miRNA profiling of biliary intraepithelial neoplasia reveals stepwise tumorigenesis in distal cholangiocarcinoma via the miR-451a/ATF2 axis

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Abstract

Distal cholangiocarcinoma (dCCA) is a biliary tract cancer with a dismal prognosis and is often preceded by biliary intraepithelial neoplasia (BilIN), representing the most common biliary non-invasive precursor lesion. BilIN are histologically well defined but have not so far been characterised systematically at the molecular level. The aim of this study was to determine miRNA-regulated genes in cholangiocarcinogenesis via BilIN. We used a clinicopathologically well-characterised cohort of 12 dCCA patients. Matched samples of non-neoplastic biliary epithelia, BilIN and invasive tumour epithelia of each patient were isolated from formalin-fixed paraffin-embedded tissue sections by laser microdissection. The resulting 36 samples were subjected to total RNA extraction and the expression of 798 miRNAs was assessed using the Nanostring® technology. Candidate miRNAs were validated by RT-qPCR and functionally investigated following lentiviral overexpression in dCCA-derived cell lines. Potential direct miRNA target genes were identified by microarray and prediction algorithms and were confirmed by luciferase assay. We identified 49 deregulated miRNAs comparing non-neoplastic and tumour tissue. Clustering of these miRNAs corresponded to the three stages of cholangiocarcinogenesis, supporting the concept of BilIN as a tumour precursor. Two downregulated miRNAs, i.e. miR-451a (−10.9-fold down) and miR-144-3p (−6.3-fold down), stood out by relative decrease. Functional analyses of these candidates revealed a migration inhibitory effect in dCCA cell lines. Activating transcription factor 2 (ATF2) and A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) were identified as direct miR-451a target genes. Specific ATF2 inhibition by pooled siRNAs reproduced the inhibitory impact of miR-451a on cancer cell migration. Thus, our data support the concept of BilIN as a direct precursor of invasive dCCA at the molecular level. In addition, we identified miR-451a and miR-144-3p as putative tumour suppressors attenuating cell migration by inhibiting ATF2 in the process of dCCA tumorigenesis.

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Introduction

Cholangiocarcinoma (CCA) can emerge at every site of the biliary tract, and is anatomically separated into three major tumour subgroups: intrahepatic CCA (iCCA), perihilar CCA (pCCA) and distal CCA (dCCA) [1]. CCA developing between the choledochal duct and approximately 1.5 cm proximal to the ampulla of Vater are classified as dCCA and are often preceded by precursor lesions [2]. The concept of epithelial tumours arising from non-invasive intraepithelial dysplasia is well-established in various human cancers [3–6]. For CCA, the fifth edition of the WHO classification of gastrointestinal tumours defines several non-invasive precursor lesions of which biliary intraepithelial neoplasia (BilIN) is the most common by far [7]. It is characterised by hyperchromasia and pseudostratification of nuclei, with cells protruding into the duct lumen in micropapillary structures (Figure 1) and is divided into low- and high-grade lesions [7]. The limited prognosis of CCA patients is generally attributed to the advanced stage of disease in which most CCAs are diagnosed [1], their high refractory nature to chemotherapy and the lack of established options for targeted therapy [8]. Subsequently, improvement in patient outcome is an urgent and unmet goal for which two tasks need to be addressed: diagnosis at an early stage of disease and augmentation of treatment options based on molecular targeted therapies. For both, it is conducive to understand the early events of cholangiocarcinogenesis via precursor lesions. Biliary precursor lesions are histologically well-described but poorly understood at the molecular level. In particular, the microscopic BilIN lesions have not so far been characterised systematically at the molecular level, probably due to technical reasons. Few studies on single gene analyses are available, e.g. KRAS mutations were identified as an early event in cholangiocarcinogenesis, occurring in 33% of BilIN lesions with concomitant iCCA [9]. Immunohistochemical analyses of BilIN revealed aberrant expression of p53, p21, cyclin D1 and EZH2, whereas the tumour suppressors Dcp4 and p16INK4A decreased [10]. Moreover, progression of BilIN is accompanied by loss of membranous β-catenin expression [11] and upregulation of MUC1, MUC2, MUC5AC [12] and CD15 [13] expression. Recent research efforts were made in iCCA but not in dCCA, although dCCA occurs about three times more frequently than iCCA [14]. To date, most available studies on dCCA have relied on immunohistochemical approaches, and so far there have been no studies that have systematically investigated miRNAs in BilIN and carcinogenesis in the distal biliary tree.

Figure 1. Dysregulated miRNA expression reflects the stepwise carcinogenesis of dCCA. Tissue samples of (A) normal biliary epithelium, (B) BilIN and (C) invasive adenocarcinoma were isolated using laser microdissection and analysis of miRNA expression was conducted using Nanostring technology. (D) Heatmap clustering of 49 miRNAs with significantly altered expression separated normal from tumour tissue samples, with most BilIN samples lining up in between. Clustering was conducted using the complete linkage method in R. The tissue sections shown were stained with haematoxylin and eosin (H&E). Scale bar: 20 μm.*p < 0.05.
In recent years, the regulation of gene expression by miRNAs has become of particular interest in cancer research. These small non-coding RNAs of 18–24 nucleotides in length bind to seed sequences at the 3′UTR of target mRNAs, leading to inhibition of protein expression. A single miRNA can interact with various mRNAs and a single mRNA can be regulated by multiple miRNAs in complex miRNA/mRNA networks [15,16]. Deregulation of miRNA expression is considered to be a common event in various human cancers. Thereby, miRNAs take either an oncogenic or a tumour suppressive role, making them potential therapeutic or diagnostic targets [17,18]. In CCA, various miRNAs with abrogated expression have been identified [19,20]. Our study aimed to contribute to the knowledge in this field by investigating miRNA expression during the consecutive stages of dCCA development in a clinicopathologically well-characterised European cohort.

Materials and methods

Patient samples

We analysed formalin-fixed and paraffin-embedded (FFPE) tissue samples from two independent dCCA cohorts of 12 patients each (Table 1, see supplementary material, Tables S1, S2) who underwent primary resection of dCCA at the time of diagnosis at the Surgical Department of Heidelberg University Hospital between 2010 and 2015. All samples were provided by the Tissue Bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in concordance with the Ethics Committee of the University of Heidelberg (ethical approval S-519/2019 and S-206/2005). For each patient of cohort 1 (see supplementary material, Table S1), tissue blocks containing non-neoplastic epithelium, high-grade BilIN and invasive tumour were selected, resulting in a total of 36 samples. All samples were histologically confirmed by a pathologist with special expertise in biliary tract cancer (BG). None of the included patients received neoadjuvant treatment and patients presented with UICC stage IIA–IIIA at the time of resection.

Staining and laser microdissection

FFPE tissue blocks were cut at 8 μm thickness, mounted on Zeiss 1.0 PEN slides (Carl Zeiss, Oberkochen, Germany) and incubated overnight at 37 °C. After dewaxing in xylene (100%) and rehydrating through decreasing concentrations of ethanol (100, 95, 75%), sections were stained in 1% cresyl violet acetate (Sigma-Aldrich, Taufkirchen, Germany). After dehydration in increasing ethanol concentrations (75, 95, 100%), tissue sections were dried and stored at 4 °C. Regions of interest (non-neoplastic epithelia, BilIN, tumour epithelia) were microdissected using a ZEISS PALM LMD laser capture microdissection unit. Isolated tissue fragments corresponding to an area of approximately 1 × 10^6 μm^2 for each sample were collected in AdhesiveCap 500 opaque tubes (Carl Zeiss). The samples were stored at −20 °C until further processing.

RNA isolation and RT-qPCR

Total RNA from microdissected tissue samples was extracted using the AllPrep DNA/RNA FFPE Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol and quantified with the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Reverse transcription of miRNAs of 50 ng total RNA was performed using the miScript II RT Kit (Qiagen). Quantitative real-time PCR (RT-qPCR) was conducted using the miScript SYBR Green qPCR Kit (Qiagen) on a StepOnePlus Real-Time PCR System.
system (ThermoFisher Scientific, Waltham, MA, USA). We amplified reversely transcribed miRNAs using Hs_miR-451_1 miScript Primer Assay and Hs_miR-144_4 miScript Primer Assay. Relative miRNA expression of three technical triplicates was assessed by the 2-delta Ct method and normalised to expression of small nuclear RNA 48 (SNORD48). All primer assays were obtained from Qiagen.

Total RNA from cell lines was isolated with TRIzol Reagent (ThermoFisher Scientific). Reverse transcription was conducted using 1 μg total RNA, random hexamer primers and RevertAid H Minus Reverse Transcriptase (ThermoFisher Scientific). Relative expression of miRNAs was measured in RT-qPCR using primaQUANT qPCR-CYBR-Master-Mix-high-ROX (Steinbrenner Laborsysteme, Wiesenbach, Germany) and primers for activating transcription factor 2 (ATF2) and ADAM10 (ADAM10) (see supplementary material, Table S3). Data were normalised to expression of serine and arginine rich splicing factor 4 (SRSF4).

Nanostring miRNA assay
We analysed the expression of 798 miRNAs with the nCounter human v3 miRNA expression assay designed by Nanostring Technology (Seattle, WA, USA). This assay allowed us to gain expression data from a low input of 100 ng total RNA. According to the manufacturer’s protocol, the examined miRNAs were attached to specific tag sequences and hybridised for 16 h at 65 °C to a capture/reporter probe pair equipped with a fluorescent barcode. These miRNA-specific barcodes were then detected by the nCounter Digital Analyzer providing count of miRNAs. We analysed the raw data using the manufacturer’s software (nSolver version 3.0). Raw data were technically normalised by the geometric mean of included positive and negative controls in order to diminish differences in hybridisation. The expression of every miRNA was then biologically normalised to the geometric mean of the top 100 most expressed miRNAs. All samples were grouped into three groups: normal, BilIN and tumour. The fold-change of miRNA expression between the groups was calculated. Comparing the expression between the normal and tumour tissue groups, a P value < 0.05 was considered statistically significant. Candidates with the highest fold-change were selected for further investigations. The dataset is available at gene expression omnibus (GSE140001).

Cell cultures
The dCCA-derived cell lines EGI-1, TFK-1 (obtained from DSMZ ACC, Braunschweig, Germany), SNU-478 and SNU-1196 (obtained from KCLB, Seoul, Republic of Korea), as well as a primary culture of normal human cholangiocytes (NHC) [21] were used in this study. Detailed information about cell cultivation, protein isolation, immunoblotting, luciferase assay, proliferation assay, colony formation assay, migration assays and microarrays is provided in Supplementary materials and methods.

miRNA overexpression and siPool knockdown
We transfected cell lines with miRNA mimics of miR-451a (5′AAACCGUUACCAUACUGAGUU3′) and miR-144-3p (5′UACAGUAUAGAAUGUAU3′) both from Qiagen using Lipofectamine RNAiMAX (ThermoFisher Scientific). AllStars (Qiagen) served as a negative control. For functional experiments, we used a lentiviral transfection system to guarantee efficient overexpression of miR-451a, miR-144-3p and combined miR-451a/144-3p. Corresponding sequences of these miRNAs (see supplementary material, Table S3) were inserted into a pTRIPZ vector using GATEWAY cloning. pDNOR201 was used as an entry vector and cloning was conducted in competent Escherichia coli MACH1. For purification of PCR products and plasmids, we used Nucleo Spin Gel, PCR Clean-up Kit and Nucleo Spin Plasmid Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. Products were tested by agarose electrophoresis and sequencing. An empty pTRIPZ vector was used as a negative control. For lentiviral transfection, pTRIPZ was transfected into HEK293T cells using polyethyleneimine. After incubation overnight at 37 °C, 5% CO₂, the medium was changed and after another 48 h the lentivirus was filtered with Millex-HA 0.45 µm filters (Merck Millipore, Tullagreen, Ireland). Mammalian cells were incubated in lentivirus-containing medium with polybrene for 24 h for transduction. Transduced cells were selected by puromycin (2 µg/ml) for 10 days and selection was repeated every 2 weeks. Doxycycline (DOX; 2 µg/ml) was used for vector induction.

To specifically knockdown endogenous ATF2 protein expression, we used a pooled mix of 24 siRNAs against ATF2 at picomolar concentrations, thereby limiting off-target effects [22] (siTOOLs Biotech, Planegg, Germany; sequences provided in supplementary material, Table S4). Cells were incubated with 0.20 µM siPool or negative control and Lipofectamine RNAiMAX as the transfection reagent for 48 h. They were then harvested and prepared for further experiments.

Statistics
Statistical analyses were conducted with R/Bioconductor [23] using the limma software package [24], GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and nSolver 3.0 (Nanostring). Student’s t-test was calculated; the significance threshold was set at 0.05 (*p < 0.05, **p < 0.001). Multiple testing was taken into account, according to Hochberg [25]. The heatmap was generated using the R packages ComplexHeatmap, RColorBrewer, dplyr and tidy. Image analysis was conducted with ImageJ 1.51j8 [26]. Potential mRNA target genes were detected using the online tools TargetScan 7.2 [27] and miRWalk 2.0 [28].
miR-451a in biliary intraepithelial neoplasia and cholangiocarcinoma

Results

Dysregulated miRNA expression reflects the carcinogenesis of dCCA

To analyse miRNA expression during the consecutive stages of cholangiocarcinogenesis in our patient cohort (cohort 1; n = 12), the epithelial components of non-neoplastic bile duct (referred to below as normal), BilIN and invasive dCCA (Figure 1A–C) were isolated by laser microdissection for each patient, corresponding to a total of 36 samples. Total RNA was extracted and expression analysis of 798 miRNAs using Nanostring technology revealed 49 miRNAs with significantly altered expression between normal and tumour tissue samples (cut-off P value <0.05; see supplementary material, Table S5). Unsupervised heatmap clustering of these miRNAs (Figure 1D) properly separated normal versus invasive dCCA tissue samples in most cases, placing the majority of BilIN samples (n = 10) between normal and invasive dCCA. Two BilIN samples lined up within the normal tissue group. One tumour sample (T10) clustered separately from all other samples.

miRNA profiling identifies potentially oncogenic and tumour suppressive candidates

For the identification of potentially oncogenic and tumour suppressive candidates, significantly altered miRNAs were screened for gradual up- or downregulation during progression from normal via BilIN to invasive tumour tissue. Ten miRNAs were gradually upregulated and 13 miRNAs were downregulated (Figure 2A,B). Sorting these candidate miRNAs by fold-change of miRNA expression between normal and tumour tissue (-2.15-fold down, p = 0.037) and a trend of downregulation of miR-144-3p (~2.8-fold down, p = 0.067) in this independent patient cohort (Figure 2E,F). Thus, we found the miRNAs miR-451a and miR-144-3p, which are co-expressed on the same transcript, to be downregulated in a stepwise manner from normal biliary epithelium via BilIN to dCCA. miR-451a represses cell migration and invasion

To analyse the functional impact of altered miR-451a/144-3p expression on cell biology, we overexpressed the corresponding miRNAs in dCCA-derived cell lines and investigated cellular behaviour. A pTRIPZ-based lentiviral transduction system was used for efficient miRNA overexpression. Lentiviral transduction of vectors including miR-451a, miR-144-3p or both miR-451a/miR-144-3p significantly increased expression of corresponding miRNAs in SNU-478 and SNU-1196 cells (Figure 3A). Cell viability was assessed over a period of 4 days. Transduction of miR-451a, miR-144-3p or both miR-451a/144-3p did not significantly influence cell viability relative to control transduced cells (Figure 3B). In addition, colony formation capacity was not altered by miR-451a, miR-144-3p or the combination of miR-451a/miR-144-3p (Figure 3C). The ability of cells to migrate and to leave the original cellular bond is a feature commonly related to a tumorigenic phenotype. Thus, we investigated the effect of miR-451a, miR-144-3p and miR-451a/144-3p on cell migration in wound healing assays. In SNU-478, transduction of miR-451a (0.66 ± 0.21), miR-144-3p (0.65 ± 0.24) and miR-451a/144-3p (0.42 ± 0.13) significantly repressed cell migration relative to control transduced cells (1.00 ± 0.05). In SNU-1196 cells, the same effect was observed for miR-451a (0.52 ± 0.23), miR-144-3p (0.53 ± 0.33) and again more pronounced for combined miR-451a/144-3p (0.34 ± 0.13) relative to control (1.00 ± 0.21; Figure 4A). Hereby, we observed that, consistent with higher downregulation of miR-451a in human dCCA tissue, miR-451a had stronger effects compared with miR-144-3p on cell migration. Thus, we focused our research on miR-451a. The capacity to migrate and invade through a Matrigel-coated membrane was assessed using a Transwell assay. Overexpression of miR-451a significantly repressed cell invasion in SNU-478 (0.58, p = 0.037) and SNU-1196 (0.37, p = 0.0003) relative to control transduced cells (Figure 4B).

miRNA-451a directly targets ATF2

To investigate downstream effects of miR-451a, we searched for specific target genes by gene expression profiling. CCA-derived SNU-478 cells were transfected with miR-451a and subjected to microarray-based gene expression profiling, which revealed downregulation of 585 genes (see supplementary material, Table S6). Matching this list with sequence-based miRNA target prediction by the online tool TargetScan (version 7.2, p = 0.007) and from normal to tumour tissue (-2.84-fold down, p = 0.001). Correlation analysis of miR-451a expression data by Nanostring assay and RT-qPCR confirmed the consistency of both methods (Pearson r = 0.91, R² = 0.83; Figure 2C). miR-144-3p expression also significantly decreased from normal tissue to BilIN (~3.85-fold down, p = 0.003), from BilIN to invasive tumour (~2.84-fold down, p = 0.002) as well as from normal to invasive tumour tissue (~10.95-fold down, p = 0.001). Correlation analysis of miR-451a expression data by Nanostring assay and RT-qPCR confirmed the consistency of both methods (Pearson r = 0.91, R² = 0.83; Figure 2C). miR-144-3p expression also significantly decreased from normal tissue to BilIN (~4.48-fold down, p = 0.006), from BilIN to tumour (~2.15-fold down, p = 0.007) and from normal to tumour tissue (~9.61-fold down, p = 0.002), implying positive correlation of RT-qPCR and Nanostring data (Pearson r = 0.65, R² = 0.43; Figure 2D). Furthermore, we included an independent dCCA patient cohort for validation of miR-451a and miR-144-3p expression (cohort 2; see supplementary material, Table S2). Similar to the data obtained for cohort 1, we observed significant downregulation of miR-451a (~8.0-fold down, p = 0.037) and a trend of downregulation of miR-144-3p (~2.8-fold down, p = 0.067) in this independent patient cohort (Figure 2E,F). Thus, we found the miRNAs miR-451a and miR-144-3p, which are co-expressed on the same transcript, to be downregulated in a stepwise manner from normal biliary epithelium via BilIN to dCCA.
see supplementary material, Table S7) resulted in an overlap of five genes: ATF2, MIF, SAMD4B, CAB39, PSMD11 (Figure 5A; see supplementary material, Figure S1A). Independent validation of these five potential direct target genes in SNU-478 and SNU-1196 showed the most significant and greatest downregulation for ATF2 upon miR-451a transfection (see supplementary material, Figure S1B). As TargetScan predicted only 31 genes to be targets of miR-451a, we also used the online tool miRWalk 2.0 for prediction, which suggested ADAM10 to be a putative target gene of miR-451a functionally related to cell migration and invasion (see supplementary material, Table S8). To further examine the effect of miR-451a on ATF2 and ADAM10, we transfected dCCA-derived cell lines EGI-1, SNU-478 and SNU-1196, as well as the NHC, with miR-451a mimics and AllStars as a negative control. The expression of ATF2 and ADAM10 mRNA was assessed by RT-qPCR. Overexpression of miR-451a significantly lowered ATF2 expression in EGI-1, SNU-478, SNU-1196 and NHC, but ADAM10 expression was only significantly decreased in SNU-478 (Figure 5B). Two
potential binding sites for ATF2 and one for ADAM10 were identified by TargetScan and direct binding of miR-451a to ATF2 and ADAM10 was tested using luciferase assays. Transduction of a pmIR reporter vector containing firefly luciferase and miR-451a binding sites corresponding to sequences in the 3’-UTR of either ATF2 or ADAM10 led to reduced luciferase activity in miR-451a-transduced HLF cells. This implicated direct binding to both target genes. Targeted mutation at the binding sites abrogated the effect of miR-451a on luciferase activity, suggesting that miR-451a directly bound the 3’-UTR of either ATF2 or ADAM10 (Figure 5C). Next, we tested the effect of miR-451a upregulation on protein level by immunoblotting. Although miR-451a attenuated ATF2 expression in both normal and tumour biliary cells, no impact on ADAM10 protein expression was observed (Figure 5D). Thus, we focused our further studies on ATF2.

ATF2 rescues miRNA-451a-mediated repression of cell migration

To investigate the role of ATF2 in the repression of cell migration, we confirmed that lentivirally transduced miR-451a repressed ATF2 expression in RT-qPCR and immunoblot assays (see supplementary material, Figure 3).
Furthermore, we studied pATF2 (Thr-71) in corresponding normal and tumour tissue sections using immunohistochemistry and observed that pATF2 expression was lower in normal compared with paired dCCA tissue in five of 11 patients (see supplementary material, Figure S3). Thus, we could show that pATF2 increased in a subgroup of dCCA patients. Next, we investigated ATF2 inhibition by pooled siRNAs (siPool), which led to reduced ATF2 levels (Figure 6A). In a wound healing assay, ATF2-siPool significantly repressed cell migration of SNU-478 (0.72 ± 0.11/C6 0.32 ± 0.11) and SNU-1196 (0.49 ± 0.32) relative to the negative control, mimicking the effect of miR-451a (Figure 6B). Finally, to verify the presumed migration-inhibiting effect of miR-451a by targeting ATF2, we conducted rescue experiments. ATF2 overexpression by a doxycycline-inducible lentiviral vector significantly enhanced cell migration in SNU-478 (1.23 ± 0.15) and SNU-1196 (1.26 ± 0.08) cells (Figure 6C). Consistent with this, miR-451a overexpression attenuated cell migration in SNU-478 (0.78 ± 0.12) and SNU-1196 (0.58 ± 0.16), whereas concurrent ATF2 upregulation reversed this effect in both cell lines to 1.02 ± 0.12 and 0.82 ± 0.13, respectively. Thus, ATF2 overexpression rescued the miR-451a-mediated repression of cell migration, suggesting that miR-451a mainly inhibits cell migration by targeting ATF2 (Figure 6C).

**Discussion**

Cholangiocarcinogenesis via the precursor lesion BilIN is a histomorphologically and immunohistochemically

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well-described process [2,7,10,29–31]. However, to date, systematic molecular analyses using current molecular array-based technologies have not been performed. Here, we examined miRNA expression with a matched sample approach, analysing non-neoplastic biliary epithelia, BilIN and invasive tumour specimens, originating

Figure 5. miRNA-451a directly targets ATF2. (A) SNU-478 cells were transfected with miR-451a mimics resulting in the downregulation of 585 genes. The data are arranged in a volcano plot. All points above the horizontal line represent significantly deregulated target genes, *p_adj < 0.05. (B) ATF2 and ADAM10 mRNA expression was assessed by RT-qPCR 48 h after transfection of miR-451a mimics in biliary cell lines. Gene expression was normalised to SRSF4 as an endogenous control and is displayed relative to AllStars-transfected cells as a negative control. Bars represent the arithmetic mean ± SD. Statistical test: unpaired t-test, *p < 0.05. SDs result from technical triplicates of two experiments. (C) Direct miRNA-451a binding to predicted sites at the 3'-UTR of ATF2 and ADAM10 was evaluated in a luciferase assay. Direct binding was indicated by reduced luciferase activity after transduction of lentiviral miRNA-451a vectors in HLF cells. Targeted mutation to the potential binding site abrogated miRNA-451a binding. (D) ATF2 and ADAM10 protein expression was investigated by immunoblotting 48 h after transfection of miR-451a mimics and AllStars as a negative control. The expression of GAPDH was assessed as an endogenous control. Protein bands of three independent transfections were quantified, *p < 0.05.
from the same patient. To ensure sample purity, all specimens were isolated by laser capture microdissection. NanoString analysis identified 49 miRNAs that were significantly altered between non-neoplastic and tumour tissue. Clustering of these miRNAs not only separated non-neoplastic from tumour tissue samples but also underlined the interposition of BilIN as the most common precursor lesion in this process. Although most BilIN samples lined up between non-neoplastic and invasive tumour tissue, a few BilIN samples were also grouped among the normal tissue group. This supports the concept of BilIN as a precursor lesion in the dynamic process of cholangiocarcinogenesis. As the development of non-neoplastic biliary epithelium to intraepithelial neoplasia and finally to invasive carcinoma is accompanied by cumulative molecular alterations [10,31,32], it fits into the concept that some BilIN samples line up with the non-neoplastic or tumour tissue groups, thereby representing less or more aggressive variants.

In accordance with the literature on miRNA expression in colorectal cancer [33], we considered significantly and stepwise up- or downregulated miRNAs as putative oncogenes or tumour suppressors, respectively. We identified 10 potentially oncogenic and 13 tumour suppressive miRNAs. The downregulated miR-451a (−10.9-fold down) and miR-144-3p (−6.3-fold down) presented with the highest fold-change and level of significance in the stepwise progression of carcinogenesis. A similar decrease in both miRNAs was recently

Figure 6. miR-451a represses cell migration by targeting ATF2. (A) Specific ATF2 knockdown was accomplished by transfection of pooled siRNAs (siPool) in SNU-478 and SNU-1196 cells and validated in immunoblots relative to a negative control (NEG). (B) Direct ATF2 inhibition by siPool led to impaired cell migration in SNU-478 and SNU-1196. (C) ATF2 was overexpressed in SNU-478 and SNU-1196 cells using a doxycycline-inducible ATF2 vector. MiR-451a overexpression was achieved by transfection of mimics and AllStars as a negative control. After doxycycline (DOX) incubation for 48 h, cell migration was assessed using a wound healing assay. Overexpression of ATF2 increased and miR-451a transfection decreased cell migration. ATF2 overexpression by DOX induction reversed the anti-migration effect of miR-451a. Bars represent the arithmetic mean ± SD of three independent experiments. Statistical test: unpaired t-test, \( p^* < 0.05 \).
was reversed by ATF2 overexpression in rescue repressed cell migration after transfection of miR-451a. Conversely, ATF2 via pooled siRNAs led to attenuated cell migration 

451a downregulation. At the functional level, silencing ATF2 via pooled siRNAs led to attenuated cell migration, mimicking the effect of miR-451a. Conversely, repressed cell migration after transfection of miR-451a was reversed by ATF2 overexpression in rescue experiments. These results imply that ATF2 plays an oncogenic pro-migrative role in dCCA that is controlled by the tumour suppressive miR-451a. Such a migration-relevant miR-451a-ATF2 axis has recently been proposed for non-small cell lung cancer [48] and hepatocellular carcinoma [46]. ATF2 as a transcription factor is part of the homo-/heterodimeric activator protein 1 (AP1) complex. It is ubiquitously expressed and regulates various cellular processes, for example the stress response [49]. In the context of cancer development, it can assume either oncogenic or tumour suppressive functions depending on its subcellular localisation. For example, when phosphorylated by PKCζ, ATF2 remains in the nucleus, promoting oncogenic functions, whereas localised in mitochondria, it acts as a tumour suppressor initiating apoptosis [50]. Because of this multiplicity of functions, stringent regulation of ATF2 is inevitable for normal cell physiology. miR-451a has been shown to regulate the stability of ATF2 transcripts by binding to the 3' -UTR, mediating tumorigenesis [49]. Thereby our study confirmed that loss of miR-451a-mediated ATF2 inhibition leads to a pro-tumorigenic phenotype by increased migration and invasion ability. We observed that miR-451a decreased stepwise during cholangiocarcinogenesis, which may result in progression to invasive dCCA.

A limitation of our study is the fact that our data are based on a rather small patient cohort. As one single miRNA potentially targets hundreds of mRNAs, it is difficult to explain the observed miRNA effect with one target alone. In particular, concerning miR-144-3p, even though we investigated potential target genes of this miRNA (see supplementary material, Figure S4), we were not able to uncover the molecular mechanism of miR-144-3p in dCCA. Overall, we identified ATF2 as a miR-451a target gene and could show its impact on cancer cell migration. This may serve as a solid basis for further investigations into the role of the miR-451a-ATF2 axis in distal cholangiocarcinogenesis.

In summary, here we present the first systematic miRNA profiling study of BillN revealing molecular alterations in the stepwise tumour progression of dCCA. In this process, we identified a significant downregulation of the tumour suppressive miR-451a/144 cluster leading to a pro-migrative cellular phenotype mediated by elevated ATF2. The miR-451a-ATF2 axis may therefore represent a potential therapeutic target for the treatment of dCCA.

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Author contributions statement

BG and SR initiated the study. MAL, JH, TA, XW, JX, CD and MK carried out the experiments. SR, MAL, JJ and BG were responsible for the study design, data analysis and data interpretation. MAL, SR and BG conceived the experiments, analysed the data and wrote the manuscript. BG collected the patient material. BG carried out the histopathological analysis. TA, JMB, MNV, APW, AM, CR, BK, CS, KH and PS revised the manuscript. All authors had final approval of the submitted and published versions.

Data availability statement

The raw data from NanoString miRNA assays are available at gene expression omnibus (GEO; GSE140001; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140001). The raw data from mRNA microarrays are available at gene expression omnibus (GEO; GSE147969; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147969).

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