miR-579-3p Controls Hepatocellular Carcinoma Formation by Regulating the Phosphoinositide 3-Kinase–Protein Kinase B Pathway in Chronically Inflamed Liver

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Chronic liver inflammation causes continuous liver damage with progressive liver fibrosis and cirrhosis, which may eventually lead to hepatocellular carcinoma (HCC). Whereas the 10-year incidence for HCC in patients with cirrhosis is approximately 20%, many of these patients remain tumor free for their entire lives. Clarifying the mechanisms that define the various outcomes of chronic liver inflammation is a key aspect in HCC research. In addition to a wide variety of contributing factors, microRNAs (miRNAs) have also been shown to be engaged in promoting liver cancer. Therefore, we wanted to characterize miRNAs that are involved in the development of HCC, and we designed a longitudinal study with formalin-fixed and paraffin-embedded liver biopsy samples from several pathology institutes from Switzerland. We examined the miRNA expression by nCounterNanostring technology in matched nontumoral liver tissue from patients developing HCC (n = 23) before and after HCC formation in the same patient. Patients with cirrhosis (n = 26) remaining tumor free within a similar time frame served as a control cohort. Comparison of the two cohorts revealed that liver tissue from patients developing HCC displayed a down-regulation of miR-579-3p as an early step in HCC development, which was further confirmed in a validation cohort. Correlation with messenger RNA expression profiles further revealed that miR-579-3p directly attenuated phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) expression and consequently protein kinase B (AKT) and phosphorylated AKT. *In vitro* experiments and the use of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology confirmed that miR-579-3p controlled cell proliferation and cell migration of liver cancer cell lines. *Conclusion:* Liver tissues from patients developing HCC revealed changes in miRNA expression. miR-579-3p was identified as a novel tumor suppressor regulating phosphoinositide 3-kinase–AKT signaling at the early stages of HCC development. (*Hepatology Communications* 2022;6:1467-1481).

Abbreviations: AKT, protein kinase B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRISPR, clustered regularly interspaced short palindromic repeats; FFPE, formalin fixed and paraffin-embedded; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miRNA/miR, microRNA; mRNA, messenger RNA; pAKT, phosphorylated protein kinase B; PI3K, phosphoinositide 3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; qPCR, quantitative polymerase chain reaction.

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Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide, and its incidence is growing at an alarming rate in the United States and Europe.[1-3] This cancer type is usually inflammation induced. Nearly 90% of patients with HCC have underlying chronic liver inflammation, which is mainly due to chronic hepatitis B (HBV) or C virus (HCV) infection, alcoholic steatohepatitis, or nonalcoholic steatohepatitis.[4,5] Chronic inflammation increases production of reactive oxygen and nitrogen species, which cause DNA damage, mutations, and epigenetic modifications, thereby raising the risk of malignant transformation.[6-8] In parallel, chronic inflammation promotes the onset of fibrosis and eventually leads to liver cirrhosis, which is regarded as a precancerous starting point of HCC.[8,9]

Nonetheless, not all patients with cirrhosis develop HCC. In fact, most patients remain tumor free for many years or even their entire lives. In Western countries, 10-year cumulative incidence rates to develop HCC for patients with cirrhosis with either chronic HCV, HBV, or alcohol-related liver disease are estimated at 21.1%, 23.2%, and 17.7%, respectively.[10] Clinical risk factors known to predispose patients with cirrhosis to HCC

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development include old age, male sex, low platelet count, and increased portal hypertension.[11] More recently, an increase in serum/plasma marker proteins (e.g., insulin-like growth factor 1) and single nucleotide polymorphisms (e.g., in transmembrane 6 superfamily 2 [*TM6SF2*] and patatin-like phospholipase domain containing 3 [*PNPLA3*]) were identified as molecular risk factors.[11,12] Latest advances in messenger RNA (mRNA) transcriptome profiling of nontumoral liver tissues further revealed associations between HCC development and the liver inflammatory microenvironment, with dysregulation of signaling pathways, including nuclear factor kappa B, interleukin-6–signal transducer and activator of transcription 3 (STAT3), and phosphoinositide 3-kinase (PI3K)–protein kinase B (AKT)–mammalian target of rapamycin, as well as abnormal production of cytokines and chemokines.[5,8,11] On this basis, the conception of a protumorigenic or antitumorigenic microenvironment has been established and a number of gene signatures have been defined, which allowed the estimation of HCC development risk in patients with chronic liver disease.[11] Similarly, changes in microRNA (miRNA/miR) levels in nontumoral liver tissue or serum (e.g., miR-122 and miR-21) have been associated with increased risk for HCC development.[13,14]

Growing evidence suggests that nontumoral liver tissue of patients with HCC is instrumental to elucidate the initial mechanisms of hepatocarcinogenesis.[15] However, most information comes from cross-sectional case-control studies that compare nontumoral liver tissue from patients with HCC to patients with cirrhosis without tumors or to nontumoral liver tissue from patients with HCC with early or late recurrence.[15] Missing in the field of HCC research are longitudinal studies that analyze the consecutive changes in the same patient from liver damage until HCC development.

We designed a longitudinal study using two cohorts of patients who either developed HCC or remained tumor free. miRNAs were extracted from formalin-fixed and paraffin-embedded (FFPE) nontumoral liver tissues obtained from two sequential biopsies from the same patient before and after HCC development. In addition, patients with cirrhosis remaining tumor free over a similar time frame served as controls. Patients who developed HCCs exhibited significant changes in miRNA expression. Furthermore, we identified a specific down-regulation of miR-579-3p and subsequent PI3K-AKT pathway activation in hepatocytes as an initial step in HCC development.

Patients and Methods

PATIENTS

We searched tissue archives for FFPE liver tissue samples from patients with HCC for a retrospective longitudinal study. FFPE nontumoral liver biopsy samples were collected at two points in time, once before HCC development (termed first biopsy) and several years later (at least 2 years) after HCC development (termed second biopsy) (Fig. 1A). Because these samples were rare, the study was performed in collaboration with six pathology institutes from Switzerland (University Hospital of Basel, University Hospital of Zürich, and the cantonal hospitals of Liestal, Locarno, Lucerne, and St. Gallen).

Patients developing HCC (Table 1; Supporting Tables S1, S5, and S6) were selected based on the following criteria: (1) if the absence of liver tumor at the time of the first biopsy was unambiguously confirmed by ultrasound, magnetic resonance imaging (MRI), or computed tomography (CT) scan (63.2% of cases); or (2) if no radiology was performed (36.8% of cases), the absence of liver tumor at the first biopsy was anticipated based on calculations using the assumption of a slow tumor growth rate,[16] the time difference between the first and second biopsy (mean ± SEM, 13.8 ± 6.6 years), and tumor size at the second biopsy (mean ± SEM, 3.4 ± 1.2 cm) for each patient individually; (3) to adjust for similar patient age between patients developing HCC and control patients (who were tumor free), patients developing HCC with age at first biopsy >66 years and at second biopsy >75 years were excluded; (4) all patients had either liver cirrhosis or severe fibrosis at the time of HCC presentation in the nontumoral liver tissue; (5) if the time difference between the first and second biopsy was at least 2 years.

Because 93% of patients with HCC were diagnosed with liver cirrhosis and 7% with severe fibrosis at the time of HCC presentation, we searched for FFPE liver biopsy samples from patients with cirrhosis who

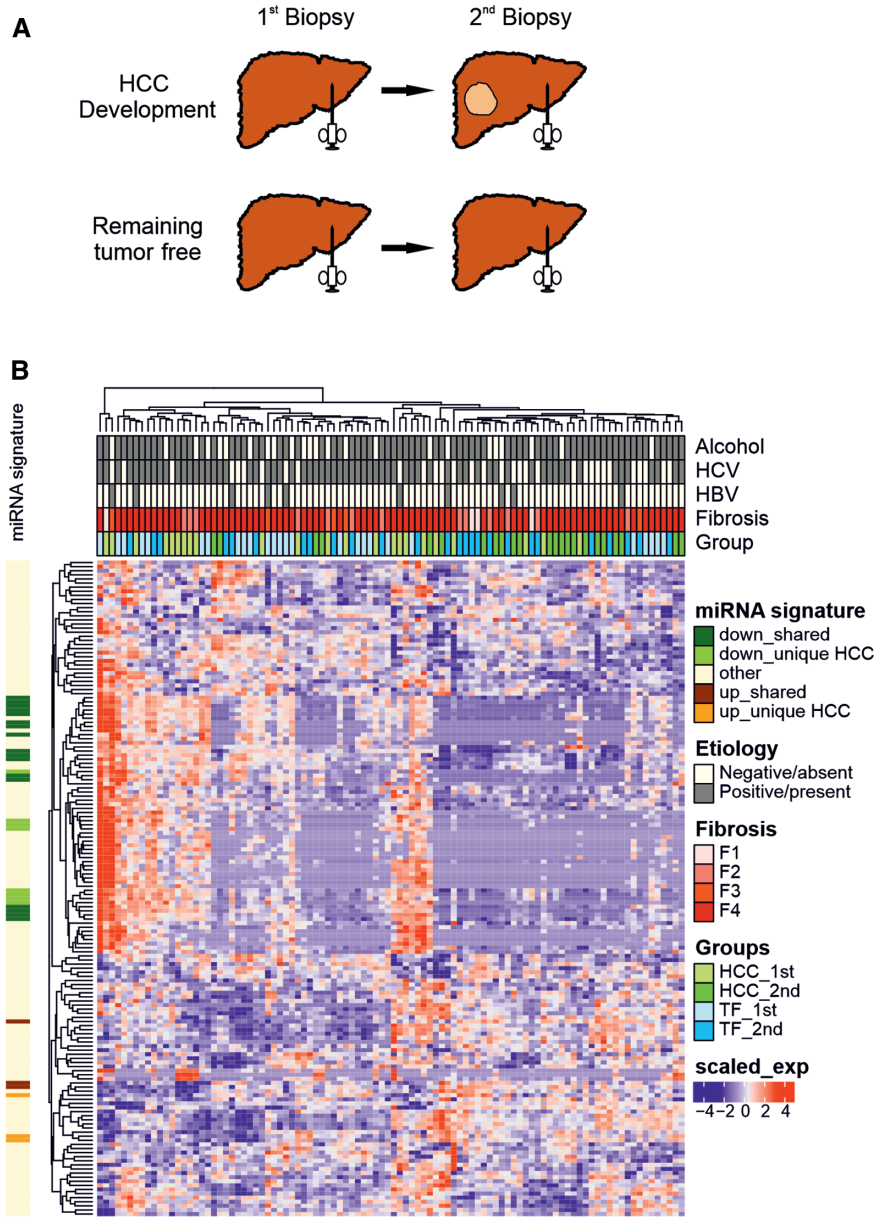


FIG. 1. Unsupervised hierarchical clustering and association of miRNAs changing significantly in patients developing HCC. (A) Scheme of longitudinal study. A patient cohort that developed HCC (n = 23) and one that remained tumor free (n = 26) were subjected to two consecutive fine-needle biopsies. The first biopsy was performed when no HCC was present in the liver. The second biopsy was taken from nontumoral liver at the time of diagnosis with HCC (HCC-developing cohort) or without HCC (cirrhosis cohort). (B) Unsupervised hierarchical clustering of HCC-developing cohort and cirrhosis cohort based on the top 160 expressed miRNA measured by the nCounterNanostring miRNA Expression panel. To the left of the heatmap, miRNAs that were significantly differentially expressed between the first and second biopsies are indicated (miRNA signature; also see Supporting Fig. S1A,B). Abbreviations: exp, expression; TF, tumor free.

were tumor free with sequential liver biopsies over a similar time line, which was once from a previous liver biopsy (first biopsy) and once at the time of cirrhosis diagnosis (second biopsy) (Fig. 1A). Patients with cirrhosis were selected (Table 1; Supporting Tables S1, S5, and S6) if (1) the absence of liver tumor at the time of the second biopsy was unambiguously confirmed by ultrasound, MRI, or CT scan; (2) patient age at the first biopsy was ≥35 years and at the second biopsy ≥48 years to adjust for similar age between

TABLE 1. CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF PATIENTS DEVELOPING HCC AND REMAINING TUMOR FREE

		HCC-Developing Cohort (n = 30)	Cirrhosis Cohort (n = 27)	*P* Value
Age at first biopsy	Mean (SEM), years	51.5 (1.5)	49.4 (1.5)	0.354
Age at second biopsy	Mean (SEM) years	61.0 (1.4)	57.4 (1.4)	0.0723
Time between first and second biopsy	Mean (SEM), years	9.5 (0.9)	7.8 (0.6)	0.144
Sex	Male	18 (60%)	22 (81%)	0.077
	Female	12 (40%)	5 (19%)	
	HCV	7 (23%)	5 (19%)	
	ASH	6 (20%)	7 (26%)	
	HCV+ASH	7 (23%)	12 (44%)	
	HCV+ASH/NASH	1 (3%)	1 (3%)	
	HBV/HBV+ASH	1 (3%)/3 (10%)	0/0	
	HBV+HCV	0	1 (3%)	
	HBV+HCV+ASH	1 (3%)	0	
	PBC/PBC+ASH	1 (3%)/1 (3%)	0	
	ASH/NASH	2 (7%)	1 (4%)	
Fibrosis at first biopsy	F0-F2	6 (20%)	9 (33%)	0.25
	F3-F4	24 (80%)	18 (67%)	
Fibrosis at second biopsy	F0-F2	0	0	0.94
	F3-F4	30 (100%)	27 (100%)	
ALT, first biopsy	Mean (SEM), IU/mL	68.4 (12.8; n = 20)	139.0 (19.0; n = 16)	0.003*
ALT, second biopsy	Mean (SEM), IU/mL	75.4 (12.8; n = 26)	73.8 (12.8; n = 24)	0.929
AST, first biopsy	Mean (SEM), IU/mL	91.4 (24.7 n = 20)	124.9 (15.1; n = 17)	0.276
AST, second biopsy	Mean (SEM), IU/mL	99.3 (17.2; n = 26)	76.7 (9.6; n = 24)	0.267
Child-Pugh score, first biopsy	Mean (SEM)	7.4 (0.9; n = 11)	5.6 (0.4; n = 12)	0.088
Child-Pugh score, second biopsy	Mean (SEM)	7.3 (0.4; n = 26)	5.7 (0.3; n = 18)	0.005*
BMI, first biopsy	Mean (SEM)	28.1 (1.7; n = 17)	25.9 (1.6; n = 11)	0.38
BMI, second biopsy	Mean (SEM)	26.7 (1.4; n = 25)	25.1 (1.4; n = 17)	0.481
Albumin, first biopsy	Mean (SEM)	35.6 (1.7; n = 20)	37.1 (1.0; n = 14)	0.502
Albmin, second biopsy	Mean (SEM)	31.3 (1.4; n = 26)	33.9 (1.4; n = 20)	0.198
Bilirubin first biopsy	Mean (SEM)	32.5 (10.9; n = 18)	24.1 (8.0; n = 14)	0.584
Bilirubin, second biopsy	Mean (SEM)	30.9 (7.6; n = 26)	24.25 (7.1; n = 20)	0.536

*Statistically significant.
Abbreviations: ASH, alcoholic steatohepatitis; BMI, body mass index; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cirrhosis.

both cohorts; (3) the time difference between the first and second biopsy was at least 2 years.

Collection and analysis of patient samples was approved by the Ethics Committee of Northern Switzerland (Study ID: 310/12) and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

NCOUNTER NANOSTRING ARRAY

nCounterNanostring miRNA Expression panel (version 2.1) and nCounterNanostringPanCancer Pathways panel (nCounterNanoString Technologies, Seattle, WA) were used for analysis of miRNA and mRNA gene expression, respectively. We used 100 ng total RNA, isolated using the AllPrep DNA/RNA FFPE extraction kit (Qiagen, Hilden, Germany), per sample and processed according to the manufacturer's instructions. The first and second biopsies of the same patient were always run on the same plate. nCounter-Nanostring miRNA Expression panels were run on the Institute of Genetics and Genomics of Geneva (IGE3) genomics platform or the Center for Cancer Research

Genomics Core Facility at the National Institutes of Health (Bethesda, MD). nCounterNanostringPanCancer Pathways panels were run on the IGE3 genomics platform (University of Geneva, Switzerland). Data were normalized and processed using the nSolver 3.0 software according to the manufacturer's instructions. No background subtraction was performed for normalization, and samples were excluded according to quality requirements given in the manufacturer's instructions. For miRNA normalization, the top 50 most expressed miRNAs were used for normalization and for the mRNA analysis housekeeping genes armadillo like helical domain containing 3 (*C10orf76*), protein inhibitor of activated STAT1 (*PIAS1*), DnaJ heat shock protein family (Hsp40) member C14 (*DNAJC14*), tetratricopeptide repeat domain 31 (*TTC31*), splicing factor 3a subunit 3 (*SFSA3*), and ERCC excision repair 3, transcription factor IIH core complex helicase subunit (*ERCC3*). Nanostring probe miR-579 corresponds to human hsa-miR-579-3p as reported in a later array version. Data analysis was performed using Qlucore software (Lund, Sweden). For miRNA analysis, a threshold of 64 was set and all values were log transformed. Data were always analyzed paired between first and second biopsy of the same patient, using patient identification as an eliminating factor for batch-effect removal. Pathways analysis was performed using Ingenuity Pathway Analysis (Qiagen). Cluster analysis was performed using R, and cluster stability was assessed using pvclust,[17] an R package for assessing the uncertainty in hierarchical clustering. Data were submitted to the Gene Expression Omnibus, National Center for Biotechnology Information and are available under GSE116045.

STATISTICAL ANALYSIS

Statistical tests were used as indicated. If not stated differently, $P < 0.05$ was considered significant. Analyses were performed using GraphPad PRISM 6 (GraphPad Software, La Jolla, CA).

Results

PATIENT COHORT

For a retrospective longitudinal study, we collected FFPE nontumoral liver biopsy samples from patients with HCC (n = 30) at two consecutive time points, before HCC development (first biopsy) and after HCC development (second biopsy) (Fig. 1A; additional information in the Patients and Methods section). All first biopsies were fine-needle biopsy samples; 83% of second biopsies were also fine-needle biopsy samples, which were taken separately at a distance of at least 2 cm from the tumor margin. In the remaining 17% of patients, the second biopsy was obtained from a fine-needle biopsy or a resection specimen at 1-2 cm adjacent to the tumor margin. At the time of HCC diagnosis, 93% of patients were diagnosed with liver cirrhosis (F4 by METAVIR scoring[18]) and 7% with severe liver fibrosis (F3). Therefore, we established a control cohort (n = 27), termed cirrhosis cohort, that included fine-needle liver biopsies from patients with cirrhosis proven to be tumor free by radiology examination. Likewise, these patients were subjected to two sequential liver biopsies (Fig. 1A).

To minimize confounding factors, the mean patient age at the first biopsy and second biopsy (51.5 vs. 49.4 years and 61.0 vs. 57.4 years, respectively) as well as time difference between the first and second biopsy (9.5 vs. 7.8 years, respectively) were similar in both cohorts (Table 1) with no statistical significance ($P > 0.05$). All patients suffered from chronic liver disease with similar etiologies, mainly abusive ethanol consumption, chronic HCV infection, or a combination of both. At the first biopsy, 80% of patients developing HCC and 67% of patients remaining tumor free had either severe fibrosis or cirrhosis (F4). At the second biopsy, 93% of patients with HCC and all control patients showed liver cirrhosis (F4). Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and bilirubin as well as Child-Pugh scores were similar in both cohorts, although ALT levels were higher in the cirrhosis cohort at the first biopsy and the Child-Pugh score was higher in the HCC-developing cohort at the second biopsy ($P = 0.003$ and 0.005, respectively). In the HCC-developing cohort, 23 of 30 patients did not eliminate the attributed causal agent between the first and second biopsy, e.g., were nonresponders to HCV treatment (none of the patients received direct-acting antivirals). Two of the remaining patients credibly stopped abusing alcohol, 1 responded to anti-HCV therapy in combination with stopping abuse of alcohol, and it was unknown for 4 whether the causal agent was eliminated. Similarly, in the cirrhosis

cohort, 24 of 27 patients did not eliminate the causal agent between the first and second biopsy, 1 patient responded to anti-HCV therapy and stopped alcohol consumption, and it was unknown for 2 patients. Thus, clinical and pathological assessments were similar in both cohorts at the first and second biopsy, raising the question why only some patients developed HCC.

SIGNIFICANT CHANGE OF MIRNAS IN PATIENTS DEVELOPING HCC

Changes in miRNA levels in nontumoral liver tissue have been associated with increased risk for HCC development. Therefore, total RNA from whole-liver sections was evaluated by the nCounterNanostring miRNA Expression panel containing 800 human miRNAs. After applying stringent quality tests, 23 patients from the HCC-developing cohort and 26 of the cirrhosis cohort were included for further analyses (Supporting Table S1). Unsupervised hierarchical clustering showed that the first biopsies in the cirrhosis cohort and the second biopsies of the HCC-developing cohort preferentially clustered at opposite ends of the dendrogram, despite the random distribution of samples in terms of etiology or fibrosis (Fig. 1B; Supporting Fig. S1A). However, it should be noted, that the top-level clusters were not stable (Supporting Fig. S1A). Differential expression analysis revealed 31 miRNAs that changed significantly ($P < 0.001$, paired t test) between the first and second biopsies in the HCC-developing cohort and 38 miRNAs in the cirrhosis cohort (Supporting Tables S2 and S3). Among these, 20 miRNAs changed in the same direction in both cohorts, whereas 11 were unique for the HCC-developing cohort and 18 were unique for the cirrhosis cohort (Supporting Fig. S1B). Because each miRNA may control expression of numerous target mRNAs, we wanted to minimize the number of miRNAs potentially involved in hepatocarcinogenesis. For this, we determined which of the 11 miRNAs unique for the HCC-developing cohort showed an interindividual difference that was changing significantly from the cirrhosis cohort (two-sample t test, $P < 0.05$; Supporting Table S4). Notably, miR-579-3p, miR-4531, miR-1322, miR-126-3p, and miR-520h fulfilled both criteria and showed significant changes from the first to the second biopsy (see exemplary miR-579-3p, Fig. 2A) as well as significant interindividual differences between the cohorts (Fig. 2B). Among these five miRNAs, miR-579-3p showed the most significant expression change and the highest fold change between the first and second biopsy (Supporting Table S2). Moreover, the expression of miR-579-3p was comparable in the first biopsies from the HCC-developing cohort and the cirrhosis cohort (Fig. 2A). Therefore, we focused on miR-579-3p for further analysis.

To confirm the significant change of miR-579-3p during HCC development, we established a validation cohort from patients developing HCC (n = 15) or remaining tumor free (n = 16). All patients displayed a comparable etiology for liver disease, fibrosis score, and clinical parameters (Supporting Table S5). For validation, miR-579-3p expression levels were measured by quantitative polymerase chain reaction (qPCR) instead of the nanostring technology. Again, miR-579-3p was significantly reduced from the first to the second biopsy in the HCC-developing cohort, whereas its levels did not change significantly in the cirrhosis cohort (Fig. 2C). Further, miR-579-3p showed a trend for an interindividual difference from the first to the second biopsy in patients with HCC compared to the cirrhosis cohort, although it did not reach statistical significance (most likely due to a small sample size) (Fig. 2D). Together, these data indicate that down-regulation of miR-579-3p is involved in HCC development.

Because miRNAs were isolated from whole-liver tissue sections composed mainly of hepatocytes (60%-70%) but also of nonparenchymal cells that could contribute to miR-579-3p down-regulation, we next performed fluorescence *in situ* hybridization on fresh-frozen liver biopsy samples from patients with cirrhosis (n = 3) and patients with no liver pathology (n = 2). In all samples, miR-579-3p was expressed almost exclusively in hepatocytes (Fig. 2E) and was only occasionally found in nonparenchymal cells. These data indicate that miR-579-3p expression levels decrease mostly in hepatocytes of patients developing HCC.

Finally, we measured miR-579-3p expression in liver cancer cell lines in comparison with a nontumoral hepatic cell line, HeparRG (Fig. 2F). Consistent with our data, miR-579-3p expression was considerably higher in the nontumoral hepatic HeparRG cells than in all nine examined liver cancer cell lines.

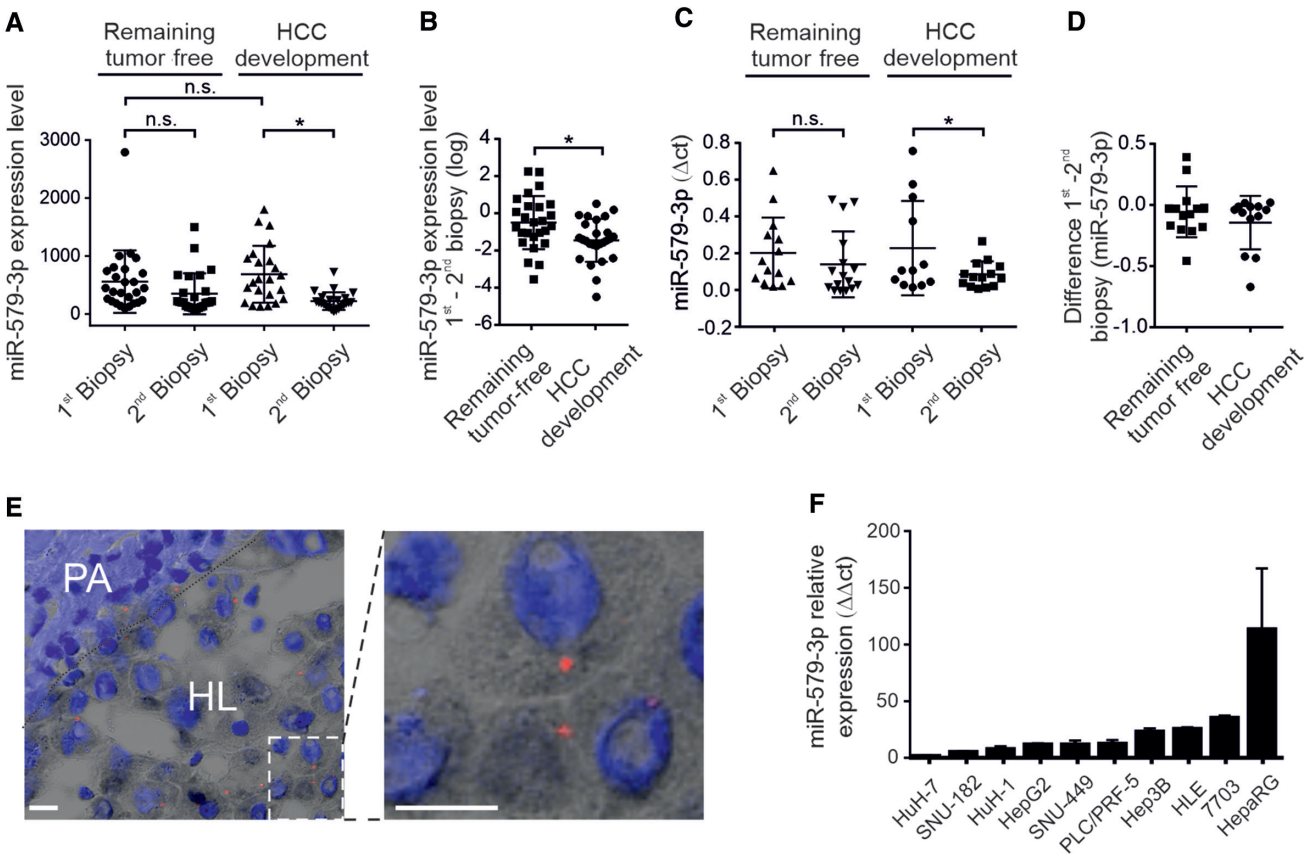
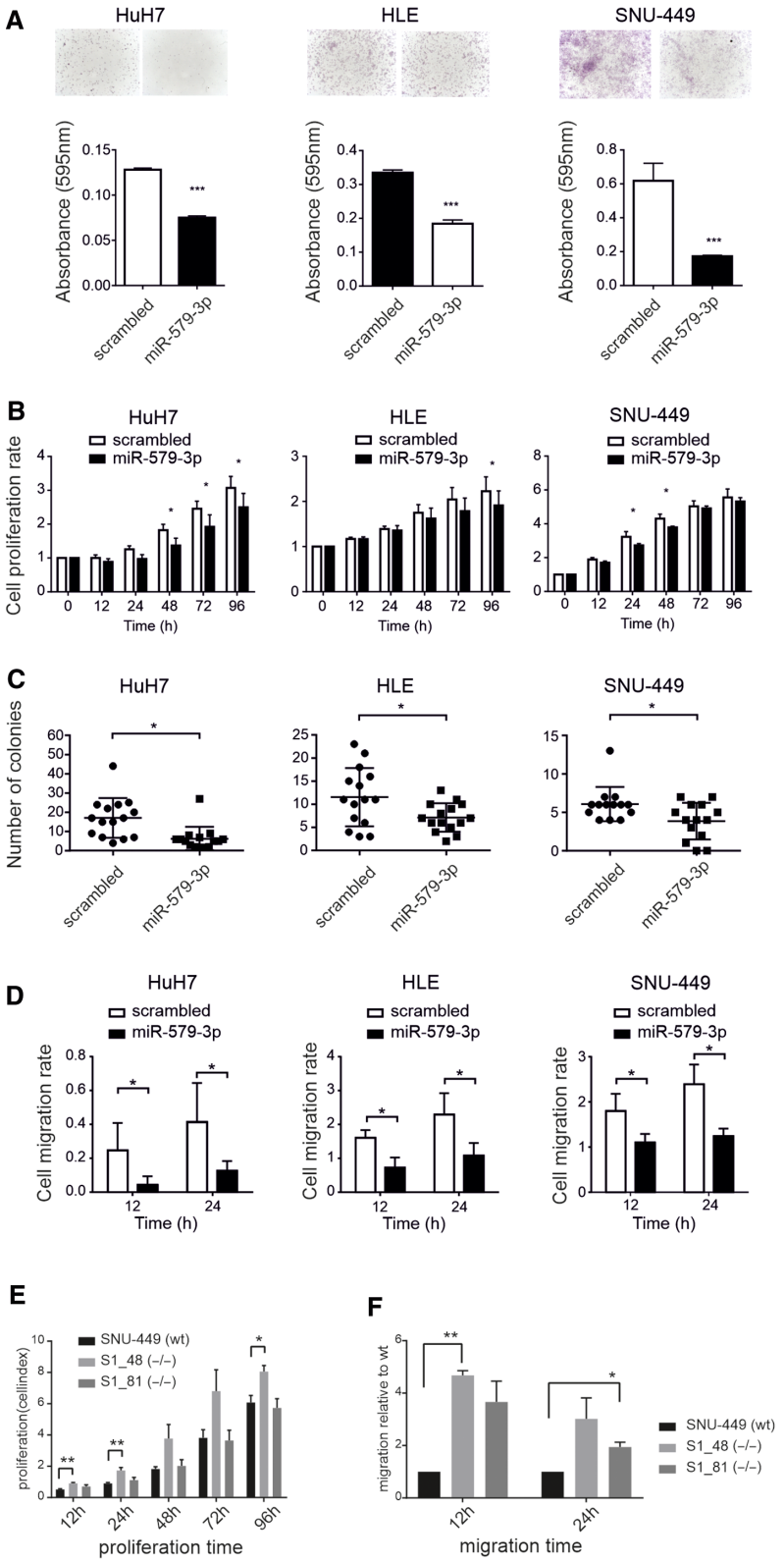


FIG. 2. Down-regulation of miR-579-3p in patients developing HCC. (A,B) Dot plots of nCounterNanostring probe intensity values representing gene expression levels of miR-579-3p in (A) each patient remaining tumor free or developing HCC and (B) interindividual differences between first and second biopsies for each patient. (C,D) qPCR analysis of miR-579-3p in the validation cohort of patients remaining tumor free and developing HCC. Each point represents 1 patient and is reported as (C) 2-dct and (D) interindividual difference between first and second biopsies. (E) Fluorescence *in situ* hybridization for miR-579-3p (blue, DAPI; red, probe for miR-579-3p; bar, 10 μm). (F) qPCR analysis of miR-579-3p expression in various liver cancer cell lines. Data are reported as 2-ddct in relation to miR-579-3p expression in HuH-7 cells. Each bar, mean ± SD of one experiment done in triplicate; **P* < 0.05. Abbreviations: DAPI, 4´,6-diamidino-2-phenylindole; dct/ddct, change (delta) in cycle threshold; HL, hepatic lobule; n.s., not significant; PA, portal area.

MIR-579-3P OVEREXPRESSION REDUCES TUMORIGENICITY

For functional analysis of miR-579-3p in HCC development, we transfected miR-579-3p into three liver cancer cell lines, HuH-7, HLE, and SNU-449 (Supporting Fig. S2A), with different basal levels of miR-579-3p expression (Fig. 2F). Overexpression of miR-579-3p reduced colony formation and kinetics of cell proliferation in all three examined cell lines (Fig. 3A,B). It also caused a consistent reduction in anchorage-independent growth in a soft agar assay (Fig. 3C; Supporting Fig. S2B) as well as inhibition of cell migration (Fig. 3D). To evaluate whether miR-579 depletion would similarly affect HCC cell proliferation and migration, we generated SNU-449 cells with deleted miR-579 by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology. The genotypes were confirmed by PCR, and miR-579-3p expression was confirmed by qPCR (Supporting Fig. S2C,D). We observed that miR-579 knockout partially resulted in enhanced proliferation (cell line S1_48; Fig. 3E) and promoted migration (cell lines S1_48 and S1_81; Fig. 3F). Together, these experiments demonstrate that high expression of miR-579-3p prevents cell proliferation and migration and conversely that down-regulation of miR-579-3p promotes hepatocarcinogenesis.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scrambled
miR-579-3p
B
Cell proliferation rate
Time (h)
C
Number of colonies
D
Cell migration rate
E
proliferation(cellindex)
SNU-449 (wt)
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migration relative to wt
migration time

FIG. 3. MiR-579-3p regulates colony formation, cell proliferation, anchorage-independent growth, and cell migration. (A-F) HuH-7, HLE, and SNU-449 cell lines were transfected with miR-579-3p or scramble control. (A) Cells were stained with crystal violet 11 days after transfection, and colony formation was measured by absorbance at 595 nm. Representative images are shown above. Original magnification ×10. (B) Kinetics of cell proliferation assessed by xCelligence proliferation assay. (C) Colony formation in soft agar measured 16 days after transfection, using ImageJ analysis. Images were taken using an optical microscope with a ×10 objective lens. Each dot represents the number of colonies in at least five different fields per well (n = 2). (D) Cell migration assessed at the indicated time after transfection using the xCELLigence migration assay. Data are reported as mean ± SD of two independent experiments. (E,F) SNU-449 wild-type cells or miR-579 depleted cells by CRISPR/Cas9 (S1_81/48 with biallelic knockout, S1_52 with monoallelic knockout) were assessed for (E) cell proliferation or (F) migration by xCelligence proliferation or migration assays. Data are reported as mean ± SEM of four or three independent experiments, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by (A,C) Mann-Whitney *t* test and (B,D) two-way ANOVA or (E,F) one-way ANOVA. Abbreviations: ANOVA, analysis of variance; h, hours; wt, wild type.

MRNA EXPRESSION CHANGES REFLECT INCREASED RISK FOR HCC DEVELOPMENT

Given the functional link between miRNAs and mRNAs, we wanted to characterize the correlation between miRNAs and mRNAs differentially expressed in patients with HCC. Therefore, we performed mRNA expression analysis by the PanCancer Pathway panel containing 730 cancer-associated genes. After applying stringent quality tests, 25 patients from the HCC-developing cohort and 24 of the cirrhosis cohort were included for further analyses (Supporting Table S6). Unsupervised hierarchical clustering showed no significant clustering, although first biopsies of the HCC-developing cohort preferentially clustered in the right half of the dendrogram and second biopsies of the HCC-developing cohort in the left half (Supporting Fig. S3). We determined the genes that changed significantly between the first and second biopsy (paired *t* test, $P \leq 0.001$). We found 82 differentially expressed genes in the HCC developing cohort and 52 genes in the cirrhosis cohort (Supporting Tables S7 and S8).

To confirm association of the differentially expressed genes in the HCC-developing cohort with an increased risk of HCC, we performed gene set enrichment analysis (GSEA) with published gene expression data from the nontumoral liver tissue of mostly patients who were HCV infected (GSE10141)[15] or HBV infected (GSE14520)[19] and undergoing curative HCC resection. The results showed that genes that were up-regulated in the 82-gene set of the HCC-developing cohort were more enriched in patients who were HCV infected with poor prognosis than those from patients remaining tumor free. Likewise, the genes that were down-regulated in the 82-gene set were more negatively enriched in patients with good prognosis than those from the 52-gene set (Supporting Fig. S4). In contrast, only down-regulated genes from the 82-gene set were more negatively enriched in patients infected with HBV with good prognosis, whereas the 52-gene set was slightly more enriched in patients infected with HBV with poor prognosis (Supporting Fig. S4). Together, these results indicate that genes changing in the HCC developing cohort between the first and second biopsies were associated with increased risk of *de novo* HCC formation in patients infected with HCV.

MIR-579-3P TARGETS THE PI3K-AKT PATHWAY

Next, we plotted the density distribution of the 82 mRNAs and 31 miRNAs that changed significantly from the first to the second biopsy in patients with HCC with available mRNA and miRNA data (n = 18), using Spearman correlation coefficients (Fig. 4A). This analysis revealed a clear enrichment of the positive and negative correlation pairs when compared to the normal distribution curve derived from the randomization of mRNA and miRNA expression values. Next, we paired miR-579-3p with all 82 mRNAs and used a correlation coefficient of 0.333, corresponding to the ninety-fifth percentile of the 100-fold random permutations, and a Spearman $P \leq 0.01$ as a cut-off threshold for positive and negative correlation. Using these criteria, we found 17 mRNAs out of 82 mRNAs that paired significantly with miR-579-3p (Supporting Table S9).

miRNAs bind the 3' untranslated regions of targeted mRNAs, resulting in degradation of the targeted mRNAs and subsequent translational repression.[20] Therefore, we used two different online web tools (Target Scan version 7.1 [http://cbio.

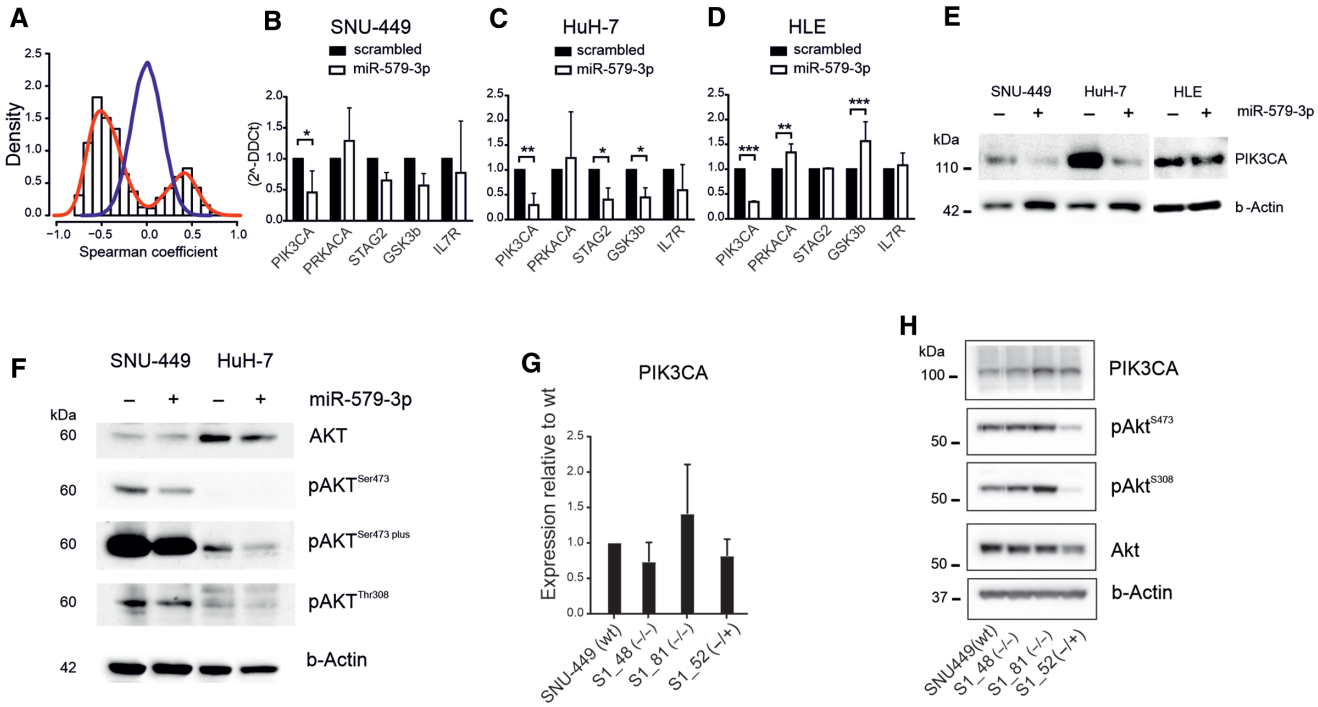

FIG. 4. MiR-579-3p targets PIK3CA. (A) Density distribution of the 82 mRNAs changing significantly from the first to the second biopsy in patients developing HCC, by Spearman correlation coefficients with miR-579-3p (red line). Normal distribution of mRNAs and miRNAs (blue line). (B-D) qPCR analysis of mRNA expression of PIK3CA, PRKACA, STAG2, GSK3β, and IL7R in (B) SNU-449, (C) HuH-7, and (D) HLE cell lines 48 hours after transfection with miR-579-3p or scrambled control. Data are reported as mean ± SD of two independent experiments. (E,F) Western blot analysis of PIK3CA in (E) SNU-449, HuH-7, and HLE cell lines, and (F) AKT, AKT^{Ser473}, and AKT^{Thr308} in SNU-449 and HuH-7 cell lines transfected with miR-579-3p for 120 hours and 96 hours, respectively. β-Actin was used as a loading control. (G) qPCR analysis of mRNA expression of PIK3CA in SNU-449 wild-type cells or miR-579 depleted cells by CRISPR/Cas9 (S1_81/48 with biallelic knockout, S1_52 with monoallelic knockout). Data are reported as mean ± SEM of four independent experiments. (H) Western blot analysis of PIK3CA, AKT, AKT^{Ser473}, and AKT^{Thr308} in SNU-449 wild-type cells or miR-579 knockout cells. β-Actin was used as a loading control. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations: GSK3B, glycogen synthase kinase 3 beta; IL7R, interleukin 7 receptor; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PRKACA, protein kinase cyclic adenosine monophosphate-activated catalytic subunit alpha; Ser, serine; STAG2, stromal antigen 2; Thr, threonine; wt, wild type.

mskcc.org/miRNA2003/miranda.html] and Miranda/microRNA.org) to search for the genes that could be predicted targets of miR-579-3p. Five genes (glycogen synthase kinase 3 beta [*GSK3B*], interleukin 7 receptor [*IL7R*], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha [*PIK3CA*], protein kinase cyclic adenosine monophosphate-activated catalytic subunit alpha [*PRKACA*], and stromal antigen 2 [*STAG2*]) were predicted by both web tools and were all negatively correlated with miR-579-3p expression in our patient cohort. We then tested if miR-579-3p regulated any of the five predicted correlative mRNAs by using liver cancer cell lines transfected with miR-579-3p. The results showed that overexpression of miR-579-3p in SNU-449, HuH-7, and HLE cells caused a down-regulation of several of the predicted genes (Fig. 4B-D). However, only PI3KCA was consistently and statistically significantly down-regulated in all three cell lines. Moreover, western blotting confirmed down-regulation of PIK3CA in SNU-449, HuH-7, and HLE cells overexpressing miR-579-3p (Fig. 4E), although to varying degrees. In addition, there was a slight down-regulation of total AKT and a more pronounced reduction of phosphorylated AKT (pAKT)$^{Serine\ (Ser)473}$ and pAKT$^{Threonine\ (Thr)308}$ in SNU-449 and HuH-7 cells (Fig. 4F) after overexpressing miR-579-3p.

To evaluate whether miR-579 depletion would similarly affect PI3KCA expression, we tested PIK3CA expression in SNU-449 cells in which miR-579 was

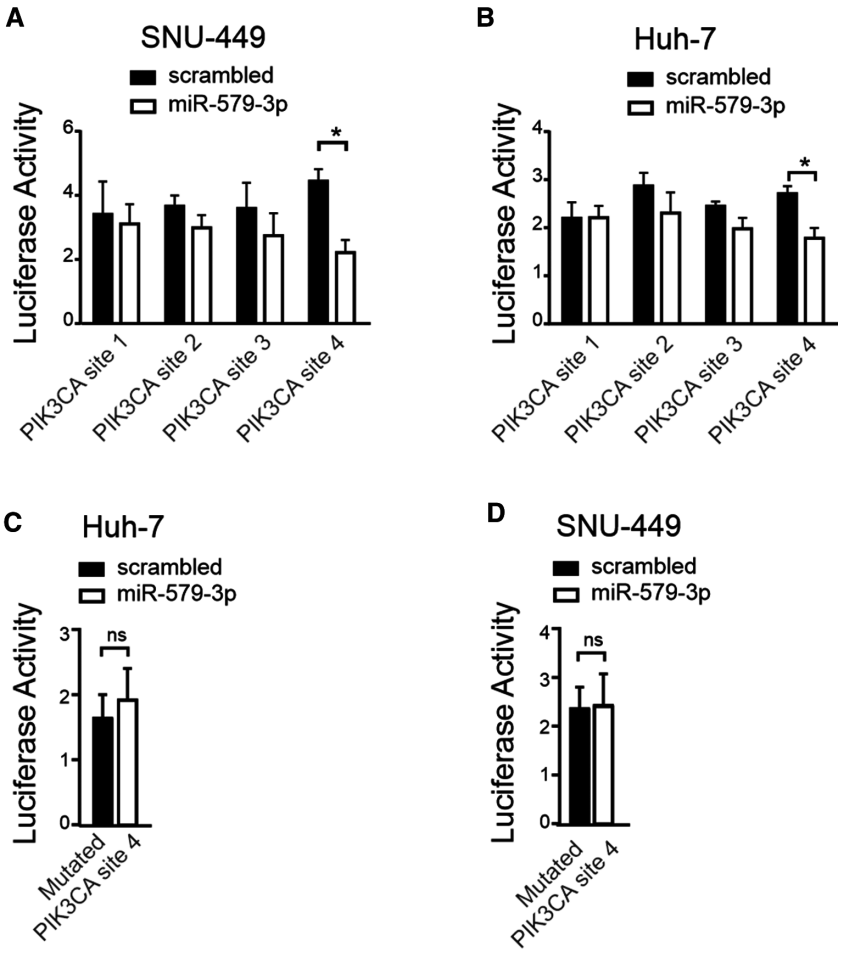

FIG. 5. PIK3CA as a direct target of miR-579-3p. (A) Luciferase assay of (A,B) four predicted binding sites of PIK3CA in SNU-449 and Huh-7 cells and (C,D) mutated binding site 4 of PIK3CA. Normalized luciferase firefly activity was assessed after 48 hours for SNU-449 and 72 hours for HuH-7 cells. Data are reported as mean ± SD of two independent experiments. *$P < 0.05$ by (B-D) ordinary one-way analysis of variance. Abbreviation: ns, not significant.

deleted by CRISPR/Cas9 technology. While no consistent increase in PI3KCA mRNA expression was observed (Fig. 4G), there was a trend for increased PI3KCA expression on the protein level (Fig. 4H). In particular, there was increased phosphorylation of Akt, which was increased in the SNU-449 cells with biallelic miR-579 depletion but not if one miR-579 allele was still present (Fig. 4H). These results further substantiate a tumor-suppressor role of miR-579-3p in the regulation of PI3K signaling and proliferation/migration phenotypes.

To validate PIK3CA as a potential direct target of miR-579-3p, we performed target scan prediction and found four binding sites for miR-579-3p at PIK3CA. Consequently, we tested all four binding sites in HuH-7 and SNU-449 cancer cell lines transfected with miR-579-3p, using the luciferase assay. The results showed that only binding site 4 was a functional target of miR-579-3p (Fig. 5A,B). Introducing a mutation in binding site 4 prevented effective binding and confirmed binding site 4 as a direct miR-579-3p target (Fig. 5C,D). These results support the importance of miR-579-3p in regulating the PI3K-AKT axis during liver cancer development.

Discussion

To the best of our knowledge, this is the first tissue-based longitudinal study comparing miRNA

expression in liver biopsies before and after HCC development in the same patient. Such studies are advantageous because confounding factors are better controlled and relevant changes can be measured at an individual level. Our approach gave novel insights, especially into miRNA involvement in HCC development, and revealed significant changes not only between the first and second biopsy in patients developing HCC but also in comparison to a tumor-free control cohort. Indeed, earlier reports indicated that changes in miRNA expression belonged to the earliest steps in hepatocarcinogenesis.[14] This may be explained by the fact that miRNA genes are often located at fragile sites or in cancer-associated genomic regions, suggesting that the genetic instability associated with chronic inflammation may especially disturb miRNAs.[21] In this case, key miRNA changes that predispose to carcinogenesis may have already occurred at the stage of cirrhosis.

miR-579-3p was identified as one of the miRNAs that changed most significantly in patients developing HCC. The functional assays further confirmed the profound effect of miR-579-3p on cell proliferation and cell migration and determined PIK3CA as a direct target of miR-579-3p. PIK3CA is a well-known oncogenic component of the PI3K signaling pathway, with a prominent role in cell proliferation and carcinogenesis in many human cancers, including HCC.[5] Consequently, miR-579-3p also regulated expression of AKT and pAKT in hepatocytes, the principal downstream targets of PIK3CA. Because miR-579-3p is absent in mice and only exists in humans, mice cannot be engineered with different miR-579-3p expression in the liver. However, several murine studies have shown that activation of PIK3CA increased AKT and pAKT in murine liver tumors and promoted murine hepatocarcinogenesis.[22] We also noticed a more profound increase in protein expression for AKT and pAKT than PIK3CA after knocking-out miR-579-3p in liver cancer cell lines. Therefore, we can't exclude that miR-579-3p also directly targeted *AKT*, as has been proposed.[23] miR-579-3p is an intronic miR located in intron 11 of the human gene zink-finger recombinase (ZFR), and a role of miR-579-3p in liver cancer has not been described.[24] However, miR-579-3p has been shown to be dysregulated in other cancer types. In melanoma, miR-579-p was down-regulated during melanoma progression from nevi to stage III/IV cancer, where it acted as an oncosuppressor and strongly controlled cell proliferation by targeting B-Raf proto-oncogene, serine/threonine kinase (BRAF) and mouse double minute 2 proto-oncogene (MDM2).[25] As in our study, miR-579-3p inhibited cell proliferation and migration in glioblastoma cell lines by regulating the PI3K-AKT signaling pathway.[23] Likewise, miR-579-3p expression inhibited cell proliferation and migration in lung cancer cell lines and was found down-regulated in colon cancer metastasis compared to nontumoral liver tissue.[26,27] In conclusion, other studies support the tumor-suppressive role of miR-579-3p, and a more detailed analysis of the mechanisms responsible for miR-579-3p down-regulation during HCC progression warrants further investigation. Besides miR-579-3p, we identified several other miRNAs (miR-4531, miR-1322, miR-126-3p, and miR-520h) potentially involved in HCC development. Among these, miR-126-3p has been shown to play an important role in several cancer types, including HCC.[28] Further studies will be necessary to address the extent that these miRNAs are involved in HCC development.

Recent analyses indicate that HCC development within damaged chronic liver tissue depends on stochastically occurring genomic aberrations that are eventually sufficient for individual clones to develop into cancer.[7,29,30] However, it seems that only the combination of several driver mutations instead of one single driver mutation is sufficient for HCC development. Therefore, to estimate the risk for HCC development in chronic liver tissue, it may be more informative to analyze gene expression in nontumoral liver tissue instead of detecting genomic aberrations.[7] Indeed, several studies indicate that gene signatures can be successfully used to estimate the risk of HCC development in patients with chronic liver disease and highlight the importance of the nontumoral hepatic inflammatory microenvironment in promoting HCC development and shaping HCC biology.[11,31-33] Surprisingly, predicting HCC recurrence based on the analysis of nontumoral liver tissue has even been more accurate than analysis of the tumor itself.[15] Our study further emphasizes the importance of nontumoral liver tissue in HCC development and shows that liver tissue from patients developing HCC goes through changes that facilitate HCC initiation. Therefore, our study helps in understanding the mechanisms of

hepatocarcinogenesis and measures to prevent HCC development, which remains the most promising strategy to reduce HCC mortality.[34] Moreover, our study will provide information to help identify patients at risk for HCC development.[11]

In conclusion, we identified miR-579-3p as an oncosuppressive miRNA and propose a novel miR-579-3p-PI3K-AKT axis that promotes HCC development. AKT1 was identified as a top, enriched, upstream regulator gene for HCC risk in a meta-analysis of human liver transcriptome,[35] and AKT activation was central in a recent murine study with liver tumor developing in chronically inflamed liver tissue.[36] Furthermore, our results suggest that miR-579-3p could be considered as a potential therapeutic candidate and may serve as a biomarker in predicting risk for HCC development in patients with chronic liver disease.

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