Non-coding RNA in cholangiocarcinoma

Massimiliano Salati[1-2], Chiara Braconi[1-3]

The Institute of Cancer Research, London, United Kingdom
University Hospital of Modena and Reggio Emilia, Modena, Italy
The Royal Marsden NHS Trust Surrey and London, United Kingdom

Correspondence to:
Chiara Braconi, MD PhD; Division of Cancer Therapeutics, The Institute of Cancer Research, 7N1 Haddow bldg, 15 Cotswold rd, Sutton SM2 5NG
Chiara.braconi@icr.ac.uk

Key words: cholangiocarcinoma, biliary cancer, microRNA, biomarker.

Abstract

Cholangiocarcinomas (CCAs) are tumors with a dismal prognosis. Early diagnosis is a key challenge because of the lack of specific symptoms, and the curability rate is low due to the difficulty in achieving a radical resection and the intrinsic chemoresistance of CCA cells. Non-coding RNAs (ncRNAs) are transcripts that are not translated into proteins but exert their functional role by regulating the transcription and translation of other genes. The discovery of the first ncRNA dates back to 1993 when the microRNA (miRNA) *lin-4* was discovered in *Caenorhabditis elegans*. Only 10 years later miRNAs were shown to play an oncogenic role in cancer cells and within 20 years miRNA therapeutics were tested in humans. Here we review the latest evidence for a role for ncRNAs in CCA and discuss the promise and challenges associated with the introduction of ncRNAs into clinical practice.

Main concepts and learning points:

- Cholangiocarcinoma (CCA) is a rare and heterogenous tumor entity with a poor prognosis and limited therapeutic options.
- Non-coding RNAs (ncRNAs) are classified according to their length into small (18 to 200 nucleotides) and long (>200 nucleotides) RNAs, and according to their function into housekeeping (e.g. ribosomal RNA and transfer RNA) and regulatory (e.g. microRNA, piwi-interacting RNA, long non-coding RNA) RNAs.
- Dysregulation of ncRNAs has been implicated in cholangiocarcinogenesis, tumor progression and chemotherapy resistance.
- microRNAs have been isolated and characterized in both the primary tumor tissue and bodily fluids (serum, plasma, urine, bile) of patients with CCA.
- microRNAs hold the potential to be introduced into the clinic as diagnostic, prognostic and predictive biomarkers for CCA. Efforts to design therapeutics that deregulate ncRNAs are ongoing.

Overview of cholangiocarcinoma

Cholangiocarcinoma (CCA) is a rare solid tumor arising from the biliary tree. CCAs are classified as intrahepatic (iCCA), perihilar (pCCA) or distal (dCCA), according to their anatomical location within the biliary tree [1-5], and each subtype has unique epidemiological, clinical and biological features. The incidence of CCA varies greatly across the world, reflecting different environmental and genetic risk factors, and has steadily increased over the last four decades, particularly that of iCCA[6]. In the Western world, the incidence of CCA varies from 0.3 to 3.5 cases per 100,000, whereas in Thailand, where it peaks, there are 80 cases per 100,000[7]. Primary sclerosing cholangitis (PSC) is the most established predisposing factor to CCA in Western countries; while hepatobiliary fluke infestation and hepatolithiasis are well-documented risk factors in Eastern countries[8]. Late diagnosis and resistance to conventional therapies largely account for an almost invariably dismal prognosis. Surgical resection followed by adjuvant therapy is the only curative approach

recommended by international practice guidelines[9]. However, these are feasible in <30% of cases, and even in these cases 5-year overall survival (OS) is disappointing ranging from 20% to 40%[10]. Most patients present with unresectable advanced disease at diagnosis and can only be offered palliative treatment, with an associated median OS of <12 months[11-12]. Huge efforts are being made by the research community to discover novel therapeutics for CCA, along with tumor biomarkers that could aid diagnosis, predict treatment efficacy or improve prognostication. In the past few years, high-throughput technologies have enabled extensive genome, exome, and transcriptome sequencing unveiling, amongst other things, the regulatory potential of non-coding RNA (ncRNA)[13]. In this article we review the role of ncRNAs in cholangiocarcinogenesis and tumor progression, as well as the potential for ncRNAs to be incorporated into the clinical management of CCA patients.

The genomic landscape of cholangiocarcinoma

The clinical heterogeneity of CCA is reflected also at the molecular level. Genome profiling studies have identified numerous genetic aberrations that affect metabolic, mitogenic, chromatin remodeling, and DNA repair signaling pathways. Notably, the mutational spectrum of CCA has been reported to vary according to the anatomical subtype and aetiology. Isocitrate dehydrogenase 1 or 2 (IDH 1/2) mutations (4.9-36%), fibroblast growth factor receptor 1-3 (FGFR 1-3) fusions, mutations and amplifications (11-45%), and BAP1 mutations (13%) occurr more frequently in iCCA; while KRAS mutations (8.3-42%), SMAD4 mutations (21%), and ERBB2/3 amplifications (11-17%) are observed more commonly in extrahepatic CCA[14]. Regarding aetiology, liver fluke-driven CCA are enriched in ERBB2 amplifications and TP53 mutations; by contrast, non-liver fluke associated CCA present high copy-number alterations and PD-1/PD-L2 expression, or epigenetic mutations (IDH1/2, BAP1) and FGFR/PRKA-related gene rearrangement[15-16].

Given the considerable genetic diversity of CCA with low penetrance driver aberrations and dependance on multiple pathways, several attempts have made to molecularly subtyping CCA to get clinical useful information. In a collaborative effort by the International Cancer Genome Consortium an integrated genomic, epigenomic and transcriptomic analysis was performed on 489 CCAs from 10 countries leading

to the identification of 4 subgroups, each harboring distinct genetic, epigenetic and clinicopathologic features[16].

Notably, this molecular clustering, thought to reflect the multifactorial interplay between genetics, epigenetics and environment in cholangiocarcinogenesis, displays a prognostic vaule (cluster 3 and 4 have a better prognosis than cluster 1 and 2) and provides an in-depth characterization of CCA beyond anatomical site (e.g. iCCA are splitting across all 4 cluster and the subtyping can be reproduced within each anatomical location separately).

Moreover, through multiplatform analyses (somatic mutations, DNA methylation, copy number, RNA expression) in a set of predominantly iCCA, The Cancer Genome Atlas group identified a molecularly distinct subtype of IDH-mutant CCA, displaying increased mithocondrial gene expression and DNA copy number and low chromatin modifier gene expression[17]. Interestingly, in the same study, the authors found 21 lncRNAs that correlated with the chromatin modifier signature, whereas one miRNA (miR-194-5p) was upregulated in the IDH-mutant subtype and negatively associated with the chromatin modifier siganture.

Non-coding RNAs

Although at least 80% of the human genome is biologically active, only roughly 3% is transcribed into protein-coding mRNAs[18]. The near totality of it, once termed “junk”, is now known to be made up of RNA that is transcribed but not translated into proteins, the so-called ncRNA. This term encompasses several classes of transcripts that can be classified according to their length into small (18 to 200 nucleotides) and long (>200 nucleotides) RNAs, and according to their function into housekeeping (e.g. ribosomal RNA and transfer RNA) and regulatory (e.g. microRNA, piwi-interacting RNA, long non-coding RNA) RNAs[19]. Non-coding RNAs are master regulators of transcription, transcript stability, and translation of protein-coding transcripts, with roles in both physiological processes and human diseases, including cancer. Among ncRNAs, microRNAs (miRNAs), transcripts of 18 to 24 nucleotides in length, are the most extensively studied. As a result of conserved seed pairing over just 7 or 8 bases on multiple mRNAs, a single miRNA has the potential to target thousands of genes[20]. miRNAs have been traditionally regarded as negative regulators of gene expression that, upon binding to the 3’ untranslated region of a

target mRNA, leads to its translational inhibition or degradation, thus downregulating the final protein output of protein-coding genes. However, over recent years, new insights into miRNA properties have emerged that are unveiling much broader regulatory functions than initially thought. Indeed, they were shown to target various non-canonical sites such as DNA promoter regions, RNA 5' untranslated region, other ncRNAs and proteins. Moreover, miRNAs can upregulate protein translational both directly, via recruitment of protein complexes, and indirectly, unleashing the translation repression exterted by inhibitory proteins. Again, there is also evidence that miRNAs can affect the Toll-like receptor signalling and downstream pathways (e.g. nuclear factor-kB pathway) through an agonist effect on Toll-like receptors[21].

With regards to their link with cancer, miRNAs have been shown to be deregulated in almost all human tumor types and to affect several steps of carcinogenesis by acting either as oncogenes or tumor suppressors[22-23]. More interestingly, signatures consisting of aberrantly expressed miRNAs have been found to have specific diagnostic and prognostic value[13]. On this basis, miRNAs have been regarded as promising tools for improving the management of cancer patients. Next, we discuss the involvement of ncRNAs in the regulation of cancer hallmarks, and the challenges associated with translating these findings into the clinic (Figure 1).

Role of miRNAs in cholangiocarcinogenesis

miRNA dysregulation has been shown to affect almost all CCA hallmarks, ranging from sustained cell proliferation to cancer-related inflammation (Tables 1 & 2).

Sustaining proliferative signaling

The earliest evidence for the role of ncRNAs in cholangiocarcinogenesis came from a study by Meng et al.[24], in which they found CCA cells had deregulated miRNA expression and that miR-21 had a predominant role in enhancing tumor cell growth. miR-21 overexpression was confirmed in independent cohorts of human primary CCA patients and its oncogenic role was linked, at least partially, to the inhibition of programmed cell death 4 and TIMP metallopeptidase inhibitor 3 expression[25]. miR-26a has also been shown to be up-regulated in human CCA, leading to increased proliferation of CCA cells via activation of beta-catenin-dependent genes[26]. It is interesting to note that miR-26 was found to be an oncosuppressor in hepatocellular

carcinoma (HCC), the other form of primary liver cancer[27], underlining the peculiarities of tissue-dependent miRNA expression. More recent evidence supports an oncogenic role for miR-191 and miR-181c in CCA, specifically their ability to sustain tumor growth and drive cancer aggressiveness[28-29]. It is likely that several miRNAs with oncogeneic and oncosuppressive properties interplay within cancer cells to promote a given phenotype. For instance, Olaru and colleagues identified a role for miR-494 as a negative regulator of cancer cell growth, by showing that miR-494 expression is down-regulated in CCA samples and that it has the ability to inhibit progression through the cell cycle[30]. Moreover, miRNAs often affect a cancer cell phenotype as a result of their ability to modulate multiple key players. For example, the expression of miR-494 induces a global deregulation of genes involved in progression through the cell cycle including cyclin dependent kinase 6, cyclin dependent kinase 4, cyclin D1, cyclin E2, histone deacetylase 1, RB transcriptional corepressor 1, polo-like kinase 1, pituitary tumor-transforming 1, cyclin B1, cyclin dependent kinase 2, and DNA topoisomerase II alpha[31].

Resisting cell death

Protection from cell death is another process in which miRNAs are involved. Apoptotic signaling pathways seem to be regulated by a combination of miRNAs with oncosuppressive or oncogenic functions. These include the oncosuppressive miR-29, whose loss in CCA is associated with an increase in the abundance of the anti-apoptotic protein myeloid cell leukemia sequence 1[32], and the oncogenic miR-25, whose high expression in CCA cells is responsible for resistance to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[33].

Activating invasion and metastasis

It was recently reported that changes in miRNA expression induce the epithelial-mesenchymal transition (EMT), a process whereby epithelial cells lose polarity and cell-to-cell adhesion in order to acquire invasiveness and migratory capabilities like mesenchymal elements. A study by Oishi et al., which employed integrative pathway analysis, linked miR-200c signaling to the EMT in a subtype of iCCA with stem-like gene expression features, providing an explanation as to why this subtype is clinically aggressive[34]. Overexpression of miR-21 was also associated with typical EMT features such as a low level of E-cadherin and high levels of N-cadherin and

vimentin, suggesting how a single miRNA can sustain tumor progression by acting on different pathways[35-36]. In addition, it has been shown that miRNAs involved in the regulation of EMT can increase the metastatic behavior of human CCA cells[37-39].

Tumor-promoting inflammation

Chronic biliary tract inflammation is thought to be the most robust factor to drive cholangiocarcinogenesis. CCA often arises in the setting of conditions such as PSC, hepatolithiasis, choledochal cysts and liver fluke infestations. Moreover, the inflammation-associated cytokine Interleukin-6 (IL-6) is increased in both the biliary tract and the systemic circulation of CCA patients, where it promotes neoplastic growth and survival through autocrine and/or paracrine mitogenic signals[40-41].

Persistent IL-6 stimulation can alter miRNA expression both *in vitro* and *in vivo* and the resulting up-regulation of let-7 contributes to IL-6-dependent signal transducer and activator of transcription 3 (STAT-3) survival signaling in malignant human cholangiocytes[42-43]. In addition, IL-6 can favor CCA growth by methylation-dependent regulation of miR-370 and associated targets such as the transcript of the oncogene mitogen-activated protein kinase kinase kinase 8 (*MAP3K8*)[44]. Enforced IL-6 expression can negatively modulate miR-148a and miR-152 in human CCA cells, leading to an increase in the level of DNA methyltransferase 1 and silencing of critical tumor suppressor genes such as Ras association domain family member 1 (*RASSF1*) and cyclin dependent kinase inhibitor 2α (*CDKN2A*, also known as *p16INK4a*)[45]. Distinct miRNA signatures were also identified in *Opisthorchis viverrini*-driven CCA, suggestive of the effect of inflammation on miRNAs[46-47]*.* This evidence strengthens the biological basis for the interplay between an inflammatory microenvironment, epigenetics and molecular events involved in cholangiocarcinogenesis, along with providing a rationale for targeting miRNAs that control the inflammatory response.

Microenvironment

The tumor microenvironment is a complex network made up of both cancer cells and reactive peritumoral stroma, the latter being enriched for various host cells including cancer-associated fibroblasts (CAF), endothelial cells, immune cells and tumor-associated macrophages[48-49]. Tumor-reactive stroma has been hypothesized to be a pivotal driver of CCA initiation and progression by affecting the behavior of cancer

cells. The loss of miR-15 was found to be specific for CCA-associated fibroblasts when compared to skin-derived fibroblasts, suggesting a key role for ncRNAs in driving the transformation of stromal cells into CAFs that have a functional role in promoting cancer growth and chemoresistance[50]. miRNAs have lately emerged as novel players in the intercellular communication network that exists between different cell types in the tumor microenvironment. miRNAs are released from donor cells in extracellular vescicles (EVs), and can induce phenotypic and functional changes in recipient target cells. Evidence suggests that the ncRNA within EVs released from non-malignant and malignant cells is different, and that tumor-specific EVs contain short or long ncRNAs that can modulate how cells respond to hypoxia, control their growth and respond to drugs[51-52] . Exosomes, a subtype of EV, can be found in the bile of rat models and influence intracellular signaling mechanisms and cholangiocyte proliferation via interaction with cholangiocyte cilia[53]. Furthermore, miRNA-loaded vesicles derived from myofibroblasts can selectively target CCA cells, influencing their neoplastic properties both *in vitro* and *in vivo*[54]. Conversely, tumor cells can release EVs that induce mesenchymal stem cells to change the local microenvironmental to enhance CCA cell growth[55]. A better understanding of such EV-mediated bidirectional interactions, and identification of the ncRNA species that are trafficked between tumor and stromal cells, could offer new CCA prevention strategies and/or therapeutic intervention in CCA patients. More importantly, the discovery that stroma-derived EVs are uniquely suited to deliver materials to CCA cells raises hope that EVs could be exploited to deliver anti-neoplastic therapies. For example, intravenous injection of miR-195-loaded fibroblast-derived EVs was shown to reach the tumor, decrease tumor size, and improve survival in a rat model of CCA[56].

Role of long ncRNAs in cholangiocarcinoma biology.

Long non-coding RNAs (lncRNAs) are a subgroup of ncRNA transcripts that are longer than 200 nucleotides and not translated into proteins. Functions and mechanisms of action of lncRNAs are different from those of miRNAs and need further characterization. LncRNAs can regulate a myriad of biological processes through interaction with various cellular macromolecules such as DNA, RNA and proteins, acting either in the nucleus or in the cytoplasm. LncRNAs are involved in

the fine regulation of gene expression acting as epigenetic modifiers through recruitement of chromatin remodelling complexes to specific genomic loci, as enhancers *in cis* of neighbouring genes, and as scaffolds for the assembly of transcriptional regulatory molecules. LncRNA can affect several aspects of the post-transcriptional regulation process, including mRNA stability, splicing, and translation. In addition, recent evidence suggests a role for lncRNAs and pseudogenes as sponges for small ncRNAs, behaving as cytoplasmic “controllers” of cell fate.

Several studies have demonstrated that a number of lncRNAs play fundamental roles in a variety of pathological processes, including cancer[57]. lncRNA expression was found to be deregulated in human iCCA, with 2,773 up-regulated and 2,392 down-regulated lncRNAs when compared to paired noncancerous tissue across a cohort of 77 human CCA patients[29]. Interestingly, these lncRNAs seem to regulate genes associated with cancer, hepatic system disease and signal transduction, supporting a role for lncRNAs in the development and progression of CCA. H19, the first lncRNA discovered, was found overexpressed in both CCA tissues and CCA cell lines, where it contributes to different steps in cholangiocarcinogenesis by promoting cell proliferation, migration, invasion, evasion of apoptosis, and EMT[58]. Similar oncogenic properties were reported for other lncRNAs such as AFAP1 antisense RNA 1 (AFAP1-AS1)[59], CPS1 intronic transcript 1 (CPS1-IT1)[60] metastasis associated lung adenocarcinoma transcript 1 (MALAT1), promoter Of CDKN1A antisense DNA damage activated RNA (PANDAR), prostate cancer associated transcript 1 (PCAT1), colon cancer associated transcript 1 (CCAT1), taurine up-regulated 1 (TUG1), urothelial cancer associated 1 (UCA1), and SOX2 overlapping transcript (SOX2-OT)[61-65]. lncRNAs are also involved in sustaining CCA progression induced by inflammation. For example, H19 and HULC are induced by oxidative stress and can deregulate the cellular host inflammatory response by acting on genes such as *IL-6* and C-X-C motif chemokine receptor 4 (*CXCR4*)[66]. Ultraconserved regions (UCRs) are non-coding genomic segments >200 nucleotides in length that are remarkably conserved beween species, with 100% sequence similarity across the human, mouse, and rat genomes. The deregulation of UCR transcription has been increasingly implicated in human cancers. It has been postulated that the conservation of UCR gene sequences between species indicates that UCRs have an essential functional role, suggesting that an alteration in their transcription may represent the first step in malignant transformation. Transcripts

derived from UCRs may be of various lengths, and their 3D structure is likely to impact on their function by affecting their interaction with ribonucleoproteins or their ability to capture small ncRNAs in "sponge-like" structures[67-68]. We and others showed that the deregulation of transcribed-UCR (T-UCR) occurs in the cancer tissues but is already present in the adjacent background tissue, suggesting that it may create a favorable environment for the progression of cancer[67,69]. Furthermore, we have shown that uc.158-can act downstream of the Wnt pathway and is likely to represent the cellular mediator that promotes CCA cell growth in response to stroma-derived Wnt signaling. More recently, lncRNAs can differentiate between T-cell classes more precisely than mRNAs, suggesting lncRNAs are potential drivers of immune cell fate and regulators of the interplay between cancer cells and those in the microenvironment[70].

Clinical implications of ncRNAs in cholangiocarcinoma

In addition to acting within cells, miRNAs are released and circulate in serum, plasma, and other bodily fluids, either as free RNAs bound to the argonaut RNA-induced silencing complex (RISC) catalytic component or in extracellular vesicles, such as exosomes or microvesicles[71]. This protects miRNAs from degradation via RNAses and gives them stability and resistance during the storage and handling of patient samples. All of these properties, together with the ability to isolate, characterize and identify disease-specific miRNA content in tissues and bodily fluids, has raised interest in miRNAs as a gold mine of biomarkers with potential in cancer diagnosis, prognosis, treatment response, and therapy[72] (Table 3).

Diagnostic role

CCA diagnosis is often challenging due to the lack of specific symptoms, the technical difficulty to access tissue, and the intense stroma reaction and paucicellular neoplastic nature of this disease. Thus, there is an urgent need for diagnostic tools that can enable early cancer detection. The pattern of miRNA expression in CCA cell lines and human tissues significantly differs from that in non-malignant counterparts, suggesting that CCA-specific miRNA expression profiles could be used to develop diagnostic assays. Differentiating between CCA and benign disorders harboring biliary strictures (e.g. PSC) poses a particular challenge

since they appear the same when imaged and because of the difficulties associated with obtaining cytological and/or histological samples of sufficient yield[73]. A combination of two circulating miRNAs (miR-222 and miR-483-5p) in the serum were proven to differentiate CCA patients from PSC patients in a retrospective study including a discovery (n=90) and a validation cohort (n=140)[74], showing the promise of circulating miRNAs. Additional experiences showed a good negative predictive value for circulating miRNA signatures that were able to correctly identify 12 out of 13 patients with PSC who did not develop CCA[75]. However, circulating miRNAs in the blood are known to be affected by other conditions, such as liver injury, cardiovascular disease, sepsis and immunological disorders, and therefore the use of circulating miRNAs should be applied with caution in the clinical practice in view of their limited positive predictive value. A way to overcome this issue is to search for CCA-specific miRNAs in the bile. Owing to the direct contact, diagnostic information could be obtained in the bile where tumor-related biomarkers may have increased concentrations and conversely non-tumor markers may be less represented[72]. Moreover, an increasing number of studies have shown that the EVs released into the bile of individuals affected by liver disease differ in functional content to those found in the bile of healthy individuals. Li et al. provided the first evidence that human bile contains miRNA-laden EVs[76], thus paving the way for the exploitation of their biomarker potential in biliary tract diseases. Using a multivariate combinatorial model, researchers were able to define a novel bile-based 5-miRNA panel (miR-191, miR-486-3p, miR-1274b, miR-16 and miR484) for CCA diagnosis that had 67% sensitivity and 96% specificity. Notably, this diagnostic tool was more accurate than the commonly available standard methods used in clinical practice (e.g. CA19-9). More interestingly, of the 11 CCA patients correctly diagnosed by the 5-miRNA panel, but misdiagnosed by CA19-9, 8 had early-stage disease (N0M0), underlining the potential of this miRNA signature approach to detect cancers in its early stages. It is worth remembering that CCA is endemic in some areas of north-eastern Thailand, where the implementation of a screening program could significantly impact on CCA-induced mortality. However, liver-fluke induced CCA has a different genetic and transcriptomic background to the CCA that arises in the western world[77]. Thus, it is likely that a miRNA-based diagnostic biomarker for CCA should be specifically targeted to this population limiting the potential of extending studies on Western populations to Eastern populations. The use of urine-based biomarkers is

likely to be implemented more successfully in screening programs than those based on the profile of miRNAs in other bodily fluids. MicroRNAs can be detected in the urine given they are filtered through the renal system due to their small size and their high stability. Silakit and coworkers have reported that miR-192 is significantly higher in the urines of *O. viverrini*-infected subjects in comparison to healthy subjects with a sensitivity of 75.0% and a specificity of 71.4%[78]. Although good, these values are not high enough to be applied to screening programs, and therefore more work is needed. The measurement of more than one miRNA is likely to represent the best approach for increasing the accuracy of the test. For example, the addition of urinary miR-21 to miR-192 resulted in a test sensitivity of 79.2% and a specificity of 76.2%, when attempting to distinguish *O. viverrini*-infected subjects from healthy ones. However, it is worthy mentioning that miR-21 is increased in a variety of tumours and non malignant diseases and lacks specificity as a diagnostic marker.

CCA is a disease with unique diagnostic challenges, the detection of dysregulated miRNAs into accessible biofluids in patients harboring a high index of suspicion for CCA offers an opportunity for earlier diagnosis and thus higher chances of curability. However, several issues remain to be addressed. Specimen collection and manipulation must be standardized in order to derive a test that can be applied in a prospective fashion. We believe that the levels of circulating miRNAs should not be normalized to those of reference transcripts (or miRNAs), as a direct count of the miRNA molecules in a given volume of biofluid is likely to represent the most accurate method for clinical implementation. The advancement in technologies with the introduction of digital PCR and RNA-sequencing should render it possible to directly count such molecules and identify a threshold that can be applied for prospective validation. However, it is certain that large patient cohorts are needed to investigate the distribution of circulating miRNAs in the population at risk, and to understand their association with CCA development in a manner that is independent of other comorbidities. In tumors with a low prevalence, such as CCA, this goal can only be achieved through a collaborative international effort. We can speculate that the detection of miRNAs within CCA-specific EVs can improve diagnostic accuracy, and that the ongoing efforts to extensively characterize EVs will identify markers enriched in CCA-specific EVs to achieve this goal [79-80] (and their ncRNA content). A more provocative question could be: “Do we need to identify a miRNA that is cancer-specific to improve early diagnosis and promote prevention?”. We have learnt from

the latest advances in oncology that the host immune response plays a driving role in containing and attacking cancer, enabling the “defeat” of cancer even in cases of multi-metastatic disease[81-83]. It is reasonable to speculate that the immune system is playing even a more important role in the early stages of malignant transformation and that detecting markers that are indicative of the failure of the host immune response may be informative for early diagnosis. Given the evidence that ncRNAs can drive the fate of immune cells and their function[70], it may be useful to start looking for changes in ncRNAs that reflect a poor immunological response rather than the presence of a cancer that has already developed.

Prognostic role

Based on the identification of ncRNAs as drivers of CCA progression, growing research efforts are attempting to identify CCA-related miRNA signatures that could inform disease prognosis and patient selection. In a cohort of 84 patients with iCCA, high expression of miR-191 was found to be independently associated with worse disease-free survival and OS[84]. miR-21 has also been consistently found to be associated with adverse clinicopathological features and survival[85]. More interestingly, circulating miR-21 levels dropped in patients undergoing curative surgery, but not in those receiving palliative resection, suggesting that the levels of miR-21 in the plasma reflect the extent of the tumor and can be used as a surrogate marker for prognosis. Likewise, high serum miR-26a expression has been significantly correlated with clinical stage, distant metastasis, differentiation status, and the poor survival of CCA patients[86]. Looking for highly-expressed miRNAs may be less technically challenging than looking for down-regulated miRNAs, where absence or low levels can also be associated with technical failure. However, recent evidence support a prognostic role for low tissue levels of miR-203, a tumor suppressor miRNA, in resected CCA[87]. With the introduction of adjuvant chemotherapy in the management of resected CCA, the discovery of a prognostic marker may represent a valuable asset for identifying those patients who are most likely to benefit from post-operative treatment. In this regard, it is likely that the study of tissue and circulating (where possible) miRNAs, in large cohorts in prospective clinical trials, will offer more insight than the investigation of the clinical value of ncRNAs in small retrospective series. The availability of tissue from resected tumors in this setting offers an additional opportunity for ncRNA detection by employing *in*

situ technologies that enable a semi-quantitative score that can be easily implemented in the clinic. We and others have previoulsy shown that cancer can be classified on the basis of ncRNA expression measured using *in situ* hybridization techniques in formalin-fixed paraffin-embedded tissue, and scored following the methods used for immunohistochemistry[68,88].

Predictive role

Chemotherapy still represents the mainstay of advanced CCA treatment, while targeted agents and immunotherapy have shown potential in selected patient populations[9,89-90]. Active chemotherapy regimens include gemcitabine, capecitabine, a combination of cisplatin and gemcitabine (CG) or of fluorouracil and oxaliplatin (FOLFOX)[11,91-92] . With an increasing number of treatment options on the horizon, the need for personalized medicine is emerging. However, the window of opportunity for treatment in CCA patients is limited, because of the rapid deterioration in patients' fitness upon chemotherapy failure. Indeed, while some patients that fail to respond to first line chemotherapy may still be sensitive to second line options a rapid drop in their performance status might preclude further lines of therapy. A better understanding of the mechanisms underpinning chemo-resistance will improve patient selection and provide important tools to enable decision-making in regard to the choice of chemotherapy sequence, achieving rapid disease control in responders and minimizing the risk of unnecessary toxicity in patients who are unlikely to benefit. Aberrantly-expressed miRNAs in cancer cells are thought to regulate the expression of genes critical for cell survival in response to chemotherapeutic stress. For instance, miR-21 expression was shown to increase following exposure to cytotoxic drugs and to modulate gemcitabine-induced apoptosis by phosphatase and tensin homolog (PTEN)-dependent activation of PI3-kinase signaling in *in vitro* and *in vivo* models[24]. In other reports, the loss of selected miRNAs (miR-29, miR-320, and miR-204) was found to mediate the resistance of CCA cells to chemotherapeutics, by acting on the apoptotic pathway[93-94]. Although preclinical, these observations link ncRNAs with the sensitivity of tumor cells to chemotherapy and suggest that ncRNAs should be investigated in the translational component of large clinical trials. The possibility of detecting circulating miRNAs in the blood of CCA patients increases the applicability of such tests in the advanced disease setting where tissue availability is limited. In addition, as described above, circulating miRNAs can be affected by other

comorbidities and thus have the potential to represent surrogate markers of the general medical status of the patient, information on which treatment choice can be based.

miRNAs can drive a number of cellular processes, and therefore have the potential to modulate cell sensitivity to a number of non-chemotherapy drugs. We, and others, have recently proven that heat shock protein 90 (HSP90) inhibition can be a promising therapeutic strategy in CCA[95-96]. The HSP90 complex interacts with a variety of client proteins which have a key role in CCA progression, such as epidermal growth factor receptor (EGFR), phosphoinositide-3-kinase, PTEN, human epidermal growth factor receptor 2, human epidermal growth factor receptor 3, and phosphoribulokinase A. Moreover, inhibition of HSP90 seems to be remarkably effective in tumors with fibroblast growth factor receptor fusions and activation of the IL-6/STAT pathway[97-98]. HSP90 controls the post-translational folding of argonaute2 (AGO2), a regulator of miRNA processing[99], suggesting that miRNAs could represent good biomarker candidates. We have shown that miR-21 can drive resistance to HSP90 inhibition by specifically affecting the multi-chaperone complex in a way that is recapitulated in human CCA cell lines, 3D organoid structures and patient-derived xenografts[95]. Given the potential of miRNAs to affect a range of signaling pathways, we suggest to include ncRNAs within future drug discovery projects expanding our pivotal experience. Patient-derived organoids (PDO) are organotypic cultures that recapitulate the architecture, genomic- and transcriptomic-profile of human tumors, and mimic their treatment response in the clinic, holding great potential in advancing drug discovery projects. We recently provided evidence that PDOs can be established from tissue derived from advanced CCA patients and can be manipulated to change miRNA expression, opening the way for the use of PDOs as a tool to personalize medicine and apply functional genomics to identify the involvement of ncRNAs in the response to drugs in CCA[95].

ncRNA-based therapeutics

Insights into the role of ncRNAs in tumor biology, alongside their ability to target multiple genes and signaling pathways simultaneously, have made them attractive targets and therapeutic tools for novel anti-cancer approaches. The two main strategies for modulating miRNA expression are based on the introduction of synthetically-derived small RNA duplexes that behave similarly to endogenous

miRNAs (miRNA mimics) or, conversely, on the delivery of molecules targeted at miRNAs (anti-miRNAs or miRNAs inhibitors). In the former case, the aim is to replenish the lost expression of miRNAs acting as tumour suppressor, whereas in the latter case the aim is to oppose the function of oncogenic miRNAs[100]. In both cases, appropriate target selection as well as strategies for tissue/cancer-specific *in vivo* delivery are critical steps in order to pursue the therapeutic goal while minimizing off-target effects and toxicities. The identification of the best miRNA targets is challenging, mainly because no miRNAs are entirely cancer-specific and conditions such as inflammation and hypoxia can affect miRNA expression, complicating the search for candidate miRNAs[101]. Moreover, the concept of temporal and spatial heterogeneity applies also to miRNAs, whose expression is dynamic over time and may vary intertumorally and intratumorally. Among the strategies being used to select key regulatory miRNAs is miRNA crosslinking and immunoprecipitation (miR-CLIP), a novel technique that can identify relevant miRNA pathways by capturing their putative targets, the so-called "targetome"[102]. The matching of these data or other from public repositories on miRNA interaction networks with prediction platforms will allow for a more accurate identification of best candidate miRNAs to be targeted. An additional strategy to look for key regulatory miRNAs is based on genome-wide high-throughput-screening technologies to evaluate libraries of miRNA mimics or inhibitors.

The degradation of naked particles by nucleases is a key issue which needs to be overcome before implementing miRNA-based therapeutics. To optimize the delivery of miRNA mimics/anti-miRNAs their nucleotide backbone can be chemically modified to improve their stability in bodily fluids and prevent degradation in serum and endocytic cellular compartments. However, oligonucleotide methylation, the introduction of locked nucleic acids, or the addition of phosphorothioate-like groups can result not only in an increase in miRNA stability but also in the loss of mRNA silencing, or in off-target effects and the production of toxic metabolites. The ideal delivery vehicle would be a non-immunogenic and biodegradable carrier, displaying target tumor specificity and robust binding. One of the most successful approaches is to encapsulate the miRNA mimics/anti-miRNAs into nanoparticles. Poly(lactide-co-glycolide) particles, neutral lipid emulsions, neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, polyethylenimine, polyethylene glycol, chitosan and n-acetyl-d-galactosamine are all promising platforms for miRNA therapeutics delivery that are

currently being tested in clinical trials. However, both the efficiency with which miRNA mimics/anti-miRNAs are delivered to the target site and their safety profile are still suboptimal, partly as a result of the net cationic charge of the carriers.

The strategy of replenishing miRNAs with tumor-suppressive function *in vivo* has recently been pursued in humans. Van Zandwijk et al. reported the results of the first-in-man phase 1 trial with a miR-16 mimic, which was administered to patients with recurrent malignant pleural mesothelioma[103]. Minicells loaded with a miR-16-based miRNA mimic and targeted to EGFR (TargomiRs) were administered to 26 patients with EGFR immunohistochemical expression that had disease progression after receiving standard chemotherapy. TargomiRs were given over a 20 minutes intravenous infusion either once or twice weekly according to a traditional 3+3 dose-escalation design across 5 dose cohorts. Among 22 assessable patients, one (5%) had a partial response and 15 (68%) had stable disease. These encouraging data show how the delivery of miRNA mimics can be a clinically feasible and effective approach. However, toxicity concerns remain, as in this study the TargomiR induced cardiotoxicity in 5 patients. The exact mechanism responsible for the cardiological toxicity is still not clear, as it may be a direct effect of the miRNA itself or the consequence of the inflammatory reaction induced by the minicell delivery vehicle. Advances in technology are likely to yield safer delivery methods, which can increase efficacy and reduce the toxicity associated with this therapeutic strategy. Moreover, pharmacological studies and the assessment of miRNA in the tumour tissue after administration of the miRNA-based therapeutics should be included in future studies to better understand the dynamics of the systemic delivery of these new therapeutics. On a different standpoint, targeting of miR-122 proved successfull in a phase 2a trial in patients with chronic HCV genotype 1 infection[104]. A 15-nucleotide locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide (Miravirsen) was given in a random fashion to 36 patients, who had been subdivided into 4 groups and given doses of 3, 5 or 7 mg per kg, or a placebo, over a 29 day period. Prolonged dose-dependent reductions in HCV RNA levels were observed, with no dose-limiting toxicities, no evidence of viral resistance or occurence of hepatocellular carcinoma. With regards to liver tumors, MRX34, a liposomal miR-34 mimic, has been the first miRNA mimic to enter clinical evaluation in patients with solid tumors, including hepatocellular carcinoma[105]. In this phase I, first-in-human, dose-escalation study, MRX34 was administered intravenously

biweekly or daily (x 5 days) to 99 patients with advanced solid tumors. MRX34 had a manageable safety profile, as the most commonly reported adverse events were mild in grade (fever, chills, fatigue, back pain, nausea), with grade 3 lymphocytopenia, thrombocytopenia, elevated AST, neutropenia, and hyponatremia as the more frequent laboratory abnormalities. This liposomal miR-34 mimic displayed anticancer activity, achieving a prolonged confirmed RECIST partial response in 3 patients (one of whom had hepatocellular carcinoma), and stable disease in 14 other cases. A phase Ia expansion cohorts is currently underway and phase II studies are being planned (Clinical trial information: NCT01829971). Recent preclinical evidence showed that this miR34 mimic can also suppress the growth of CCA cells[106], suggesting that this investigation could be extended to CCA patients.

Conclusion

CCA is characterized by late diagnosis and poor response to standard treatment resulting in an almost invariably dismal prognosis. Therefore, there is an urgent need for earlier diagnosis, a better prediction for tumor aggressiveness and response to treatment as well as novel therapeutic options. The better understanding of CCA biology has revealed ncRNAs as pivotal players affecting CCA initiation, progression and treatment resistance. On these premises, ncRNAs are being studied intensively as promising tumor biomarkers to incorporate in the clinical management for improving the outcome of CCA patients. Preliminary findings are encouraging, even though further research is required to confirm their reliability and clinical reproducibility.

Acknowledgments

Chiara Braconi is the recipient of an Institute of Cancer Research Clinician Scientist Fellowship, an Early Diagnosis Award from Pancreatic Cancer Action and a NIHR Royal Marsden/ICR Biomedical Research Centre project grant. This work was supported in part by LILT Modena Onlus. We would like to thank Nicky Evans for her editorial assistance.

Conflict of interest

The authors have no conflict of interest to declare.

Figure legend

Figure 1. Selected ncRNAs interacting with their relative targets within major oncogenic signaling pathways altered in CCA. Upwards black arrow shows upregulated ncRNAs, while downwards black arrow refers to downregulated ncRNAs. Continous arrows shows an activating function, while dashed line refers to inhibitory function.

References

1. Braconi C, Patel T. Cholangiocarcinoma: new insights into disease pathogenesis and biology. Infect Dis Clin North Am 2010; 24:871e884, vii
2. Marcano-Bonilla L, Mohamed EA, Mounajjed T, Roberts LR. Biliary tract cancers: epidemiology, molecular pathogenesis and genetic risk associations. Chin Clin Oncol 2016;5:61
3. Saha SK, Zhu AX, Fuchs CS, Brooks GA. Forty-Year Trends in Cholangiocarcinoma Incidence in the U.S.: Intrahepatic Disease on the Rise. Oncologist. 2016 May;21(5):594-9. doi: 10.1634/theoncologist.2015-0446. Epub 2016 Mar 21
4. Global Burden of Disease Cancer Collaboration. The Global Burden of Cancer 2013. JAMA Oncol. 2015;1(4):505–527
5. Rizvi S, Khan SA, Hallemeier CL, Kelley RK, Gores GJ.Cholangiocarcinoma - evolving concepts and therapeutic strategies.Nat Rev Clin Oncol. 2018 Feb;15(2):95-111
6. Patel T. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. Hepatology. 2001 Jun;33(6):1353-7
7. Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, et al.Guidelines for the diagnosis and management of intrahepatic chol-angiocarcinoma. J Hepatol 2014;60:1268–1289
8. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology. 2013 Dec;145(6):1215-29

9. Valle JW, Borbath I, Khan SA, Huguet F, Gruenberger T, Arnold D. Biliary cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016;27:v28–37
10. Razumilava N, Gores G.J. Cholangiocarcinoma. Lancet. 2014; 383: 2168–2179
11. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med. 2010; 362: 1273–1281
12. Valle JW, FuruseJ, Jitlal M, Beare S, Mizuno N, Wasan H,et al. Cisplatin and gemcitabine for advanced biliary tract cancer: a meta-analysis of two randomised trials. Ann Oncol. 2014; 25: 391–398
13. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet (2009) 10(10):704–14
14. Valle JW, Lamarca A, Goyal L, Barriuso J, Zhu AX. New Horizons for Precision Medicine in Biliary Tract Cancers.Cancer Discov. 2017 Sep;7(9):943-962.
15. Chan-On W, Nairismägi ML, Ong CK, Lim WK, Dima S, Pairojkul C, et al. Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers. Nat Genet. 2013 Dec;45(12):1474-8.
16. Jusakul A, Cutcutache I, Yong CH, Lim JQ, Huang MN, Padmanabhan N, et al. Whole-Genome and Epigenomic Landscapes of Etiologically Distinct Subtypes of Cholangiocarcinoma. Cancer Discov. 2017 Oct;7(10):1116-1135. doi: 10.1158/2159-8290.CD-17-0368
17. Farshidfar F, Zheng S, Gingras MC, Newton Y, Shih J, Robertson AG, et al. Integrative Genomic Analysis of Cholangiocarcinoma Identifies Distinct IDH-Mutant Molecular Profiles. Cell Rep. 2017 Jun 27;19(13):2878-2880
18. Pennisi E. Genomics. ENCODE project writes eulogy for junk DNA. Science. 2012 Sep 7;337(6099):1159, 1161
19. Dozmorov MG, Giles CB, Koelsch KA, Wren JD . Systematic classification of non-coding RNAs by epigenomic similarity. BMC Bioinformatics. 2013;14 Suppl 14:S2

20. Lewis BP, Burge CB, Bartel DP .Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005 Jan 14;120(1):15-20
21. Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov. 2013 Nov;12(11):847-65
22. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005 Jun 9;435(7043):834-8
23. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006 Feb 14;103(7):2257-61. Epub 2006 Feb 3
24. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology. 2006 Jun;130(7):2113-29
25. Selaru FM, Olaru AV, Kan T, David S, Cheng Y, Mori Y, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3.Hepatology. 2009 May;49(5):1595-601
26. Zhang J, Han C, Wu T.MicroRNA-26a promotes cholangiocarcinoma growth by activating β-catenin. Gastroenterology. 2012 Jul; 143(1):246-56.e8
27. Wang XW, Heegaard NH, Orum H. MicroRNAs in liver disease. Gastroenterology. 2012 Jun;142(7):1431-43
28. Li H, Zhou ZQ, Yang ZR, Tong DN, Guan J, Shi BJ, et al. MicroRNA-191 acts as a tumor promoter by modulating the TET1-p53 pathway in intrahepatic cholangiocarcinoma. Hepatology. 2017 Jul;66(1):136-151
29. Wang J, Xie H, Ling Q, Lu D, Lv Z, Zhuang R, et al. Coding-noncoding gene expression in intrahepatic cholangiocarcinoma. Translational Research. 2016; 168:107–121
30. Olaru AV, Ghiaur G, Yamanaka S, Luvsanjav D, An F, Popescu I, et al. MicroRNA down-regulated in human cholangiocarcinoma control cell cycle

through multiple targets involved in the G1/S checkpoint. Hepatology. 2011 Dec;54(6):2089-98

31. Yamanaka S, Campbell NR, An F, Kuo SC, Potter JJ, Mezey E, et al. Coordinated effects of microRNA-494 induce G_2/M arrest in human cholangiocarcinoma. Cell Cycle. 2012 Jul 15; 11(14): 2729–2738
32. Mott JL, Kobayashi S, Bronk SF, Gores GJ.mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene. 2007 Sep 13;26(42):6133-40. Epub 2007 Apr 2
33. Razumilava N, Bronk SF, Smoot RL, Fingas CD, Werneburg NW, Roberts LR, et al.miR-25 targets TNF-related apoptosis inducing ligand (TRAIL) death receptor-4 and promotes apoptosis resistance in cholangiocarcinoma. Hepatology. 2012 Feb;55(2):465-75
34. Oishi N, Kumar MR, Roessler S, Ji J, Forgues M, Budhu A, et al. Transcriptomic profiling reveals hepatic stem-like genesignatures and interplay of miR-200c and epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma. Hepatology. 2012; 56(5): 1792–1803
35. Liu Z, Jin ZY, Liu CH, Xie F, Lin XS, Huang QMicroRNA-21 regulates biological behavior by inducing EMT in human cholangiocarcinoma Int J Clin Exp Pathol. 2015 May 1;8(5):4684-94
36. Liu CH, Huang Q, Jin ZY, Zhu CL, Liu Z, Wang C. MiR-21 and KLF4 jointly augment epithelial-mesenchymal transition via the Akt/ERK1/2 pathway. Int J Oncol. 2017 Apr;50(4):1109-1115
37. Li B, Han Q, Zhu Y, Yu Y, Wang J, Jiang X.Down-regulation of miR-214 contributes to intrahepatic cholangiocarcinoma metastasis by targeting Twist. FEBS J. 2012 Jul;279(13):2393-8.
38. Li J, Yao L, Li G, Ma D, Sun C, Gao S, et al. MiR-221 Promotes Epithelial-Mesenchymal Transition through Targeting PTEN and Forms a Positive Feedback Loop with β-catenin/c-Jun Signaling Pathway in Extra-Hepatic Cholangiocarcinoma. Plos One. 2015; 10(10): e014116
39. Wang Q, Tang H, Yin S, Dong C. Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9. Oncology Reports. 2013; 29(5): 2046–2052

40. Park J, Tadlock L, Gores GJ, Patel T.Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. Hepatology 1999; 30(5):1128-1133
41. Meng F, Yamagiwa Y, Ueno Y, Patel T. Over-expression of interleukin 6 enhances cell survival and transformed cell growth in human malignant cholangiocytes. J Hepatol 2006;44(6):1055-1065
42. Lin KY, Ye H, Han BW, Wang WT, Wei PP, He B, et al. Genome-wide screen identified let-7c/miR-99a/miR-125b regulating tumor progression and stem-like properties in cholangiocarcinoma. Oncogene. 2016;35:3376–86
43. Meng F, Henson R, Wehbe-Janek H, Smith H, Ueno Y, Patel T.The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. J Biol Chem. 2007 Mar 16;282(11):8256-64
44. Meng F, Wehbe-Janek H, Henson R, Smith H, Patel T.Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. Oncogene. 2008 Jan 10;27(3):378-86
45. Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology. 2010 Mar;51(3):881-90. doi: 10.1002/hep.23381
46. Plieskatt JL, Rinaldi G, Feng Y, Peng J, Yonglitthipagon P, Easley S, et al. Distinct miRNA signatures associate with subtypes of cholangiocarcinoma from infection with the tumourigenic liver fluke Opisthorchis viverrini. J Hepatol. 2014 Oct;61(4):850-8
47. Chusorn P, Namwat N, Loilome W, Techasen A, Pairojkul C, Khuntikeo N, et al. Overexpression of microRNA-21 regulating PDCD4 during tumorigenesis of liverfluke-associated cholangiocarcinoma contributes to tumor growth and metastasis,Tumour Biol. 34 (3) (2013) 1579–1588
48. Raggi, C., Invernizzi, P. & Andersen, J. B. Impact of microenvironment and stem-like plasticity in cholangiocarcinoma: molecular networks and biological concepts. J. Hepatol. 62, 198–207 (2015)
49. Mertens JC, Fingas CD, Christensen JD, Smoot RL, Bronk SF, Werneburg NW, et al. Therapeutic effects of deleting cancer-associated fibroblasts in cholangiocarcinoma. Cancer Res. 2013 Jan 15;73(2):897-907

50. Utaijaratrasmi P, Vaeteewoottacharn K, Tsunematsu T, Jamjantra P, Wongkham S, Pairojkul C, et al. The microRNA-15a-PAI-2 axis in cholangiocarcinoma-associated fibroblasts promotes migration of cancer cells. Mol Cancer. 2018 Jan 18;17(1):10.
51. Kogure T, Yan IK, Lin WL, Patel T. Extracellular Vesicle-Mediated Transfer of a Novel Long Noncoding RNA TUC339: A Mechanism of Intercellular Signaling in Human Hepatocellular Cancer. Genes Cancer. 2013;4:261–272
52. Kogure T, Lin WL, Yan IK, Braconi C, Patel T. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology. 2011;54:1237–1248
53. Masyuk AI, Huang BQ, Ward CJ, Gradilone SA, Banales JM, Masyuk TV, et al. Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia. Am J Physiol Gastrointest Liver Physiol. 2010. 299:G990–G999
54. Sirica AE. The role of cancer-associated myofibroblasts in intrahepatic cholangiocarcinoma. Nat Rev Gastroenterol Hepatol.2012;9:44 54
55. Haga H, Yan IK, Takahashi K, Wood J, Zubair A, Patel T.Tumour cell–derived extracellular vesicles interact with mesenchymal stem cells to modulate the microenvironment and enhance cholangiocarcinoma growth J Extracell Vesicles. 2015 Jan 2;4:24900
56. Li L, Piontek K, Ishida M, Fausther M, Dranoff JA, Fu R, et al. Extracellular vesicles carry microRNA-195 to intrahepatic cholangiocarcinoma and improve survival in a rat model. Hepatology. 2017 Feb;65(2):501-514
57. Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways Cancer Cell. 2016;29:452–463
58. Xu Y, Wang Z, Jiang X, et al. Overexpression of long noncoding RNA H19 indicates a poor prognosis for cholangiocarcinoma and promotes cell migration and invasion by affecting epithelial-mesenchymal transition. Biomed Pharmacother. 2017 Aug;92:17-23
59. Lu X, Zhou C, Li R, Deng Y, Zhao L, Zhai W. Long Noncoding RNA AFAP1-AS1 Promoted Tumor Growth and Invasion in Cholangiocarcinoma. Cell Physiol Biochem. 2017;42(1):222-230
60. Ma SL, Li AJ, Hu ZY, Shang FS, Wu MC . Co-expression of the carbamoyl-phosphatesynthase 1 gene and its long non-coding RNA correlates with poor

prognosis of patients with intrahepatic cholangiocarcinoma. Molecular Medicine Reports. 2015; 12(6):7915–7926

61. Wang C, Mao ZP, Wang L, Wu GH, Zhang FH, Wang DY, et al. Long non-coding RNA MALAT1 promotes cholangiocarcinoma cell proliferation and invasion by activating PI3K/Akt pathway. Neoplasma. 2017;64(5):725-731
62. Xu Y, Jiang X. Upregulated long noncoding RNA PANDAR predicts an unfavorable prognosis and promotes tumorigenesis in cholangiocarcinoma. Onco Targets and Therapy. 2017; 10:2873–2883
63. Jiang XM, Li ZL, Li JL, Zheng WY, Li XH, Cui YF, et al. LncRNA CCAT1 as the unfavorable prognostic biomarker for cholangiocarcinoma. Eur Rev Med Pharmacol Sci. 2017 Mar;21(6):1242-1247
64. Xu Y, Leng K, Li Z, Zhang F, Zhong X, Kang P, et al. The prognostic potential and carcinogenesis of long non-coding RNA TUG1 in human cholangiocarcinoma. Oncotarget. 2017 Jul 22;8(39):65823-65835
65. Zhang F, Wan M, Xu Y, Li Z, Leng K, Kang P, et al. Long noncoding RNA PCAT1 regulates extrahepatic cholangiocarcinoma progression via the Wnt/β-catenin-signaling pathway. Biomed Pharmacother. 2017 Oct;94:55-62
66. Wang WT, Ye H, Wei PP, Han BW, He B, Chen ZH, et al.LncRNAs H19 and HULC, activated by oxidative stress, promote cell migration and invasion in cholangiocarcinoma through a ceRNA manner. J Hematol Oncol. 2016 Nov 3;9(1):117
67. Carotenuto P Fassan M, Pandolfo R, Lampis A, Vicentini C, Cascione L, et al. Wnt signalling modulates transcribed-ultraconserved regions in hepatobiliary cancers. Gut. 2017 Jul;66(7):1268-1277.
68. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, et al. The landscape of long noncoding RNAs in the human transcriptome. Nat Genet. 2015 Mar;47(3):199-208 .
69. Braconi C, Valeri N, Kogure T, Gasparini P, Huang N, Nuovo GJ, et al. Expression and functional role of a transcribed noncoding RNA with an ultraconserved element in hepatocellular carcinoma. Proc Natl Acad Sci U S A. 2011 Jan 11;108(2):786-91
70. Ranzani V, Rossetti G, Panzeri I, Arrigoni A, Bonnal RJ, Curti S, et al. The long intergenic noncoding RNA landscape of human lymphocytes highlights

the regulation of T cell differentiation by linc-MAF-4. Nat Immunol. 2015 Mar;16(3):318-325

71. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A (2011) 108 (12):5003–8
72. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nat Rev Clin Oncol. 2011 Jun 7;8(8):467-77.
73. Patel T. Extracellular vesicle noncoding RNA: new players in the diagnosis and pathogenesis of cholangiocarcinoma. Hepatology (2014) 60(3):782–4
74. Bernuzzi F, Marabita F, Lleo A, Carbone M, Mirolo M, Marzioni M, et al. Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Clin Exp Immunol. 2016 Jul;185(1):61-71
75. Voigtländer T, Gupta SK, Thum S, Fendrich J, Manns MP, Lankisch TO et al. MicroRNAs in serum and bile of patients with primary sclerosing cholangitis and/or cholangiocarcinoma PLoS One. 2015 Oct 2;10(10):e0139305
76. Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. Hepatology. 2014 Sep;60(3):896-907
77. Chan-On W, Nairismägi ML, Ong CK, Lim WK, Dima S, Pairojkul C, et al. Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers. Nat Genet. 2013 Dec;45(12):1474-8
78. Silakit R, Loilome W, Yongvanit P, Thongchot S, Sithithaworn P, Boonmars T, et al. Urinary microRNA-192 and microRNA-21 as potential indicators for liver fluke-associated cholangiocarcinoma risk group. Parasitol Int. 2017 Aug;66(4):479-485
79. Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology. 2017 Oct;66(4):1125-1143
80. Julich-Haertel H, Urban SK, Krawczyk M, Willms A, Jankowski K, Patkowski W, et al. Cancer-associated circulating large extracellular vesicles in

cholangiocarcinoma and hepatocellular carcinoma. J Hepatol. 2017 Aug;67(2):282-292

81. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010 Aug 19;363(8):711-23
82. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 2015;372:320-330
83. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med 2015;372:2521-2532
84. Li H, Zhou ZQ, Yang ZR, Tong DN, Guan J, Shi BJ, et al. MicroRNA-191 acts as a tumor promoter by modulating the TET1-p53 pathway in intrahepatic cholangiocarcinoma. Hepatology. 2017 Jul;66(1):136-151
85. Wang LJ, He CC, Su X, Zhou CY, Ma JL, et al. MiR-21 promotes intrahepatic cholangiocarcinoma proliferation and growth in vitro and in vivo by targeting PTPN14 and PTEN. Oncotarget. 2015; 6:5932-5946
86. Wang LJ, Zhang KL, Zhang N, Ma XW, Yan SW, Cao DH, Shi SJ. Serum miR-26a as a diagnostic and prognostic biomarker in cholangiocarcinoma. Oncotarget. 2015;6:18631–18640
87. Li J, Gao B, Huang Z, Duan T, Li D, Zhang S, et al.Prognostic significance of microRNA-203 in cholangiocarcinoma. Int. J. Clin. Exp. Pathol. 2015, 8, 9512–9516
88. Valeri N, Gasparini P, Fabbri M, Braconi C, Veronese A, Lovat F, et al. Modulation of mismatch repair and genomic stability by miR-155. Proc Natl Acad Sci U S A. 2010 Apr 13;107(15):6982-7
89. Goyal L, Saha SK, Liu LY, Siravegna G, Leshchiner I, Ahronian LG, et al. Polyclonal secondary FGFR2 mutations drive acquired resistance to FGFR inhibition in patients with FGFR2 fusion-positive cholangiocarcinoma.Cancer Discov. 2017 Mar;7(3):252-263
90. Javle M, Lowery M, Shroff RT, Weiss KH, Springfeld C, Borad MJ, et al. Phase II Study of BGJ398 in Patients With FGFR-Altered Advanced Cholangiocarcinoma. J Clin Oncol. 2018 Jan 20;36(3):276-282.

91. Novarino, A.M.T., Satolli, M.A., Chiappino, I. Giacobino A, Napoletano R, Ceccarelli M, et al. FOLFOX-4 regimen or single-agent gemcitabine as first-line chemotherapy in advanced biliary tract cancer. Am J Clin Oncol. 2013; 36: 466–471
92. He S, Shen J, Sun X, Liu L, Dong J.A phase II FOLFOX-4 regimen as second-line treatment in advanced biliary tract cancer refractory to gemcitabine/cisplatin. J Chemother. 2014 Aug;26(4):243-7
93. Okamoto K, Miyoshi K, Murawaki Y. miR-29b, miR-205 and miR-221 enhance chemosensitivity to gemcitabine in HuH28 human cholangiocarcinoma cells. Plos One. 2013; 8(10): e77623.
94. Chen L, Yan HX, Yang W, Hu L, Yu LX, Liu Q, et al. The role of microRNA expression pattern in human intrahepatic cholangiocarcinoma. Journal of Hepatology. 2009; 50(2): 358–369
95. Lampis A, Carotenuto P, Vlachogiannis G, Cascione L, Hedayat S, Burke R, et al. MIR21 Drives Resistance to Heat Shock Protein 90 Inhibition in Cholangiocarcinoma.Gastroenterology. 2018 Mar;154(4):1066-1079.e5
96. Shirota T, Ojima H, Hiraoka N, Shimada K, Rokutan H, Arai Y, et al. Heat Shock Protein 90 Is a Potential Therapeutic Target in Cholangiocarcinoma. Mol Cancer Ther. 2015 Sep;14(9):1985-93.
97. Lin H, Kolosenko I, Bjorklund AC, Protsyuk D, Österborg A, Grandér D, et al. An activated JAK/STAT3 pathway and CD45 expression are associated with sensitivity to Hsp90 inhibitors in multiple myeloma. Exp Cell Res 2013;319:600-11
98. Acquaviva J, He S, Zhang C, Jimenez JP1, Nagai M1, Sang J, et al. FGFR3 translocations in bladder cancer: differential sensitivity to HSP90 inhibition based on drug metabolism. Mol Cancer Res 2014;12:1042-54
99. Johnston M, Geoffroy MC, Sobala A, Hay R, Hutvagner G.HSP90 protein stabilizes unloaded argonaute complexes and microscopic P-bodies in human cells. Mol Biol Cell 2010;21:1462-9
100. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases.Nat Rev Drug Discov. 2017 Mar;16(3):203-222

101. Rupaimoole R, Wu SY, Pradeep S, Ivan C, Pecot CV, Gharpure KM, et al. Hypoxia-mediated downregulation of miRNA biogenesis promotes tumour progression. Nat Commun. 2014 Oct 29;5:5202

102. Imig J, Brunschweiger A, Brümmer A, Guennewig B, Mittal N, Kishore S, et al. miR-CLIP capture of a miRNA targetome uncovers a lincRNA H19-miR-106a interaction. Nat Chem Biol. 2015 Feb;11(2):107-14

103. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, et al. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: A first-in-man, phase 1, open-label, dose-escalation study. Lancet Oncol. 2017, 18, 1386–1396

104. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med. 2013 May 2;368(18):1685-94

105. Hong DS, Kang YK, Brenner AJ,Sachdev JC, Ejadi S, Borad MJ, et al. MRX34, a liposomal miR-34mimic, in patients with advanced solid tumors: final dose-escala-tion results from a first-in-human phase I trial of microRNA therapy.J Clin Oncol.2016;34:suppl:abstr2508

106. Han Y, Meng F, Venter J, Wu N, Wan Y, Standeford H, et al. miR-34a-dependent overexpression of Per1 decreases cholangiocarcinoma growth. J Hepatol. 2016 Jun;64(6):1295-304