EpCAM^+$ cell population in both HuH7 and HuH1 cells (Fig 5B, HuH7; Supplementary Fig 8A, HuH1). We have previously found that about 1–2% of HuH7 or HuH1 cells could form spheroids, a cell self-renewal feature (3, 5). Consistently, miRZip-155 was able to inhibit spheroid formation of HCC cells (Fig 5C, HuH7; Supplementary Fig 8B, HuH1). In addition, miRZip-155 was able to suppress colony formation, cell migration and invasion in HCC cells (Fig 5D, HuH7; Supplementary Fig 8C–E, HuH1). All these findings demonstrated that HCC cells with suppressed miR-155 had reduced HCC malignant features.

Since miR-155 may have multiple cellular targets, we searched its potential targets that are associated with the $EpCAM^+AFP^+$ HCC subtype using TargetScan and MiRDB. This analysis revealed a total of 789 predicted targets. Class comparisons revealed that 780 genes were significantly down-regulated in $EpCAM^+AFP^+$ HCC subtype compared to $EpCAM^-AFP^-$ HCC subtype. Venn-diagram analysis revealed 27 overlapping genes between predicted targets and differentially expressed genes (Fig 6A, Supplementary Table 2). Among them, CEBPB, SMAD1 and MYLK have been reported as miR-155 targets (42–44). We determined if miR-155 can regulate CEBPB in HCC cells due to the abundant level of CEBPB, and found that miRZip-155 could induce CEBPB expression in HuH7 and HuH1 cells (Fig 6B). To further determine if miR-155 may be functionally linked to the features of

EpCAM$^+$AFP$^+$ HCC subtype, we performed hierarchical clustering of 27 genes. We found that these genes could separate HCC into two main subgroups (Cohort 1) with one group having an overall low level of these 27 genes (Fig 6C). Furthermore, HCC cases with lower levels of these genes had a worse overall survival and a shortened time to recurrence (Fig 6D). In addition, the association between low levels of these genes with poor HCC prognosis has been further validated in Cohort 2 (Fig 6E–F). As a control, the randomly selected 27 genes from 780 genes with down-regulation in EpCAM$^+$AFP$^+$ HCC had no significant association with either overall survival or time to recurrence in HCC cases (Supplementary Fig 9). Taken together, these results suggested that the identified 27 potential miR-155 targets were likely a part of a miR-155 signaling pathway that was fundamentally associated with malignant features of hepatic CSCs.

Discussion

Cancer stem cells have been identified in many tumor types, including HCC, the fifth most common and second most deadly malignancy with observable heterogeneity (1). Numerous studies have shown that hepatic CSCs could be enriched via different cell surface markers including EpCAM (1, 5). Functional characterizations of hepatic CSCs have revealed several deregulated signaling pathways, such as Wnt/beta-catenin, AKT, TGF-beta pathways to be critical at inducing “stemness” of HCCs and in promoting self-renewal. Noticeably, these signaling pathways were also important in normal hepatic stem cells. Following the identification and characterization of hepatic CSCs, more and more studies have demonstrated that hepatic CSCs are responsible for metastasis and recurrence after HCC resection due to their ability to give rise to a new tumor at a local or distant site. The development of strategies specifically targeting hepatic CSCs may provide new methods that could be used to improve HCC patients’ survival. However, since the similarity of cancer and normal stem cells, one of the most therapeutically important challenges is a selective targeting and eradication of CSCs without sacrificing normal stem cells. There is a central need to understand the biological difference of hepatic CSCs and normal stem cells. The unique changes in hepatic CSCs compared to HCC cells with differentiated hepatocyte features as well as to normal hepatic stem cells and hepatocytes hold the hope as the potent therapeutic target to specifically eliminate hepatic CSCs.

We have previously found that EpCAM is a biomarker for both normal hepatic stem cells and hepatic CSCs. Here, we focus on the difference between EpCAM$^+$ hepatic CSCs and EpCAM$^+$ normal hepatic stem cells. Small RNA next-generation sequencing was used to globally and unbiasedly detect and quantify miRNAs. Our study compared the stemness-related miRNAs in EpCAM$^+$ HCC cells vs. EpCAM$^+$ hepatic normal stem cells. We have found the difference of cancer and normal hepatic stem cells according to their miRNA expression profiling, and that a small group of miRNAs were remarkably altered in EpCAM$^+$ HCC cells compared to EpCAM$^-$ HCC cells, but not in normal stem cells compared to hepatocytes. EpCAM$^-$ HCC cells have been showed to have less malignancy features but mature hepatocyte features (3, 5). Meanwhile, the gene surrogates of these EpCAM$^+$ HCC cells specific miRNAs were significantly associated with clinical outcome of HCC patients. The presence of these miRNAs suggests the biological distinctions between

CSCs and normal stem cells, and the possibility of specifically targeting hepatic CSCs without sacrificing hepatic normal stem cells.

In the current study, we found that miR-155 is a miRNA highly related to $EpCAM^+$ hepatic CSCs. It was highly expressed in $EpCAM^+$ HCC cells compared to $EpCAM^-$ HCC cells, normal hepatic stem cells, hepatocytes, as well as normal fetal and adult livers. Functionally, silencing miR-155 resulted in altered $EpCAM^+$ cell populations, and a reduced spheroid formation, colony formation, migration and invasion. The predicted miR-155 target genes, that were associated with $EpCAM^+AFP^+$ HCC subtype, could predict HCC survival in two independent cohorts. Thus miR-155 may be a novel molecular target for a stem-like HCC subtype. Further investigations should allow us to develop novel therapies to specifically eliminate hepatic CSCs without sacrificing normal stem cells to avoid unwanted side effect. Interestingly, miR-155 has been reported due to its potential role as a therapeutic target in B-cell lymphoma (45).

The strength of our study is that we have used a sensitive small RNA deep sequencing method to identify miRNAs specific to hepatic CSCs. Our study design included the use of freshly isolated primary HCC cells to avoid potential artifacts derived from cultured cell lines. The limitation has been that we were able to analyze only two HCC donors due to the difficulties in obtaining fresh primary HCC tumor specimens for cell sorting of live $EpCAM^+$ and $EpCAM^-$ cells. To allow our data being more representative, we have used the stringent criteria to screen the most significant candidate miRNAs (Fig 2B–C). To compensate and to strengthen our findings, we have utilized the available miRNA microarray data from a large cohort of HCC specimens to validate our results, and have used two HCC cohorts with a total of 335 HCC cases to examine whether the functional gene surrogates of these candidate miRNAs were associated the stem cell-related features. To further address the concern on the representation of our results from only two sequenced HCC samples, we have also investigated the function of miR-150 (another candidate miRNA) in regulating HCC malignancies. As shown in Supplementary Fig 10, suppressing miR-150 consistently led to a reduced $EpCAM^+$ proportion in both HuH1 and HuH7 cells, and a decreased spheroid formation, colony formation and cell migration. However, the two sequenced samples and most of samples in two HCC cohorts were hepatitis B or C virus-related patients. It remains unknown whether our data are also significant in other etiology-related HCCs, such as alcoholic and nonalcoholic steatohepatitis -related HCC. In addition, many biomarkers besides EpCAM have been used to isolate the enriched hepatic CSC population and it remains unknown of the hierarchical relationship of these CSCs (1). We are planning to perform more work to test the association of miR-155 with these different hepatic CSCs from patients with various etiologies, as well as explore whether miR-155 can serve as an effective therapeutic target in eliminating CSCs in HCC.

In summary, we globally compared the miRNA transcriptome between $EpCAM^+$ and $EpCAM^-$ cells from HCC specimens and normal livers by a small-RNA deep sequencing technology. We found that a small group of miRNAs were uniquely altered in $EpCAM^+$ HCC cells. In addition, we validated the specific high level of miR-155 in $EpCAM^+$ HCC cells, and found that targeting miR-155 could significantly suppress malignant features of HCC cells. Our results indicate that miR-155 may serve as an effective therapeutic target to

specifically eliminate EpCAM$^+$ CSCs thereby preventing metastasis and tumor relapse in HCC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: This work was supported by the intramural research program of the center for cancer research, the U.S. National Cancer Institute (Z01-BC 010313 and Z01-BC010876). Collaborators at the UNC School of Medicine (Chapel Hill, NC) funded by an SRA from Vesta Therapeutics (Bethesda, MD), by an NIH grant (R21CA182322), and with core services support via several center grants: NIH (P30DK34987); NIDDK (DK34987); and a Cancer Center grant (CA016086).

List of Abbreviations

CSC	cancer stem cell
EpCAM	epithelial cell adhesion molecular
(qRT-PCR)	quantitative reverse transcription polymerase chain reaction
HpSC	hepatic stem cell
HB	hepatoblast
HCC	hepatocellular carcinoma
miRNA	microRNAs
LCI	Liver Cancer Institute

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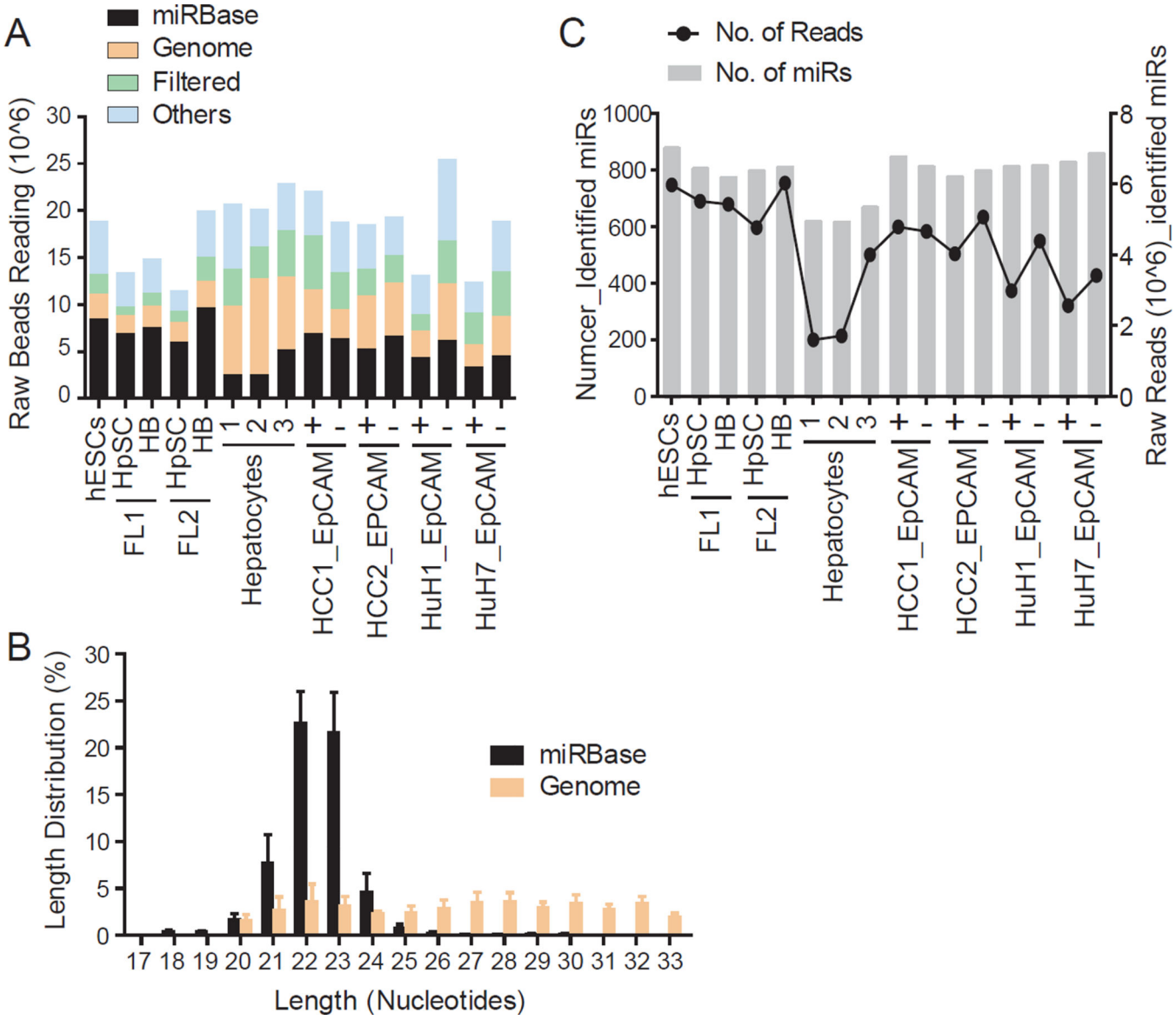

Figure 1. The raw sequencing data of small RNA transcriptome in 16 samples

(A) Histogram of all the reads from each output file of 16 sequenced samples. FL refers to fetal liver. (B). Read length distribution of reads aligned to miRBase (Black) and reads aligned to Genome (Orange) in 16 sequenced samples. The results are shown as mean ± standard deviation (SD). (C) The total number of identified miRNAs (grey bars) and the total raw reads number for identified miRNAs (Black dots) in each sample. An in-house pipeline was used to process the aligned reads. Details are shown in Materials and Methods.

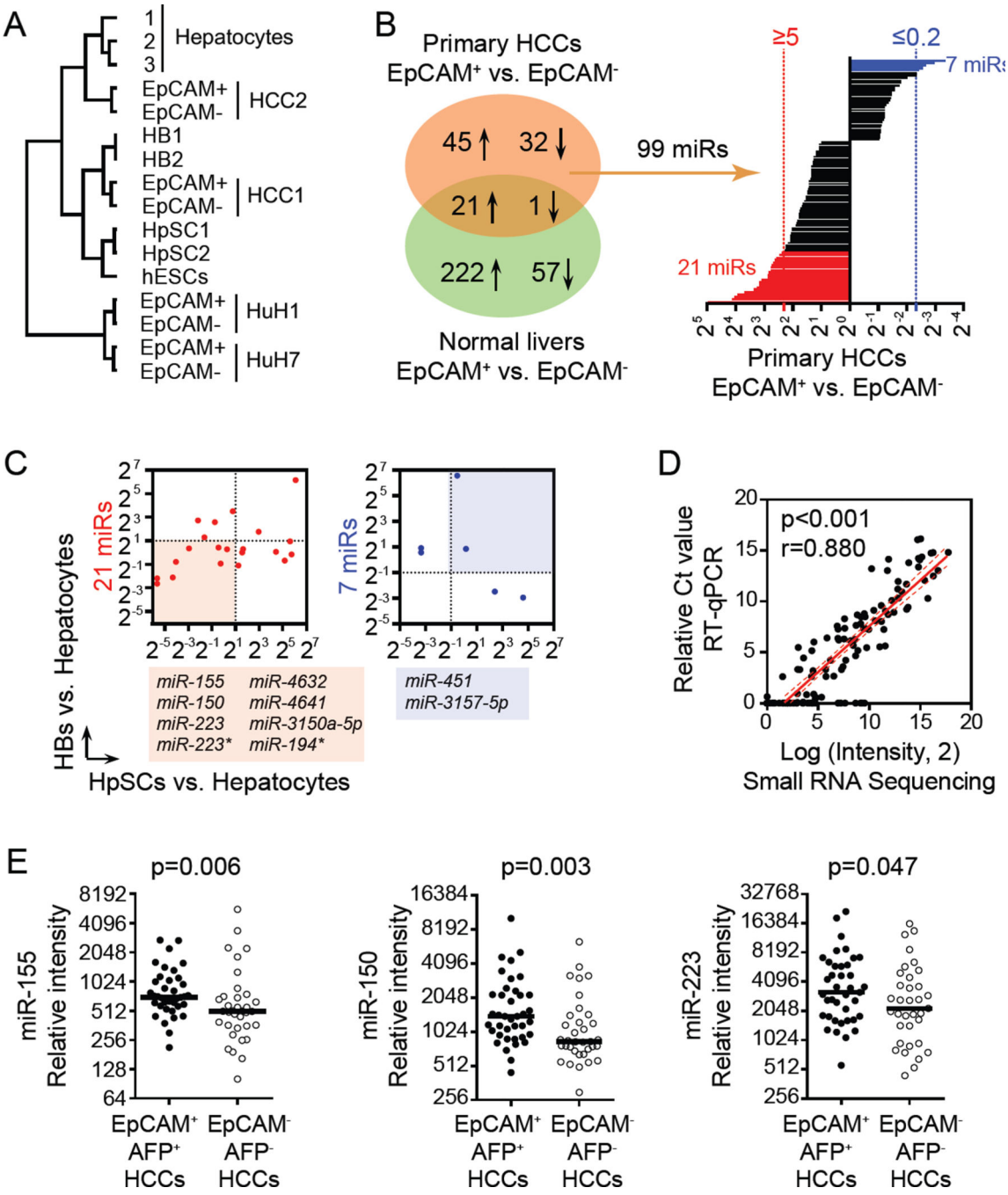

Figure 2. Aberrant high expression of three miRNAs in EpCAM$^+$ HCC cells

(A) Unsupervised clustering of 16 sequenced samples based on the expression of 600 miRNAs with relative high abundance. (B) Venn-diagram of miRNAs with ≥ 2-fold alteration in the comparison of EpCAM$^+$ and EpCAM$^-$ HCC cells and miRNAs from the comparison of EpCAM$^+$ and EpCAM$^-$ normal liver cells. The right panel shows the bar graph of 99 miRNAs with ≥ 2-fold changes in the comparison of EpCAM$^+$ and EpCAM$^-$ HCC cells. Red bars: n=21, ≥5-fold of EpCAM$^+$ HCC cells vs. EpCAM$^+$ HCC cells. Blue bars: n=7, ≤0.2-fold of EpCAM$^+$ HCC cells vs. EpCAM$^-$ HCC cells. (C) Scatter plot of 21

miRNAs (left channel, red spots) and 7 miRNAs (right channel, blue spots) from the right panel of Fig 2B to display their expression in $EpCAM^+$ hepatic stem cells and hepatoblasts compared to $EpCAM^-$ hepatocytes. The red spots shadowed in red refer to miRNAs with ≥ 5-fold of $EpCAM^+$ HCC cells vs. $EpCAM^-$ HCC cells and ≤ 2-fold of $EpCAM^+$ hepatic progenitors vs. $EpCAM^-$ hepatocytes. The blue spots shadowed in blue refer to miRNAs with ≤ 0.2-fold of $EpCAM^+$ HCC cells vs. $EpCAM^-$ HCC cells and ≥ 0.5-fold of $EpCAM^+$ hepatic progenitors vs. $EpCAM^-$ hepatocytes. (D) Pearson correlation analysis of $EpCAM^+$ HCC cells-related miRNAs expression data (log2) from small RNA sequencing and from qRT-PCR validation in all 16 samples. The $EpCAM^+$ HCC cells - related miRNAs were listed in (C). (E) miRNA array data for three miRNAs in extreme $EpCAM^+AFP^+$-HCCs and $EpCAM^-AFP^-$-HCCs. Y-axis refers to the log2 intensity. Student t-test was performed.

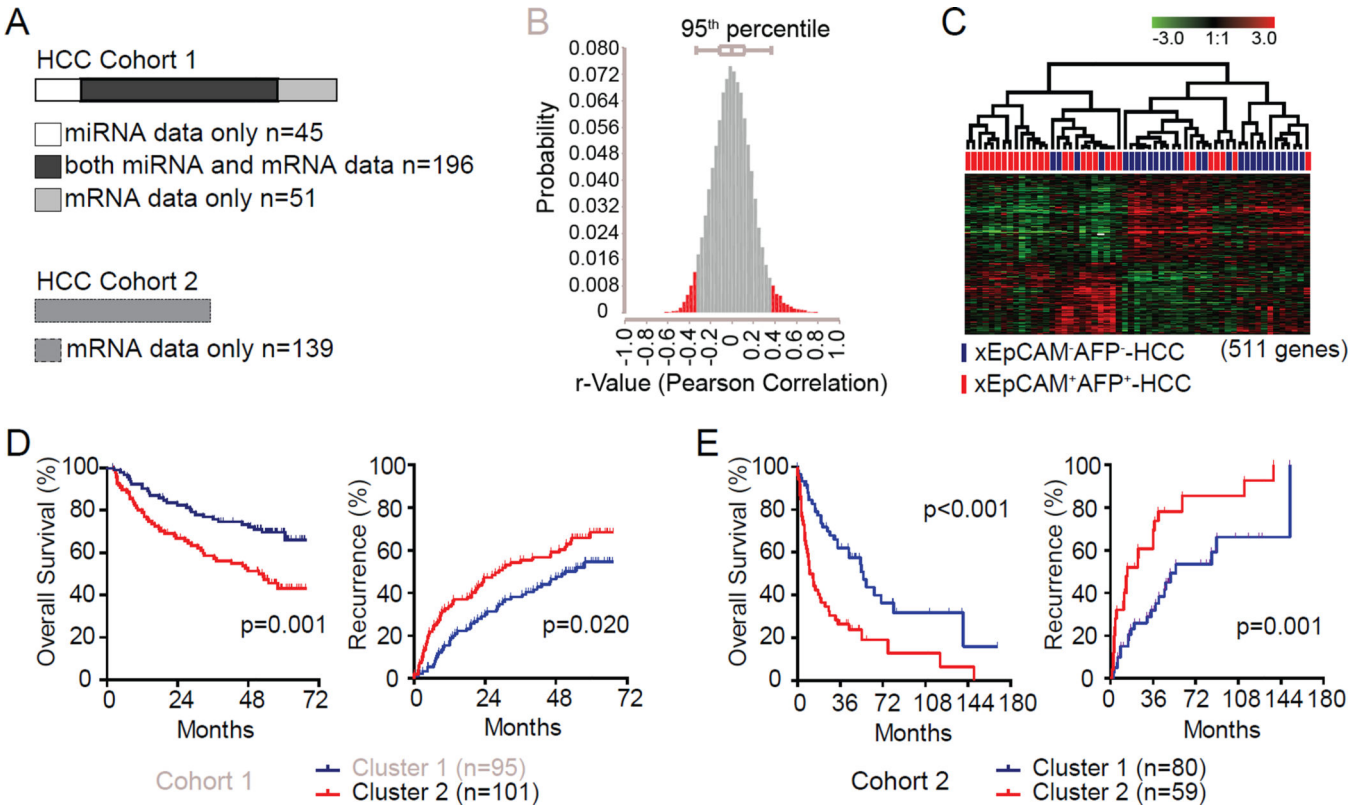

Figure 3. Gene surrogates of EpCAM$^+$ HCC cells-related miRNAs were associated with HCC prognosis

(A) We have used two HCC cohorts, i.e., HCC cohort 1 (n= 292) and HCC cohort 2 (n=139). In cohort 1, 196 HCC cases had the available miRNA and mRNA array data, while 45 patients have miRNA array data only and 51 patients have mRNA array data only. All patients in HCC cohort 2 have mRNA array data only. (B) Pearson correlation was performed between three EpCAM$^+$ HCC cells-miRNAs and ~13,000 genes in 196 HCC cases with available miRNA and mRNA array from cohort 1. 511 gene surrogates of EpCAM$^+$ HCC cells-related miRNAs were identified with the criteria described in Materials and Methods. (C) Unsupervised hierarchical clustering based on these gene surrogates for extreme EpCAM$^+$AFP$^+$ and EpCAM$^-$AFP$^-$HCCs with both miRNA and mRNA expression data (n=57). (D) Kaplan-Meier analysis of overall survival and recurrence in HCC cases from Cohort 1 (n=196, cases used for detecting gene surrogates) based on the classification of cluster 1 and cluster 2 by 511 genes. (E) Kaplan-Meier analysis of overall survival and recurrence in HCC cases from cohort 2 (n=139) based on the classification of cluster 1 and cluster 2 by 511 genes. Log-rank test was performed.

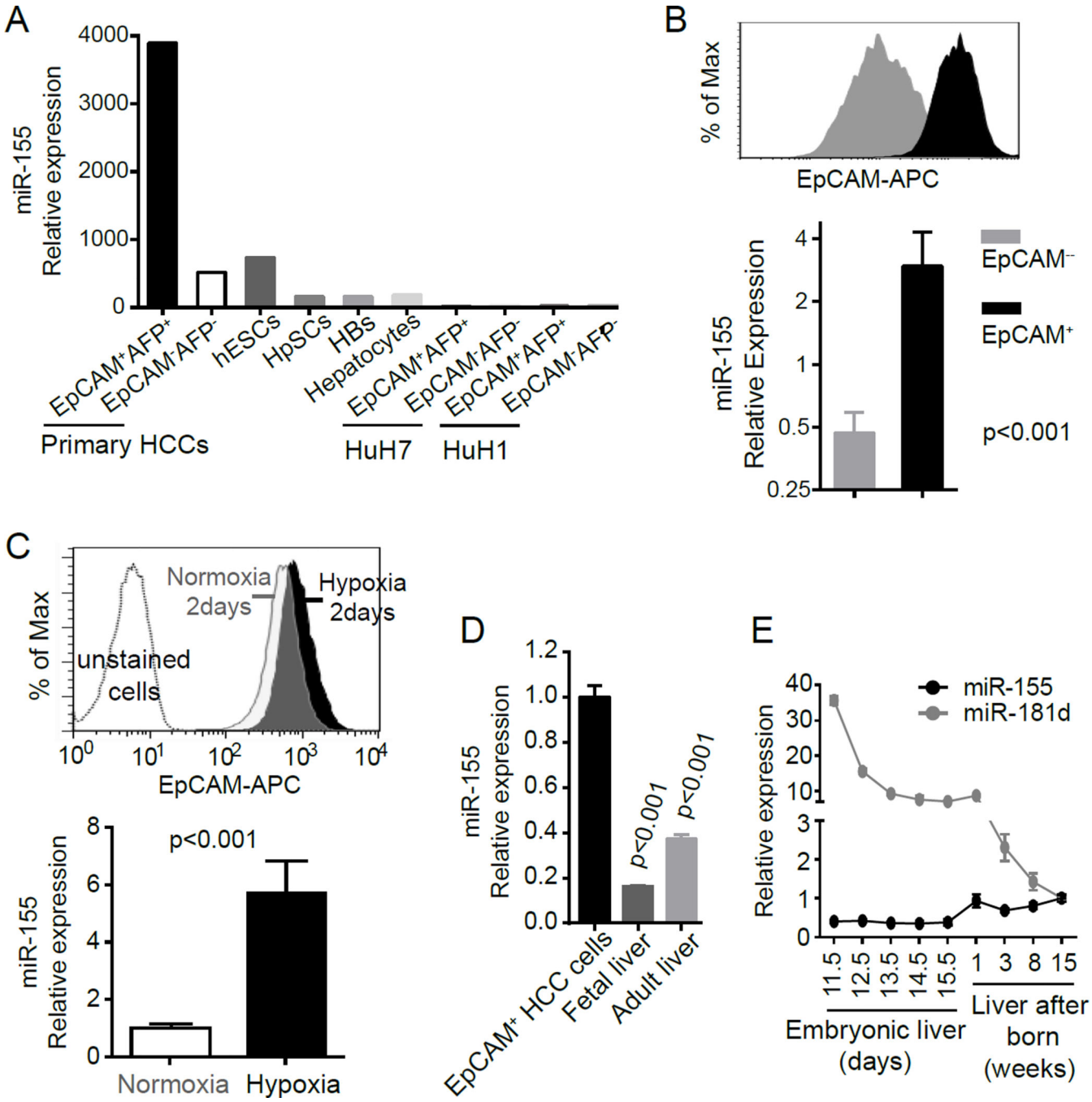

Figure 4. miR-155 was specifically highly expressed in EpCAM$^+$ HCC cells

(A) The sequencing data of miR-155 in EpCAM$^+$ and EpCAM$^-$ cells from HCC primary samples and normal livers. (B) qRT-PCR data of miR-155 in enriched EpCAM$^+$ and EpCAM$^-$ cells from HuH7 HCC cell line. EpCAM$^+$ cells were enriched via KnockOut medium/Serum Replacement culture and cells under regular media were used as control. EpCAM$^-$ cells were enriched via EpCAM silencing technology and control siRNA was used as controls. MiR-155 expression in these cells was compared to the corresponding controls. The top channel is the FACS data showing the enriched EpCAM$^+$ and EpCAM$^-$ population.

APC-conjugated EpCAM antibody was used. (C) $EpCAM^+$ cell distribution (upper channel, FACS) and miR-155 expression (lower channel, qRT-PCR) in HuH7 cells under normoxia and hypoxia condition for two days. For FACS analysis, APC-conjugated EpCAM antibody was used. (D) qRT-PCR data of miR-155 in human fetal livers and normal livers. $EpCAM^+$ HCC cells enriched from KnockOut medium/Serun Replacement culture were used as a control. (E) miR-155 expression during the development of mouse liver from embryonic stage to adult stage. MiR-181d was also examined as a positive control. (B,C,D) Student t-test was performed.

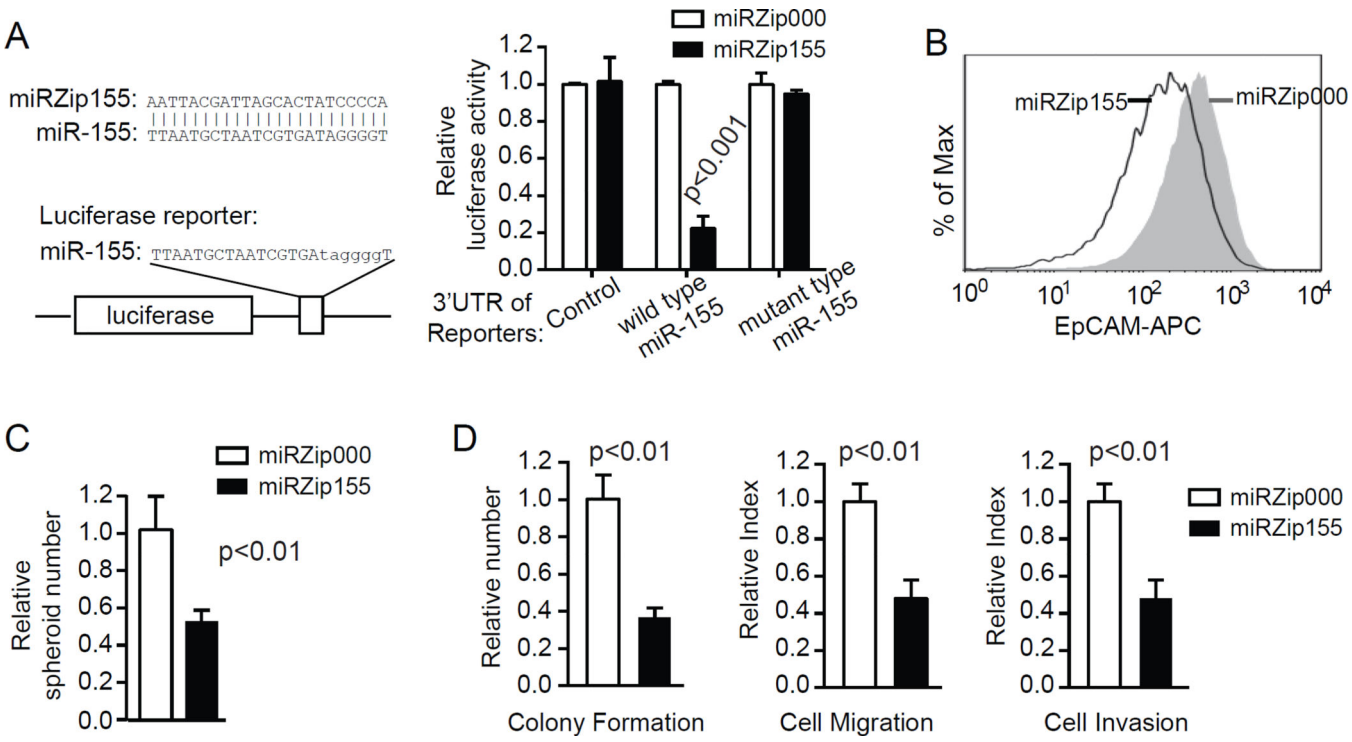

Figure 5. Suppressing miR-155 reduced HCC malignancy features *in vitro*

(A) The duplex between anti-miR-155 expressed from miRZip-155 and miR-155, and the reporter plasmid for miRZip-155 with mature miR-155 in the 3'UTR region of luciferase (left channel). The lower cases in mature miR-155 were mutated to ATCCCC in reporter plasmid with mutant miR-155. Luciferase activities of reporter plasmids with wild type and mutant miR-155 in 3'UTR region was measured in HuH7 cells co-transfected with miRZip-000 or miRZip-155 (right channel). The luciferase activity was shown as the mean ± SD. (B) FACS analysis of HuH7 infected with miRZip-155 and control virus for 2 days under hypoxia condition. APC-conjugated EpCAM antibody was used. (C) HuH7 cells infected with miRZip-155 and control virus for 2 days under hypoxia condition were seeded in ultra-low attachment plates to assay spheroid formation. (D) Assays on colony formation, migration and invasion were performed in HuH7 infected with miRZip-155 and control virus for 2 days under hypoxia condition. (A,C,D) Student t-test was performed.

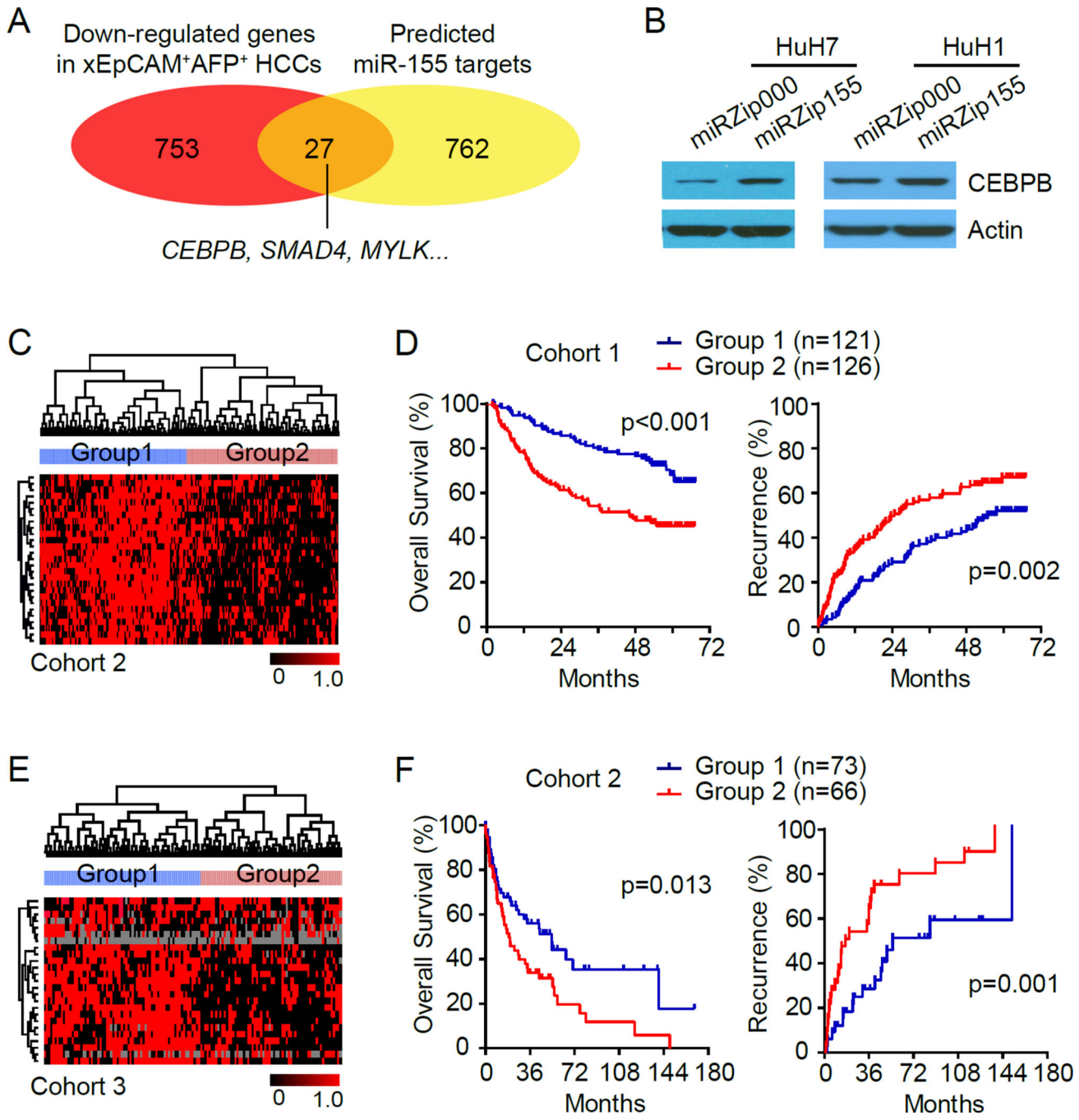

Figure 6. The reduced level of miR-155 potential targets was associated with poor overall survival and short time to recurrence in HCC patients

(A) Venn-diagram of down-regulated genes in extreme EpCAM$^+$ HCC cases and predicted miR-155 targets. 27 genes were overlapped, in which three genes were reported as miR-155 targets. (B) The expression levels of CEBPB and Actin in HuH7 and HuH1 cells infected with miRZip-155, as determined by Western blotting. (C) Hierarchical clustering of 27 genes in cohort 1 (n=247) predicted two HCC subgroups, i.e., group 1 (n=121) and group 2 (n=126). The reduction of these genes was noticed in group 2. (D) Kaplan-Meier analysis of overall survival and recurrence in HCC cases from cohort 1 based on the classification of

group 1 and group 2 by 27 genes. (E) Hierarchical clustering of 27 genes in cohort 2 (n=139) could predict two HCC subgroups, i.e., group 1 (n=73) and group 2 (n=66). The reduction of these genes was noticed in group 2. (F) Kaplan-Meier analysis of overall survival and recurrence in HCC cases from cohort 2 (n=139) based on the classification of group 1 and group 2 by 27 genes. Log-rank test was performed.

Table 1

Clinical characteristics of 16 samples used for small RNA sequencing

No.	Cells for Sequencing[#]	Samples		Age	Sex	Virus[*]	AFP
		Resource	Histology				
#1–2	EpCAM$^+$/EpCAM$^-$ cells	HCC Patient 1	HCC	75 (y)	F	HCV	+
#3–4	EpCAM$^+$/EpCAM$^-$ cells	HCC Patient 2	HCC	79 (y)	F	HBV	+
#5–6	EpCAM$^+$/EpCAM$^-$ cells	HuH1	HCC	53 (y)	M	HBV	+
#7–8	EpCAM$^+$/EpCAM$^-$ cells	HuH7	HCC	57 (y)	M	−	+
#9–10	HpSCs/HBs	Fetal liver 1	Fetal liver	E17 (w)	/	−	+
#11–12	HpSCs/HBs	Fetal liver 2	Fetal liver	E17 (w)	/	−	+
#13–15	Hepatocytes	Normal liver 1–3	Hepatocyte	/	/	−	−
#16	hESCs	H1/H9	hESC	/	/	/	/

[#]: EpCAM$^+$ HCC cells and EpCAM$^-$ HCC cells were isolated by FACS from primary AFP$^+$ HCC tissues and cultured AFP$^+$ HCC cell lines. HpSCs (hepatic stem cells, EpCAM$^+$ cells) and HBs (hepatoblasts, EpCAM$^+$ AFP$^+$ cells) were isolated by FACS from fetal livers.

[*]: Virus refers to either HCV or HBV.