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Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma

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Cholangiocarcinoma (CCA), which is a poor prognosis malignancy that arises from the malignant transformation of cholangiocytes, is associated with chronic inflammation of the biliary epithelium. Thus far, the molecular mechanisms of the origin and neoplastic processes of CCA that are promoted by inflammation are still unclear and need to be fully elucidated. Here using small RNA sequencing to determine the microRNA (miRNA) expression profiles in CCA, we found that let-7c, miR-99a and miR-125b, which are three miRNAs of the same cluster, were downregulated in CCA and targeted interleukin 6 (IL-6), IL-6R and type 1 insulin-like growth factor, which are important cytokines and receptors of the IL-6/signal transducer and activator 3 (STAT3) pathway and have key roles in inflammation and CCA initiation. We also found that enforced expression of let-7c, miR-99a or miR-125b could reduce the activity of STAT3 and further suppress CCA tumorigenicity *in vivo* and inhibit the migration and invasion of CCA cells *in vitro*. Surprisingly, let-7c/miR-99a/miR-125b cluster also significantly decreased the ability of CCA cells for cancer stem cell-like mammosphere generation by downregulating CD133 and CD44, which suggests the pivotal roles of let-7c, miR-99a and miR-125b in CCA by regulating both inflammation and stem-like properties. Our findings showed potential links between miRNAs and inflammation, and provide a potential treatment strategy for developing an miRNA-based therapy via IL-6/STAT3 targeting for CCA.

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INTRODUCTION

Cholangiocarcinoma (CCA) is the second-most common primary hepatobiliary malignancy and is generally believed to be a result of malignant transformation of cholangiocytes that line the intrahepatic and extrahepatic biliary tract.[1] The incidence of CCA is increasing worldwide, and the overall survival rate is $< 5\%$ of patients surviving to 5 years.[2] Currently, conventional chemotherapy and radiation therapy have been notably ineffective in improving long-term survival, and the only curative treatment for CCA is surgical resection of the tumor.[2] Therefore, an understanding of the cellular mechanisms underlying CCA pathogenesis is urgently needed to develop efficient treatments. The known risk factors account for only a few cases of CCA and are associated with chronic inflammation of the biliary system; these include hepatolithiasis, liver fluke infection, primary sclerosing cholangitis and choledochal cysts.[3] In recent years, accumulating evidences also suggest the potential roles of CCA stem cells in CCA pathology. Some membrane glycoproteins, including CD133 and CD44, have pivotal roles in cancer stem cells of hepatobiliary malignancy, and have been used as stem cell biomarkers for stem-like cells, however, the underlying mechanisms of CCA stem cells are still not clear yet.[4–7]

Inflammation, one of the most studied risk factors of CCA, favors tumorigenesis by stimulating angiogenesis, damaging DNA, maintaining stem cells in tumor microenvironment and chronically stimulating cell proliferation.[8,9] Thus, studies of inflammation-associated cytokines on the pathogenesis and growth of CCA are of great significance.[10] Interleukin 6 (IL-6) is one of the most important multifunctional cytokines in the response of hepatobiliary epithelia to inflammation.[11] An increased concentration of IL-6 in the bile and serum has been identified as contributing to cholangeitis and abscessus.[12–15] The production of IL-6 through an autocrine–paracrine mechanism by biliary epithelial cells or cholangiocytes mediates the acute phase and immune responses,[16] and can influence the growth of normal and tumor cells and contribute to tumor formation.[17] IL-6 imparts its signal into the cell by forming a complex with the plasma membrane proteins IL-6R and gp130, which results in the activation of Janus kinases (JAKs). The JAKs phosphorylate signal transducer and activator (STAT) proteins, predominantly STAT3 for IL-6, at residue tyrosine 705. The phosphorylated STAT3 dimerizes and translocates to the nucleus, where it binds to DNA and triggers the transcription of target genes, including oncogenes, survival genes, cell cycle regulators, cytokines and molecular modulators of angiogenesis and migration.[18–21] The activation of STAT3 promotes tumorigenesis and progression to aggressiveness. The receptor for type 1 insulin-like growth factor (IGF1R) has also been identified as an important factor for cellular transformation, malignant progression and cancer cell survival.[22] Cross-activation of IGF1R signaling and the IL-6/STAT3 pathway have also been described.[23–25] Several studies suggest an important role for an activated IL-6/STAT3 pathway in the pathogenesis or progression of CCA.[10,11,20,26] However, the mechanism by which aberrant

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activation of the IL-6/STAT3 pathway contributes to tumorigenesis is largely unknown.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs of approximately 22 nucleotides in length that can silence specific target genes through translational repression or direct mRNA degradation by binding to 3′ untranslated regions (3′ UTR).[27] These exist stably in various tissues and control a number of fundamental biological processes. In addition, aberrant expression of miRNAs is reported in various diseases, including CCA. However, the deregulation of miRNAs in CCA remains unclear, and the mechanisms that regulate the inflammatory pathway in the pathogenesis and progression of CCA are poorly understood. In this study, we investigated the miRNA expression profiles in human CCA using small RNA sequencing and found that downregulation of let-7c, miR-99a and miR-125b, which belong to the same miRNA cluster, were associated with activation of the IL-6/STAT3 pathway. Exogenous expression of these miRNAs reduced the activity of STAT3 and inhibited the malignant transformation of CCA cells and tumorigenicity in mice. These results suggest that these miRNAs may serve as pivotal pathogenesis factors, at least in part, by regulating the IL-6/STAT3 signaling pathway.

RESULTS

RNA sequencing identified a novel set of miRNAs that were differentially expressed in CCA

To assess the deregulation of global miRNA expression, we investigated the miRNA spectrum from the pools of three moderately poorly differentiated and three well-differentiated CCA tumors (T), as well as pools of their peritumoral (N) tissues. Overall, 1733 miRNAs, including miRNA's, were evaluated, and the relative expression of each miRNA was examined: 652 were detected in sequencing samples, and only 125 miRNAs had >200 r.p.m. in at least one sample. To confirm the library sequencing, we tested the expression of some randomly selected miRNAs in the pooled samples using quantitative reverse transcriptase–PCR. The results from the two detection methods were coherent (Supplementary Figure 1).

Of the 125 miRNAs, with fold changes >2.0 and *P*-values <0.001 when compared with the normal group, only 10 or 33 miRNAs were upregulated and 21 or 42 miRNAs were downregulated in the well- or moderately poorly differentiated tumor groups, respectively (Supplementary Table 2). Eight miRNAs were differentially expressed in the well and moderately poorly differentiated CCA samples compared with the normal sample: three of the miRNAs (miR-31, miR-200c and miR-141) were upregulated, and five miRNAs (miR-451, miR-486-5p, miR-101, miR-130a and miR-122) were downregulated in the well- and moderately poorly differentiated CCA. To further investigate the expression patterns of miRNAs in CCA, we used quantitative reverse transcriptase–PCR to determine the expression of the miRNAs with important roles in carcinoma, especially in hepatobiliary cancer, which were differentially expressed based on the sequencing data. We found that miR-122, miR-32, miR-101, let-7c, miR-99a and miR-125b were significantly ($P<0.05$) downregulated, whereas miR-200c, miR-21 and miR-221 were significantly ($P<0.05$) upregulated in 24 paired tumor tissues compared with normal tissue (Figure 1). Therefore, these miRNAs may have important roles in CCA.

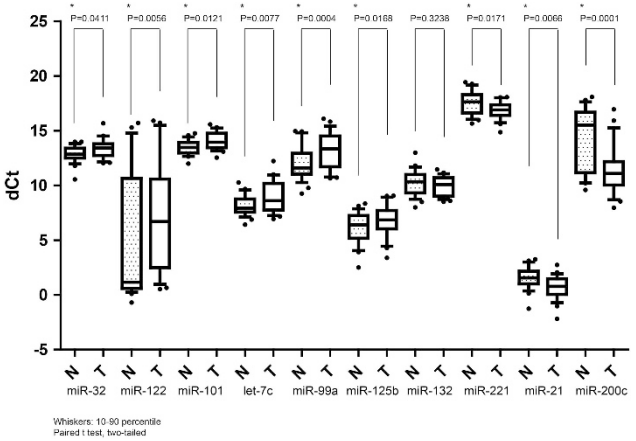


Figure 1. miRNAs are differentially expressed in CCA and adjacent tissues. miR-32, miR-122, miR-101, let-7c, miR-99a and miR-125b were significantly downregulated ($P<0.05$), whereas miR-200c, miR-21 and miR-221 were significantly upregulated ($P<0.05$). $dCt = Ct_{microRNA} - Ct_{internal\ control}$. All indicated *P*-values were determined using a two-tailed, paired *t*-test.

The IL-6/ STAT3 pathway was found to be a prime target for deregulated miRNAs in CCA

Inflammation-associated cytokines and their receptors have been reported having pivotal roles in CCA and most of them serve as important risk factors.[10,28] To screen and investigate whether the differentially expressed miRNAs identified above directly target to the inflammation-associated genes or pathway, we focused on the most common CCA-associated inflammation cytokines include IL-6,[10,26] IL-8,[29] IGF1,[30] transforming growth factor-β[31–33] and VEGF,[33] and predicted the targeting relationship between the deregulated miRNAs in CCA and these cytokines and receptors using Starbase v2.0, which is a database that combines prediction algorithms of miRNA-mRNA interactions and large-scale CLIP-Seq data.[34] The results showed that most of these miRNAs could interact with at least one of the cytokines or receptors, as shown in Figure 2, which implies that many miRNAs may be involved in CCA by regulating these inflammation-associated cytokine pathways. Interestingly, among the deregulated miRNAs, we found that miR-99a, let-7c and miR-125b, which belong to the same cluster located on chromosome 21q21,[35] could regulate all of the mentioned inflammation-associated cytokine pathways that are involved in CCA. As miRNAs that originate from the same genomic cluster may be co-expressed and involved in pathways that cooperatively contribute to cell progression, we speculated that the let-7c/miR-99a/miR-125b cluster may be an important regulator involved in CCA by regulating cytokine-induced inflammatory pathways.

To investigate whether the let-7c/miR-99a/miR-125b cluster acts synergistically in CCA, we first detected the expression levels of miR-99a, let-7c and miR-125b in cancerous tissues of CCA patients, and analyzed the correlation relationships of three miRNAs using Pearson correlation test. We found that these miRNAs were concordantly expressed, that is, a patient with a high expression level of any one of let-7c/miR-99a/miR-125b cluster also showed high expression levels of the other two (Figure 3a). Next, we investigate whether these three co-expressed miRNAs were involved in cooperative pathways. As IL-6 is one of most important inflammation cytokines involved in pathogenesis and growth of CCA,[10,11] and the interaction of IL-6 with IL-6R that results in the activation of STAT3 have been demonstrated importance in inflammation and cancer initiation,[18] we chose the IL-6/STAT3 inflammatory pathways to experimentally address the miRNA target interactions with inflammation cytokines and receptors. As shown in Figure 2, the inflammation factor IL-6 was predicted to be a target of let-7c, and its receptor, IL-6R, was a potential target of let-7c and miR-125b. In addition, IGF1R was a predicted target of miR-99a. IGF signaling could activate the STAT3 signaling pathway.[22–24] We co-transfected the individual miRNAs (miR-99a, let-7c or miR-125b) with a Renilla luciferase reporter construct containing the wild type (WT) or mutated versions of the putative mRNA 3′ UTR fragment into HEK-293T cells (RNA sequences in

Figure 3b). Relative to the nonspecific control miRNA, co-transfection with each miRNA significantly suppressed luciferase activity of the vector containing the WT 3′ UTR compared with the empty vector and the mutated version (histograms in Figure 3b). Moreover, overexpression of let-7c, miR-99a or miR-125b in lentivirus-infected CCA cell lines (Supplementary Figure 2) reduced the protein expression of their corresponding targets (let-7c targets IL-6, let-7c/miR-125b targets IL-6R and miR-99a targets IGF1R) (Figure 3c). Together, these data demonstrate that let-7c and miR-125b are able to regulate IL-6R expression, let-7c can also target IL-6, and IGF1R is the bona fide target of miR-99a.

As IL-6, IL-6R and IGF1R are associated with IL-6/STAT3 signaling, we further tested whether let-7c, miR-99a and miR-125b could affect the activity of STAT3. Treatment of CCA cells with IL-6 for the indicated time induced and maintained STAT3 phosphorylation in control CCA cells. Previous studies suggested that phosphorylated STAT3 is not expressed without IL-6 stimulation and peaked on 0.5 h after IL-6 stimulation,[20,36] thus in the study we only detected STAT3 phosphorylation level on 0.5, 1 and 2 h after IL-6 stimulation. We found that exogenous let-7c, miR-99a and miR-125b promoted the reduction of phosphorylated STAT3 and eliminated the activity of STAT3 (Figure 4a). These experiments further support let-7c/miR-99a/miR-125b having important roles in the IL-6/STAT3 signaling pathways.

We next investigated whether downregulation of miRNAs in clinical CCA samples resulted in the upregulation of their targets. We evaluated the protein expression levels of IGF1R, IL-6R and IL-6 as well as STAT3 phosphorylation in 18 randomly paired protein available CCA samples using western blotting. The results showed that IL-6R, IGF1R and pSTAT3 were elevated in approximately 77.8% (14/18), 55.6% (10/18) and 72.2% (13/18) of tumor tissues, respectively (Figure 4b and Supplementary Figure 3). Unfortunately, the expression of IL-6 was barely detectable, which could be caused by excretion out of the cell and maintenance in tumor tissues.

All together, these data demonstrated that the IL-6/STAT3 pathway is under the control of let-7c/miR-99a/miR-125b, and that aberrant activation of the IL-6/STAT3 pathway by the downregulation of let-7c/miR-99a/miR-125b may cause the pathogenesis of CCA.

Enforced expression of let-7c/miR-99a/miR-125b inhibited CCA mammosphere formation *in vitro*

Many studies have revealed that the activation of IL-6/STAT3 signaling is an important contributor to inflammation-induced cancer.[18–20] To verify the impact of let-7c/miR-99a/miR-125b on the pathogenesis of CCA, we first used a mammosphere culture system to evaluate the function of these miRNAs on the capability of CCA cells in malignancy transformation.[37] Single-cell suspensions of CCA cells were grown in conditions that do not allow for adherence to a substratum. Most CCA cells died under these conditions, but a small number survived and generated floating, spherical colonies, and these cells showed increased potential for self-renewal and differentiation and presented more stem-like, malignant cells.

MZ-cha-1 and SK-cha-1 cells were cultured in suspension (plated on ultra-low attachment plates) to generate mammospheres, but not under adherent conditions (Figure 5a). The expression levels of let-7c/miR-99a/miR-125b were all substantially downregulated in mammosphere cells compared with non-mammosphere cells (Figure 5a) in both cell lines, implying the inhibitory roles of let-7c/miR-99a/miR-125b in mammosphere generation. To further test whether low levels of let-7c/miR-99a/miR-125b could control mammosphere generation and, thus, regulate cancer cell self-renewal, we studied the effect of enforced let-7c/miR-99a/miR-125b expression using the mammosphere

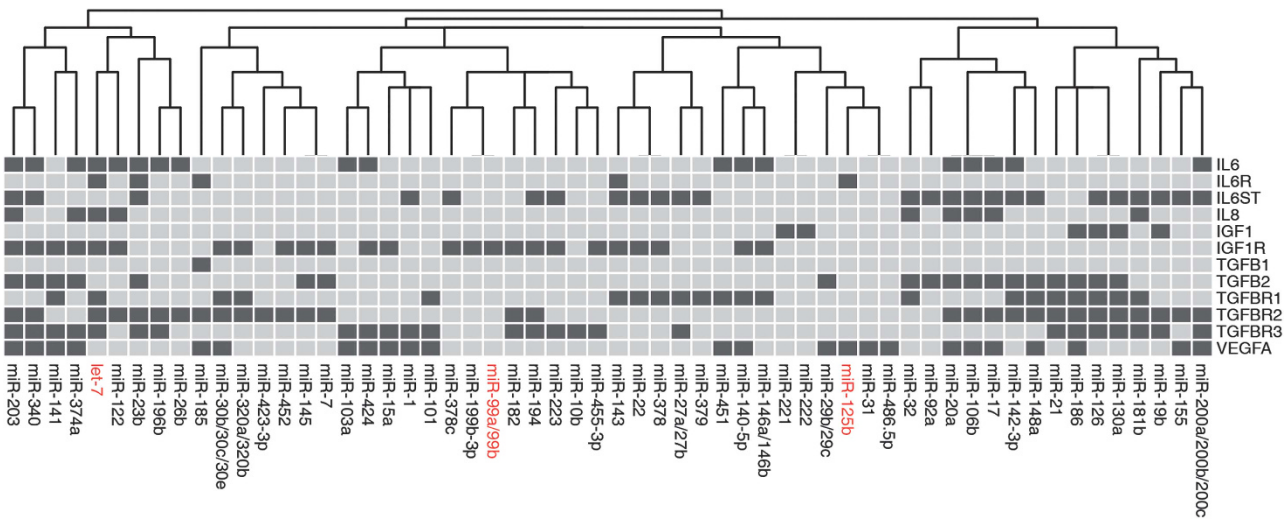
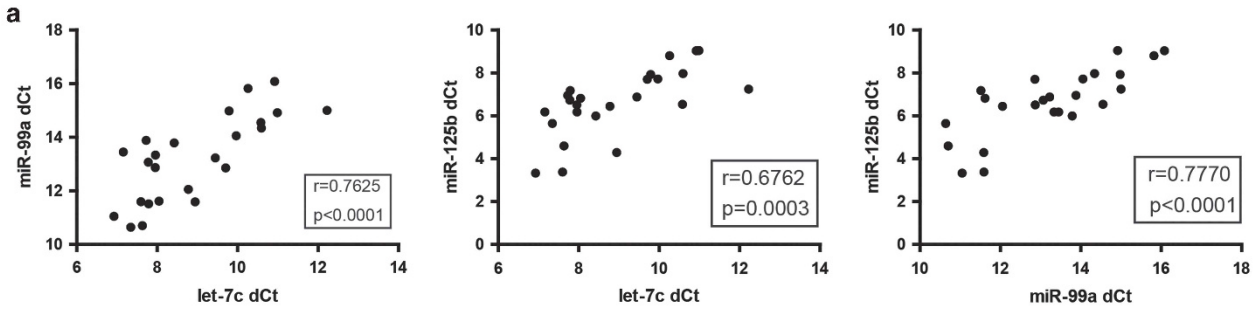
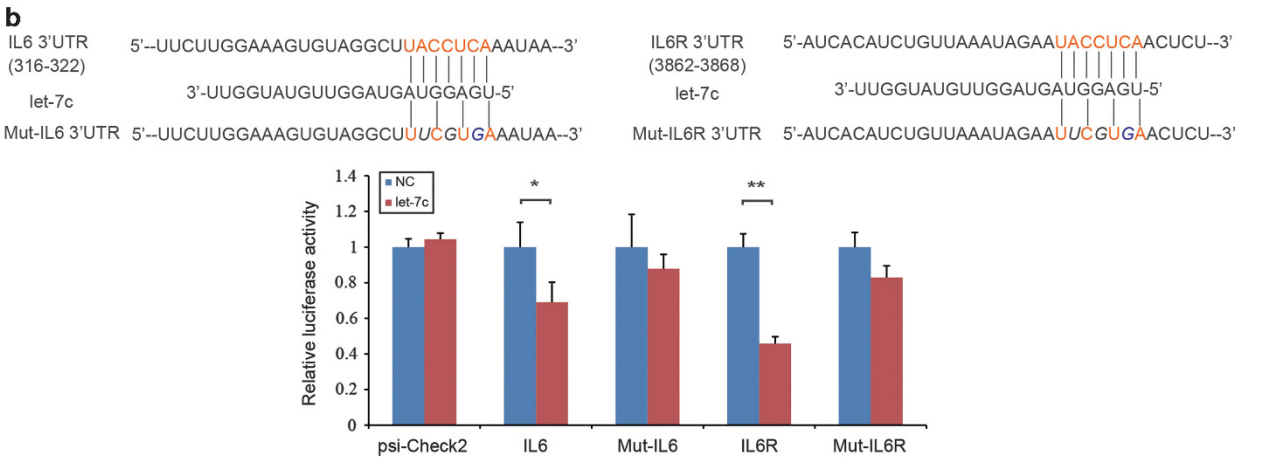
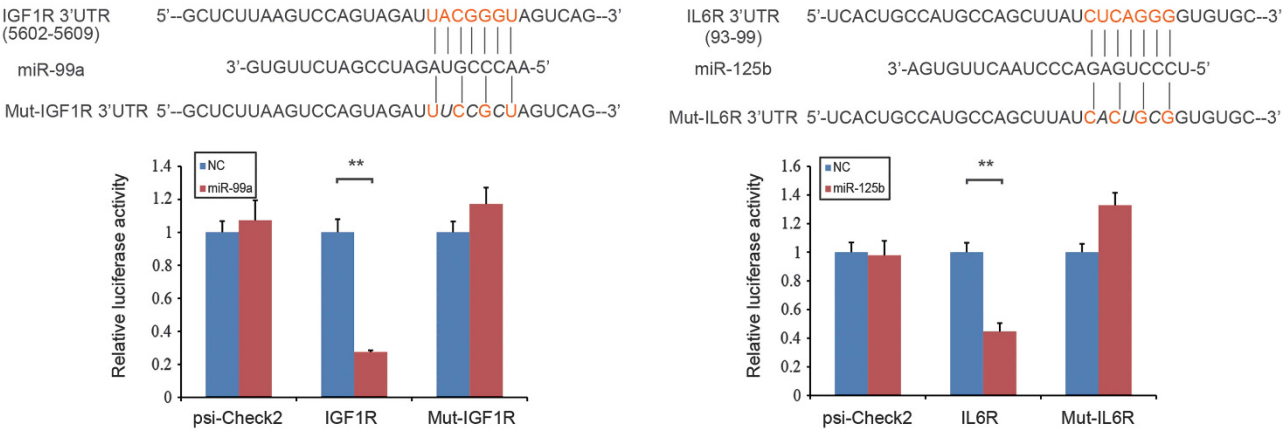


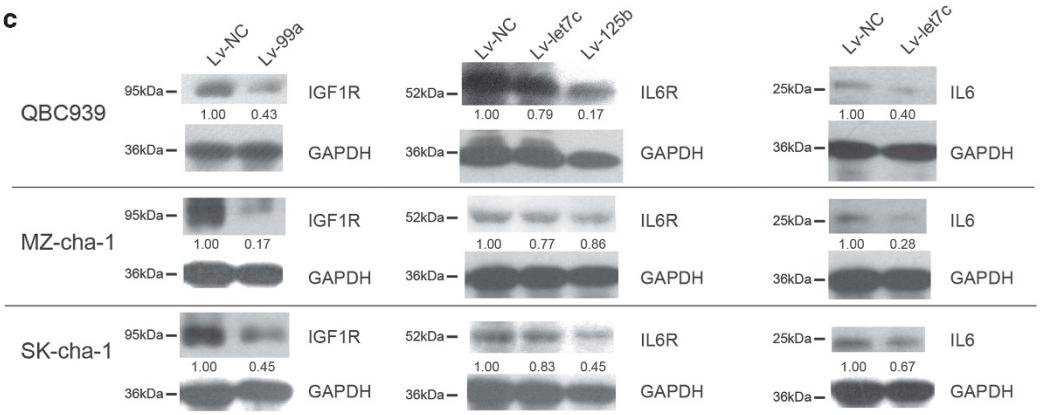
Figure 2. The targeting relationships are predicted between the deregulated miRNAs and common, CCA-associated cytokines and receptors. The dark points represent at least one miRNA target site that was predicted using Starbase v2.0 within the 3' UTR of the genes.

Figure 3. IGF1R, IL-6R and IL-6 are direct targets of let-7c/miR-99a/miR-125b. (**a**) The positive correlation among miR-99a, let-7c and miR-125b, correlation analysis was determined using the Pearson product–moment correlation. (**b**) Schematic representation of the interaction of an miRNA with the 3′ UTR of its corresponding target. Each predicted MRE 3′ UTR was inserted into a psiCHECK-2 vector immediately downstream from the Renilla luciferase gene. Luciferase reporter assays analyzing the putative targets of the three miRNAs. HEK-293T cells were co-transfected with psiCHECK-2 plasmids with the 3′ UTR of the target genes or the target genes with mutated miRNA-binding sites (Mut) and miRNA mimics or negative control mimics (NC) using Lipofectamine 2000. Firefly luciferase activity was normalized to Renilla luciferase activity, and the results were expressed relative to the control. (**c**) QBC939, MZ-cha-1 and SK-cha-1 cells were infected by lentivirus overexpressing miRNA or negative controls, and cells lysates were prepared for western blotting with antibodies against IGF1R, IL-6R or IL-6. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression served as a loading control, and densitometric ratios were recorded. Experiments were performed in triplicate, and all indicated *P*-values were determined using *t*-test. $*P < 0.05$; $**P < 0.01$.









assay. Consistent with our expectations, the sphere-forming capacity was significantly reduced by miRNAs compared with the negative controls (26.7% for let-7c, 71.1% for miR-99a and 44.4% for miR-125b in MZ-cha-1; and 38.1% for let-7c, 24.8% for miR-99a and 31.4% for miR-125b in SK-cha-1; Figures 5b and c), and the volume of the mammospheres was smaller in let-7c/miR-99a/miR-125b overexpressing MZ-cha-1 and SK-cha-1 cells than in control cells (Figures 5b and c). To address whether the effects on mammosphere formation are indeed because of the suppression of stem cell-like properties, we measured the level of CD133 and CD44, markers of CCA stem cells, in mammospheres. In addition, we found that the percentage of $CD133^{+}CD44^{+}$ cells is reduced in MZ-cha-1 and SK-cha-1 cells with let-7c/miR-99a/miR-125b overexpression (Figures 5b and c, right lanes). These data demonstrate that let-7c/miR-99a/miR-125b has an important suppressing role in CCA cell malignancy transformation, and these miRNAs may also be involved in the maintenance and proliferation of tumor stem cells in CCA, which further provides potential effective targets for CCA treatment.

Enforced expression of let-7c/miR-99a/miR-125b inhibited CCA cell migration and invasion *in vitro*

The activation of STAT3 has also been linked to the progression of cancer by promoting tumor migration.[38,39] As let-7c/miR-99a/miR-125b regulated the activation of IL-6/STAT3 pathway in CCA cells, we postulated that ectopic expression of let-7c/miR-99a/miR-125b would suppress malignancy transformation and impede the migratory and invasive abilities of CCA cells. We therefore used cell migration and cell invasion assays to confirm this speculation. Consistent with our expectation, overexpressed let-7c/miR-99a/miR-125b significantly decreased the migration ability of MZ-cha-1 and SK-cha-1 cells (Figure 6a) and decreased the cell invasion of MZ-cha-1 and SK-cha-1 cells (Figure 6b). We performed cellular viability and apoptosis assays to determine whether the inhibition on the migratory and invasive potential is due to a reduction in survival and proliferation, and no significant repression was observed with these miRNAs overexpression (Supplementary Figure 4), which suggested that re-forced let-7c/miR-99a/miR-125b expression helped inhibit the migration and invasion capabilities of the cells, and these inhibitions are not due to altered proliferation or apoptosis rates. It is also noteworthy that overexpressed let-7c/miR-99a/miR-125b showed similar functions as STAT3 inhibitors in CCA,[39] which further suggested that let-7c/miR-99a/miR-125b helps to inhibit the capability of tumor migration by affecting the IL-6/STAT3 pathway.

Let-7c/miR-99a/miR-125b suppress tumorigenicity *in vivo*

To examine whether let-7c/miR-99a/miR-125b also inhibited the capability of malignant transformation and tumor migration of CCA cells and tumor growth *in vivo*, let-7c/miR-99a/miR-125b-overexpressing and control MZ-cha-1 and SK-cha-1 cells were injected subcutaneously into the flanks of nude mice, and the animals were closely monitored for tumor growth.

Consistent with the *in vitro* results, a significant and sustained suppression of tumor growth was found in animals injected with let-7c/miR-99a/miR-125b-overexpressing cells compared with the control group, suggesting tumor-suppressing roles for all three miRNAs (Figures 7a and b and Supplementary Figures 5A and B). Let-7c/miR-99a/miR-125b also significantly reduced the tumor weight compared with that of the control group in mice (Figure 7c and Supplementary Figure 5C), whereas no significant changes in body weight among groups. All these data further confirmed the tumor-suppressing roles of the let-7c/miR-99a/miR-125b cluster in CCA pathogenesis.

As we found that the let-7c/miR-99a/miR-125b cluster controlled tumorigenesis by targeting IL-6, IL-6R and IGF1R and affecting the IL-6/STAT3 inflammatory pathway *in vitro*, we asked whether this mechanism also worked *in vivo*. To examine this, we then evaluated expression of the inflammatory cytokine IL-6, as well as the two cytokine receptors IL-6R and IGF1R, in mice tumor tissues. The results showed that the protein level of IL-6, IL-6R and IGF1R was repressed in most miRNA overexpressing tumor tissues and, more importantly, that phosphorylation of STAT3 as well as activated phosphorylated IGF1R were also reduced in these tissues (Figure 7d, Supplementary Figures 5D and 6). These findings indicated that these inflammatory cytokines and

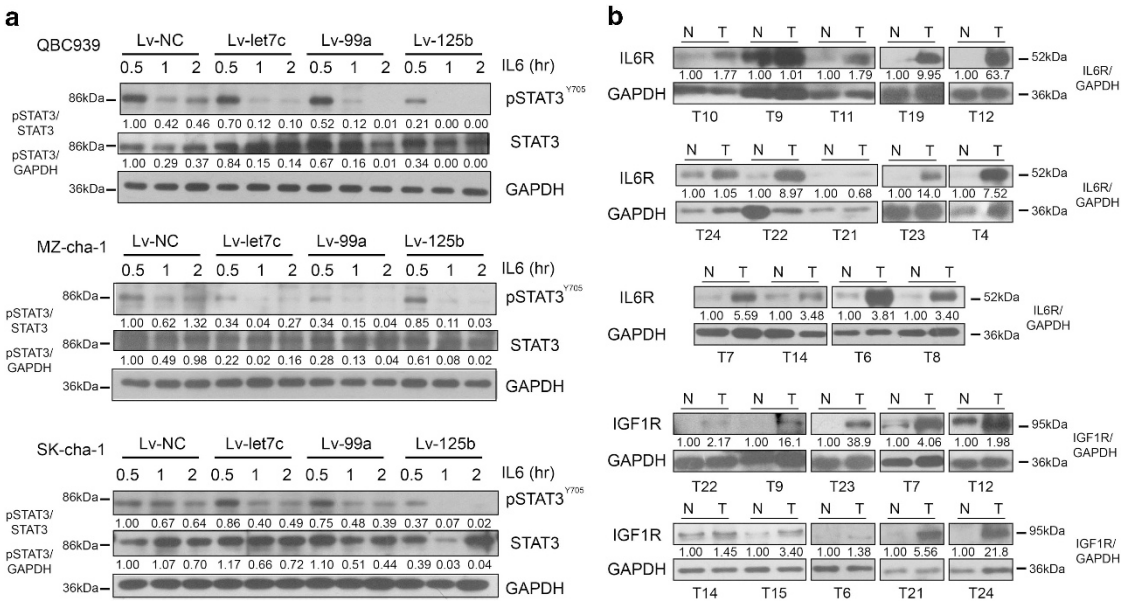


Figure 4. The IL-6/STAT3 pathway is regulated by let-7c/miR-99a/miR-125b in CCA cell lines and clinical samples. (**a**) Treatment of QBC939, MZ-cha-1 and SK-cha-1 cells with IL-6 for 0.5, 1, or 2 h, respectively, induced and maintained STAT3 phosphorylation; and exogenous let-7c, miR-99a and miR-125b eliminated the activity of STAT3. (**b**) Downregulation of let-7c/miR-99a/miR-125b in clinical CCA samples resulted in the upregulation of IL-6R and IGF1R. GADPH expression served as a loading control and densitometric ratios were recorded. Experiments were performed in triplicate.

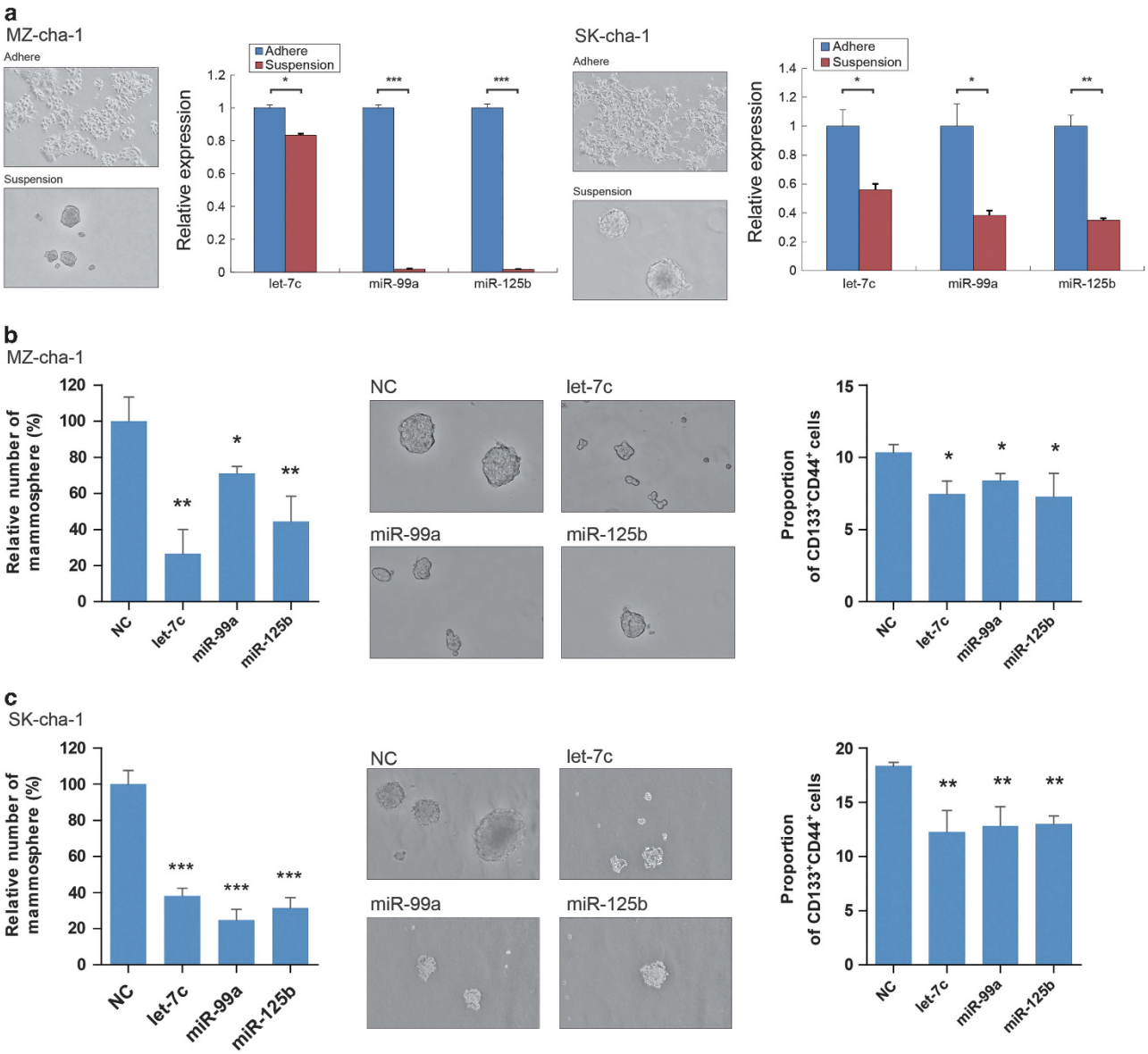


Figure 5. let-7c/miR-99a/miR-125b inhibited CCA cell transformation and migration *in vitro*. (**a**) The let-7c/miR-99a/miR-125b cluster is significant downregulated in mammospheres cultured in suspension (*t*-test). (**b**) Enforced let-7c/miR-99a/miR-125b expression suppressed mammosphere generation and proportion of $CD133^+CD44^+$ cells in MZ-cha-1 cells. (**c**) Enforced let-7c/miR-99a/miR-125b expression suppressed mammosphere generation and proportion of $CD133^+CD44^+$ cells in SK-cha-1 cells. An average of three times of experiments was showed in each experiment, and one-way analysis of variance test was used. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

receptors were regulated by the overexpressed miRNAs, which led to inhibition of the IL-6/STAT3 pathway in CCA cells. Further immunofluorescence analysis of CD44 demonstrated the stemness of CCA cells was repressed by let-7c/miR-99a/miR-125b in xenograft tumor specimens (Supplementary Figure 7). Therefore, the regulated inflammatory pathway blocked the pathogenesis and progression of CCA *in vivo*.

DISCUSSION

The importance of some deregulated miRNAs have been recognized in CCA;[40] however, little is known about the link between miRNAs and inflammation of the biliary epithelium, which is one of the most important risk factors of CCA.[41] Here, small RNA sequencing was selected to screen the deregulated miRNAs in CCA tissues. Among the ectopic miRNAs, the family members of the let-7c/miR-99a/miR-125b cluster were found similarly downregulated and targeted the IL-6/STAT3 pathway. Furthermore, overexpressed let-7c/miR-99a/miR-125b reduced the activation of STAT3 and inhibited the malignant transformation of CCA cells *in vitro* and tumorigenicity *in vivo*.

MiRNAs have critical roles in carcinogenesis, and also have been regarded as potential biomarkers for cancer diagnosis and targets for anticancer therapeutics in various cancers.[42,43] However, not all of the ectopic miRNAs in cancer tissues have roles in oncogenesis; only the most abundant miRNAs in a cell mediate target suppression, and miRNAs that are expressed below ~100 copies per cell have little regulatory capacity.[44,45] Therefore, our work revealed the deregulation of miRNAs with >200 r.p.m. in well- and moderately poorly differentiated CCA tissues compared with normal tissues using high-throughput sequencing. These data provide global and, more importantly, promising functional profiling of miRNAs in the pathogenesis and progression of CCA.

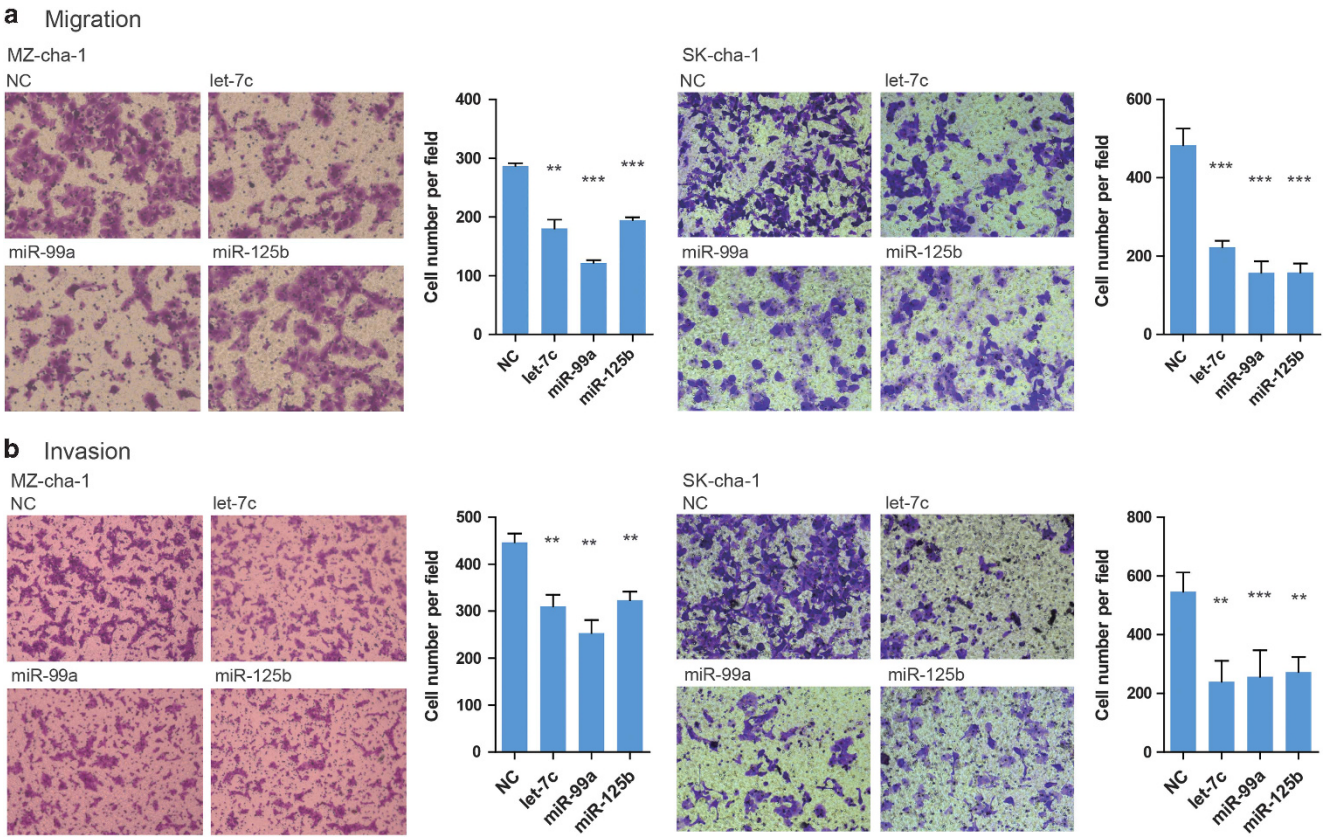


Figure 6. let-7c/miR-99a/miR-125b inhibited CCA cell migration and invasion *in vitro*. Enforced let-7c/miR-99a/miR-125b expression decreased the ability of (**a**) migration and (**b**) invasion of MZ-cha-1 and SK-cha-1 cells. Five random sights in each sample were selected to analyze cell count, and the mean of triplicate experiments was exhibited. $^{**}P < 0.01$; $^{***}P < 0.001$.

In this article, we found that that let-7c, miR-99a and miR-125b are downregulated in CCA patients. Overexpression of let-7c/miR-99a/miR-125b suppresses CCA tumorigenicity *in vivo* and inhibits the migration and invasion by targeting important cytokines and receptors (IL-6, IL-6R and IGF1R) of the IL-6/STAT3 inflammation pathway. Surprisingly, let-7c/miR-99a/miR-125b also significantly decreased the ability of CCA cells for cancer stem cell-like mammosphere generation. The members of the let-7, miR-99/100 and miR-125 family are organized into three clusters in the human chromosome. These miRNAs are often co-expressed and participate in the same biological pathways. Our previous studies have revealed that miR-125b and miR-100 are upregulated in acute myeloid leukemia and block myeloid cell differentiation.[46–48] A recent study also indicated that miR-99a and miR-100 are downregulated in acute lymphoblastic leukemia and promote cell apoptosis induced by glucocorticoids.[49] Henson *et al.*[50] reported that miR-125b and miR-100 are downregulated in oral squamous cell carcinoma, and exogenous expression of miR-125b and miR-100 significantly reduced cell proliferation. In prostate cancer, the androgen receptor binds to the host gene of the let-7c/miR-99a/miR-125b cluster, LINC00478, and this cluster is repressed by the chromatin remodeler EZH2 when the androgen receptor is stimulated.[35] Meng *et al.*[51] reported that miR-99a is reduced by overexpression of IL-6 in CCA cells, which indicates that the let-7c/miR-99a/miR-125b cluster may be repressed by the IL-6 inflammation factor and may be involved in inflammatory signaling pathways.

Inflammation is a complex response, and inflammatory cytokines, including ILs, T-cell growth factor and chemokines, contribute to control of the magnitude of the inflammatory responses. These cytokines are often regarded as important mediators in CCA. Here, we found many miRNAs that were deregulated in CCA could regulate various inflammatory cytokines, inflammation-related cytokines and their receptors, which implies that these deregulated miRNAs are involved in multiple inflammatory signaling pathways. Among these pathways, IL-6-mediated STAT3 activation, which is aberrantly sustained in CCA cells,[26] is one of the well-established, classical pathways. To our knowledge, this is the first report to demonstrate the contribution of the let-7c/miR-99a/miR-125b cluster to the pathogenesis of CCA.

Notably, the let-7c/miR-99a/miR-125b cluster showed significant inhibition of tumorigenicity *in vivo* and of cell progression *in vitro*, which further raised the potential of the let-7c/miR-99a/miR-125b cluster serving as molecular targets for CCA treatment. Furthermore, pSTAT3 inhibition was prolonged when cells were treated with let-7c/miR-99a/miR-125b, which may counteract many biological effects of STAT3-mediated tumorigenesis pathways. Moreover, previous reports have shown that inhibition of STAT3 by small interfering RNA effectively inhibited tumor growth and overcame drug resistance,[52–55] thus, the endogenous miRNAs that target STAT3 may provide a more natural and secure choice for treatment, and our research provides an alternative strategy for developing an miRNA-based therapy via IL-6/STAT3 targeting for CCA.

In conclusion, this study screened the miRNA profiles of CCA and provided a first glimpse of the functional role of the let-7c/miR-99a/miR-125b cluster by targeting pivotal inflammatory factors such as IL-6, IL-6R and IGF1R, which further suppress the activation of STAT3 and inhibit tumor pathogenesis and progression. These results indicate the potential roles of the let-7c/miR-99a/miR-125b cluster as serving as therapeutic targets in CCA.

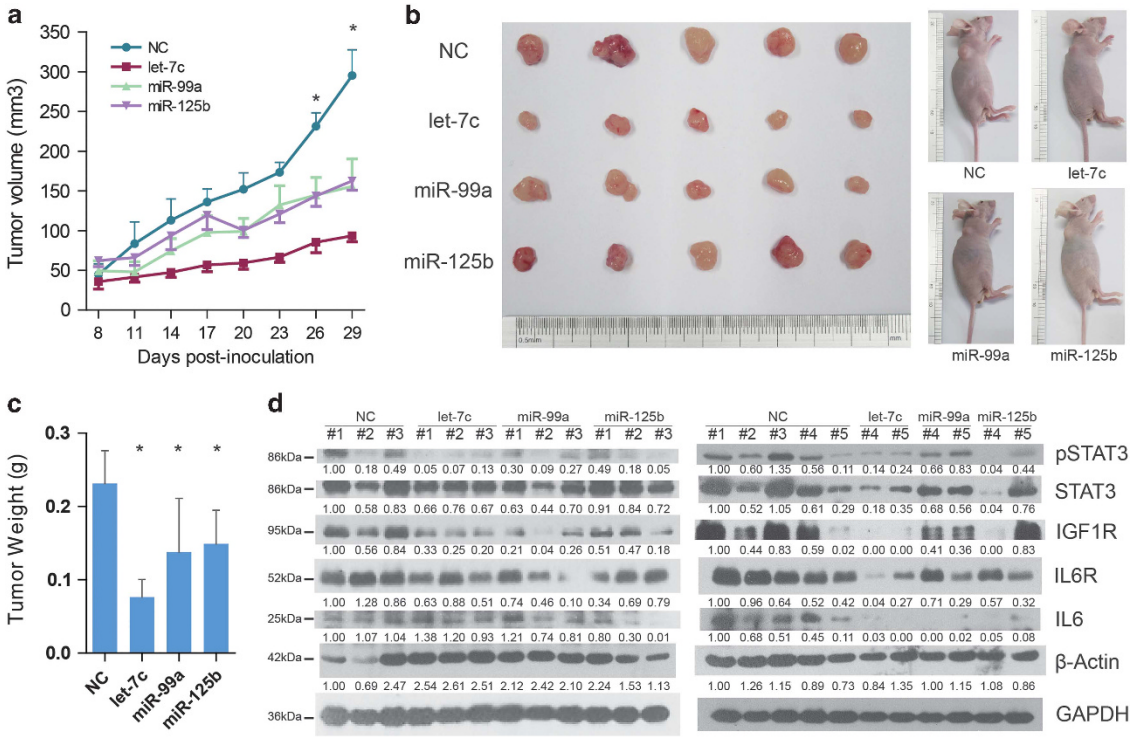


Figure 7. Let-7c/miR-99a/miR-125b suppressed tumorigenicity of CCA cells *in vivo*. let-7c, miR-99a and miR-125b reduced (**a**, **b**) tumor volume (mean ± s.e.m., one-way analysis of variance) and (**c**) tumor weight in nude mice (mean ± s.d., one-way analysis of variance, five mice per group). (**d**) In tumors overexpressing the miRNAs, IL-6, IL-6R and IGF1R as well as the phosphorylation of STAT3 were suppressed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin served as loading controls, and densitometric ratios of objective protein/GAPDH were recorded. *$P < 0.05$.

Table 1. Clinicopathological characteristics of CCA patients

Code	*Location*	*Age*	*Gender*	*Size (cm)*	*Hepatitis*	*CA 19-9 (U/ml)*	*Differentiation*	*Stage (TNM)*
T1	E	60	F	2.5	HBV−; HCV−	3154	M	T3N0M0
T2	E	47	F	4.0	HBV+; HCV−	82.9	W	T2N1M0
T3	E	76	M	4.6	HBV−; HCV−	32 394	W	T?N0M0
T4	I	63	F	2.4	HBV−; HCV−	55.9	W	T2N1M0
T5[a]	E	33	F	4.0	HBV−; HCV−	3.2	M-P	T4N1M0
T6	NA	71	F	5.0	HBV−; HCV−	2518	P	T3N1M0
T7[a]	I	50	M	5.0	HBV+; HCV−	2098.3	W	T3N0M0
T8[a]	I	62	M	10.0	HBV+; HCV−	14 047.5	M-P	T3N1M0
T9	E	73	M	4.0	HBV−; HCV−	164.1	M	T1N0M0
T10	I	71	F	7.9	HBV−; HCV−	68.2	M-P	T1N0M0
T11	I	56	M	5.0	HBV+; HCV−	145.9	M	T4N?M0
T12	E	63	F	3.0	HBV−; HCV−	2041	P	T3N1M0
T13[a]	I	59	M	7.0	HBV+; HCV−	18 706	M-P	T3N1M0
T14	E	50	M	1.5	HBV−; HCV−	158.8	W	T2N0M0
T15[a]	I	45	F	8.5	HBV−; HCV−	17 929.9	W	T3N0M0
T16	NA	49	M	1.5	HBV−; HCV−	946	M	T?N1M0
T17[a]	E	86	M	2.0	HBV−; HCV−	46.3	W	T?N0M0
T18	E	57	M	3.8	HBV−; HCV−	160.6	W	T4N1M0
T19	E	46	M	1.2	HBV−; HCV−	NA	W	T2N0M0
T20	I	62	F	3.0	HBV−; HCV−	1922.2	M	T2N1M0
T21	E	61	M	3.5	HBV+; HCV−	0.9	W	T3N1M0
T22	I	66	M	5.0	HBV−; HCV−	0	W	T3N1M0
T23	E	67	M	2.5	HBV−; HCV−	606.4	M-P	T3N1M0
T24	E	62	M	2.7	HBV−; HCV−	29.5	M	T4N0M0

Abbreviations: Age: age at surgery; CCA, cholangiocarcinoma; Differentiation: W, well, M, moderately, M-P, moderately poorly and P, poorly differentiated; Location: I, intrahepatic, E, extrahepatic; Gender: M, male, F, female; NA, not available; Size: measured in centimeters (cm) at the time of resection; Stage (TNM): unable to evaluate; TNM: Topography Lymph Node Metastasis. [a]Deep-sequencing sample.

MATERIALS AND METHODS

Patient samples and cell lines

Human CCA and peritumoral (designated as normal) tissues were obtained with informed consent between 2011 and 2013 from Sun Yat-sen Memorial Hospital. Sample collection was approved by the Hospital's Protection of Human Subjects Committee. Twenty-four pairs of normal peritumoral specimens and pathologically diagnosed biopsy specimens were obtained from the same patients with CCA who underwent surgical resection. The clinicopathological characteristics of the CCA patients are summarized in Table 1.

QBC939 human CCA cells were obtained from Shuguang Wang (The Third Military Medical University, Chongqing, China), whereas SK-cha-1 and MZ-cha-1 human CCA cells were kindly provided by Dr Chundong Yu (Xiamen University, Fujian, China).

Library preparation, quantitative reverse transcription real-time PCR and RNA sequencing

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and quantitative reverse transcription real-time PCR was performed as reported previously.[46,56] dCt (delta Ct) values of specific gene were normalized with those obtained from the amplification of the internal control, in detail, $dCt = Ct_{gene} - Ct_{internal\ control}$, and the relative expression of gene $= 2^{-(dCtgene\ -\ dCtcontrol)}$. The primers for real-time PCR are shown in Supplementary Table 1. Small RNA library preparation and sequencing were performed using Solexa sequencing Technology (BGI, Shenzhen, China), and sequencing data analysis was performed as previously described.[57]

Luciferase assays

HEK-293T cells were co-transfected with 200 ng of psiCHECK-2-derived reporter vectors and 50 nM of miRNA mimics or the mimic control and then applied to a luciferase reporter assay, as previously described.[46]

Lentiviral vector production and stably transduced cells

For lentiviral vector construction, a genomic fragment containing the pre-miRNA (~200 bp) was inserted into the pGreenPuro vector (System Biosciences, Johnstown, PA, USA). A fragment that could form a hairpin structure that was processed into a small interfering RNA that targeted firefly luciferase was inserted into the same vector as a negative control. Primers and other oligonucleotides are shown in Supplementary Table 1.

Immunoblotting and immunofluorescence analysis

Cells were lysed in RIPA buffer. The proteins were detected with anti-IL-6 (Santa Cruz, Dallas, TX, USA, sc-7920), anti-IL-6R (Abcam, Cambridge, UK, ab128008), IGF1R (Sigma-Aldrich, St Louis, MO, USA, SAB1104892), anti-STAT3 (Santa Cruz, sc-482), anti-phoshpo-STAT3 (Cell Signaling, Danvers, MA, USA, 9145), anti-phoshpo-IGF1R (Cell Signaling, 2969), anti-beta-actin (Cell Signaling, 4970) or anti-glyceraldehyde 3-phosphate dehydrogenase (Protein Tech Group, Chicago, IL, USA, 10494-1-AP). Immunoreactivity was determined using the ECL method (Millipore, Boston, MA, USA) according to the manufacturer's instructions.

Migration and invasion assays

A 24-well transwell plate (8-μm pore size, Corning, NY, USA) was used to measure migratory and invasive ability. For the transwell migration assays, 5×10^4 lentiviral stably transduced MZ-cha-1 cells were plated in the top chamber that was lined with a non-coated membrane. For the invasion assays, the chamber inserts were coated with a 1:8 deliquation of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Then, 2.5×10^5 cells were plated in the top chamber. After incubation at 37 °C for 24 h, the cells located in the lower chamber were fixed, stained with 0.1% crystal violet and counted using a microscope. The mean of triplicate assays for each experimental condition was determined.

Proliferation and apoptosis assays

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8). After transfection, 5×10^3 cells per well were plated in 96-well sterile plastic culture plates, and the CCK-8 assay (Dojindo Molecular Technologies, Shanghai, China) was performed after 0, 24, 48, 72, and 96 h. To assess the rate of apoptosis, the Annexin V-PI Kit (Nanjing Keygen, Nanjing, China) was used according to the manufacturer's guidelines. The detection was performed with a FACS Calibur using CellQuest software (BDIS, San Jose, CA, USA).

Mammosphere culture

Cells (1500 cells/ml) were cultured for 8 days in serum-free DMEM-F12 (BioWhittaker, Radnor, PA, USA) supplemented with B27 (1:50, Invitrogen), 20 ng/ml EGF (Invitrogen), 20 ng/ml bFGF (Invitrogen) and Antibiotic-antimycotic (1:100, Invitrogen) in suspension in Corning Costar 3471 6-well plates.

Flow cytometry

Flow cytometry for CD133 and CD44 expression was performed using phycoerythrin-conjugated monoclonal mouse anti-human CD133/1 (Miltenyi Biotec, Bergisch Gladbach, Germany) and antigen-presenting cell-conjugated mouse anti-human CD44 (Miltenyi Biotec). Samples were analyzed on a BD FACS Calibur (BD Biosciences) and data were analyzed using Flowjo software (BD Biosciences).

Animal model

Six-week-old male nude mice were maintained under specific, pathogen-free conditions in the Laboratory Animal Center of Sun Yat-sen University. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiments. Mice were randomly assigned to four groups with five mice, and investigators were blinded to the group allocation. In each group, lentiviral stably transduced MZ-cha-1 or SK-cha-1 cells (2.5×10^6) were subcutaneously injected into the dorsal right flanks of the mice, and the mice were monitored each 3 days for tumor growth.

Statistical analysis

Two-tailed Student's *t*-test was performed to determine statistically significant differences between two groups, and multiple comparisons were done using one-way analysis of variance, and a *P*-value of < 0.05 was considered significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Variation within each group were estimated, and the statistically comparison would not perform unless the variance was similar between groups. If not mentioned, all data were expressed as the mean ± s.d. of three independent experiments. The linear correlation between miRNAs was measured by Pearson product–moment correlation test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

K-YL, HY and B-WH conceived and carried out the experiment, wrote the paper; W-TW, P-PW and X-JL performed mammosphere formation experiments and migration and invasion assays; Y-QC conceived the experiment and cowrote the paper.

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