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Chapter 4

Noncoding RNAs in Gallbladder Cancer

Panagiotis Paliogiannis, Gavinella Latte and Karim Bel Imam

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Abstract

Gallbladder cancer (GBC) is the most frequent malignancy of the biliary tract, representing about 85–90% of the cancers involving this anatomical district; it is characterized by high mortality rates with less than 10% of the sufferers surviving more than 5 years. Extensive scientific research is needed in order to identify biomarkers for early diagnosis, improve the treatment options available, and assess new effective therapies. Consistent improvements have been made in recent years in the field of noncoding RNAs. More than 90% of the human genome is constituted by a noncoding portion that actively transcribes an enormous and complex amount of RNA, while only approximately 2% represents the coding genes. Noncoding RNAs are divided into two categories in accordance with their dimensions: small RNAs, which are made by less than 200 nucleotides, and long RNAs, which are bigger. MicroRNAs (miRNAs) and long noncoding RNA (lncRNAs) are the main subclasses, respectively, which concentrate consistent scientific efforts in recent times with promising results in several diseases, including cancer. In this review, we summarize the roles of miRNAs and lncRNAs in gallbladder cancer pathophysiology and their possible translational implication in the diagnosis and treatment of this aggressive disease.

Keywords: biliary tract, gallbladder, cancer, long noncoding RNA, lncRNAs, miRNAs

1. Introduction

Gallbladder cancer (GBC) is the most frequent malignancy of the biliary tract, representing about 85–90% of the cancers involving this anatomical district. Furthermore, it is the main cause of death among biliary tract tumors [1]. More than 76,000 cases of gallbladder cancer have been estimated worldwide in 2012; two thirds were registered in less developed areas of the globe [2]. At the same time, more than 60,000 deaths were estimated worldwide, evidencing

that incidence and mortality rates are very close [2]. Indeed, the absence of specific clinical manifestations in the early stages of the disease, along with the lack of specific biological markers, makes the prompt diagnosis challenging, and a great part of the patients presents with advanced stage local or metastatic lesions. Most of those who receive surgery, chemotherapy, and/or radiotherapy develop early recurrences or do not respond to treatments; as a result, the overall survival is poor, with less than 10% of the sufferers surviving more than 5 years [3]. This makes necessary further scientific efforts in order to identify trustful biomarkers for early diagnosis, improve the treatment options available, and assess new effective therapies.

Interesting developments were made in recent years in the study of noncoding RNAs (ncRNAs) and their involvement in cancer development, growth, and dissemination. Results of the human genome project and other next-generation sequencing studies evidenced that the approximately 20,000 protein-coding genes represent approximately 2% of the human genome, while more than 90% is made by a noncoding portion that actively transcribes an enormous and complex amount of RNA [4]. This part of the transcriptome has been called "dark matter" in the past because it has been interpreted as transcriptional debris; nevertheless, recent advantages confirmed that this huge amount of ncRNA displays numerous roles in the normal cellular biology, as well as in many pathological processes.

The group of ncRNAs is commonly divided into two further categories, according to their size. The first one includes small ncRNAs, like the recently discovered microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), and others, in addition to the classical cellular RNAs (ribosomal, transfer, and other RNAs). miRNAs are RNAs approximately 22 nucleotides long, which function as intricate components of cellular networks involved in the specific regulation of both protein-coding and noncoding genes, generally by posttranscriptional silencing [5, 6]. The **Figure 1** summarizes the main types of ncRNAs currently known. Noncoding RNAs greater than 200 nucleotides represent the remaining category, including molecules defined long noncoding RNA (lncRNAs). This merely dimensional definition of lncRNAs has some limitations, like the arbitrary cutoff value and the real protein coding potential, and this reflects the complexity of this group of molecules [7].

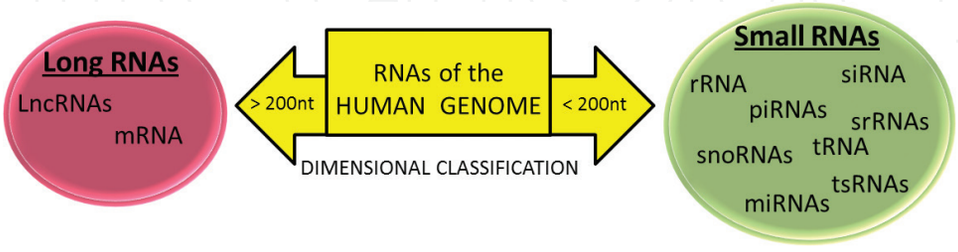

Figure 1. According to the length of RNA chain, RNA molecules of the human genome can be classified in small RNA and long RNA. Generally, small RNAs are shorter than 200 nucleotides (nt) in length, while long RNAs are made by more than 200 nt. Long RNAs, also called large RNAs, include mainly the long noncoding RNAs (lncRNAs) and the messenger RNA (mRNA). Small RNAs mainly include ribosomal RNA (rRNA), transfer RNA (tRNA), microRNAs (miRNAs), small-interfering RNAs (siRNAs), small-nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNAs), and small rDNA-derived RNAs (srRNAs).

2. MicroRNAs in gallbladder cancer

MicroRNAs (miRNAs) are endogenous noncoding RNAs that bind to the 3′ untranslated region (UTR) of a target messenger RNA (mRNA), specifically in a sequence called miRNA recognition element (MRE), which can be fully or partially complementary. They are essential posttranscriptional regulators of multiple genes and determine the function of the cells under physiological and in several pathological conditions. Since 1993, when they were discovered, hundreds of miRNAs have been characterized, and they are being widely studied as an important biological compound with promising prospects as diagnostic and prognostic biomarkers and as therapeutic targets. A number of studies on the roles of several miRNAs in the pathogenesis of GBC have been recently published; numerous miRNAs exhibit expression changes, with most of them being upregulated in neoplastic cells and tissues, and further evidences confirmed their biological effects as either oncogenes or tumor suppressors (**Table 1**).

2.1. Onco-suppressor miRNAs in gallbladder cancer

Ten different miRNAs have been demonstrated to have onco-suppressive properties in recent studies (**Table 1**). miRNA-1 and miRNA-145 were analyzed in a study in which a significance analysis of microarrays (SAM) algorithm was employed to identify a set of 36 miRNAs consistently downregulated in GBC compared to normal gallbladder tissue. The real time (RT-PCR)

miRNA	Main effect	Interactions	References
miRNA-1	Onco-suppressor	VEGF-A and AXL	[8]
miRNA-145	Onco-suppressor	AXL	[8]
miRNA-135a-5p	Onco-suppressor	VLDLR	[9]
miRNA-26a	Onco-suppressor	HMGA2	[10]
miRNA-34a	Onco-suppressor	PNUTS	[11]
miRNA-355	Onco-suppressor		[12]
miRNA-130a	Onco-suppressor	HOTAIR, cMyc	[13]
miRNA-218-5p	Onco-suppressor	BMI1, CCT1	[14]
miRNA-146b-5p	Onco-suppressor	EGFR	[15]
miRNA-143	Onco-suppressor		[16]
miRNA-155	Oncogenic		[19]
miRNA-20a	Oncogenic	Smad7	[17]
miRNA-182	Oncogenic	CADM1	[18]
miRNA-21	Oncogenic	PTEN	[20]
miRNA-187	Oncogenic		[21]
miRNA-122	Oncogenic		[21]

Table 1. The miRNAs most studied in gallbladder cancer.

analysis confirmed the statistically significant reduced expression of miRNA-1 and miRNA-145 in tumors and GBC cell lines [8]. The ectopic expression of miRNA-1 and miRNA-145 in NOZ cell lines of GBC significantly repressed cell viability and colony formation, while only miRNA-1 reduced gene expression of known oncogenes, such as the vascular endothelial growth factor A (VEGF-A) and AXL receptor tyrosine kinase (AXL), suggesting that these miRNAs act as tumor suppressors in GBC [8].

Also miRNA-135a-5p has been demonstrated to be an onco-suppressor in gallbladder cancer. Its levels have been found to be significantly downregulated in GBC tissues and were correlated with the histological grade of the tumors [9]. Furthermore, the transfection of a miRNA-135a-5p mimetic inhibited proliferation and colony formation of GBC cells by G1/S phase cell-cycle block; lentivirus-mediated overexpression of miRNA-135a significantly reduced the proliferation of GBC cells. In addition, xenografts from miRNA-135a–infected cells in nude mice were significantly smaller compared to controls [9]. These evidences suggested the onco-suppressive role of this miRNA in GBC.

Also, miRNA-26a has a similar role. The expression of miRNA-26a was associated with the pathological stage of GBC in a recent study, which also demonstrated that miR-26a contributed in reducing neoplastic cell proliferation. The authors found that the introduction of high-mobility group AT-hook 2 (HMGA2), whose expression is inversely related to the levels of miRNA-26a, eliminated its effect on GBC cells [10]. In other words, the alterations of neoplastic cell proliferation induced by miRNA-26a in GBC appear to be intermediated by HMGA2 [10].

In another recent article, the levels of miRNA-34a and the telomere length were evaluated in 77 GBCs and 36 peri-tumoral tissues by RT-PCR [11]. The study evidenced a significantly reduced expression of miRNA-34a and longer telomere length in GBC tissues. Furthermore, it was found that the reduced expression of miRNA-34a was a negative prognostic factor. Remarkably, induced overexpression of miRNA-34a *in vitro* reduced the colony-forming capacity of GBC stem-like cells and repressed xenograft neoplastic growth *in vivo* [11].

The reduced expression of miRNA-335 has been found to be associated with aggressive clinical and pathological properties of GBC, specifically with high histologic grade, advanced clinical stage, and positive lymph node metastasis [12]. Furthermore, a reduced expression of miRNA-335 in GBC patients was associated with poor prognosis [12].

Also, miRNA-130a was found to be significantly downregulated in cancer tissues, compared with adjacent normal tissues; furthermore, its levels were negatively correlated to a lncRNA, HOX transcript antisense RNA (HOTAIR), which has been shown to be correlated with the metastatic progression of several carcinomas, and as a consequence, to be a negative prognostic factor [13]. We will return in this interaction later in this chapter, talking about lncRNAs in gallbladder cancer. A similar interaction is also displayed between miRNA-218-5p and lncRNA CCT1; the later negatively regulates miRNA-218-5p which, in turn, inhibits GBC cell invasion, migration, and proliferation by targeting the B-cell–specific moloney murine leukemia virus integration site 1 (Bmi1) [14].

The expression level of miRNA-146b-5p was similarly downregulated in GBC tissues compared with that in adjacent healthy tissues and was significantly correlated with tumor size and development in a study published by Cai et al. [15]. Moreover, high levels of miRNA-146b-5p in gallbladder neoplastic cells repressed malignant growth by provoking apoptosis and G1 phase cell-cycle block. In addition, the authors established that the amounts of epidermal growth factor receptor (EGFR) mRNA and those of miRNA-146b-5p were inversely related; this led them to the conclusion that EGFR can be considered as a mediator of the oncologic functions of miRNA-146b-5p in GBC [15].

Finally, miRNA-143 was found to be downregulated in studies performed by miRNA microarray analysis in GBC tissues, in comparison to adjacent healthy tissues [16]. Using blood samples from 40 GBC patients and healthy volunteers, the aberrant expression pattern of miRNA-143 was confirmed, and it was also evidenced that its expression levels were correlated with lymph node metastasis and the pathological TNM stage of the disease.

2.2. Oncogenic miRNAs in gallbladder cancer

Six miRNAs with an oncogenic activity in GBC have been reported in recent studies (**Table 1**): miRNA-155, miRNA-20a, miRNA182, miRNA-21, miRNA187, and miRNA-122 [17–21]. All of them have been found to be upregulated in neoplastic tissues in comparison to healthy tissues, while miRNA-187 and miRNA-122 have been determined also in blood samples. Some of them display interesting interactions with other molecular networks. For example, miRNA-20a was evidenced to play an essential role in the metastatic progression and poor survival of GBC by targeting the mothers against decapentaplegic homolog 7 (Smad7)-β-catenin axis [17]. Downregulation of miRNA-20a by a specific antagonist effectively restored the expression of Smad7 in GBC cells *in vitro* and *in vivo* and weakened transforming growth factor (TGF)-β–induced cell metastasis.

A similar situation was observed regarding the miRNA-182. In a recent study, it was found that the TGF-β–induced overexpression of miRNA-182 promoted GBC cell migration and invasion, while its inhibition produced the arrest of neoplastic progression [18]. Furthermore, the reduction of miR-182 expression by means of a specific inhibitor *in vivo* had a negative impact on the incidence of GBC lung metastases. Interestingly, the cell adhesion molecule 1 (CADM1) gene was identified as a novel molecular target of miRNA-182; its ectopic expression in GBC cells led to decreased tumoral invasion [18].

3. LncRNAs in gallbladder cancer

The first lncRNA, lncRNAH19, has been discovered in 1990 by Brannan et al. [22]. Since then, a great number of further lncRNAs have been discovered, and several digital databases provide information about their molecular features and their biological functions [7]. More than 6700 lncRNA genes have been identified in the human genome in recent times [23]. Generally, their length reaches 100 kilobases, without significant open reading frames (ORF); they are

transcribed by RNA polymerase II or III and can be polyadenylated or not, spliced or not, nuclear or cytoplasmic. Their expression levels are usually lower than those of the protein-coding genes, and a certain tissue-specificity has been described.

Several classifications of lncRNAs, based on different criteria, have been proposed. A first classification, based on their location on the genome, divides the lncRNAs into five groups: (a) sense, when they overlap with the exons of a different transcript on the same strand, (b) antisense, when they overlap with the exons of a different transcript on the opposite strand, (c) intronic, when they originate from an intron of a different transcript, (d) bidirectional, when the lncRNA and an adjacent transcript on the opposite strand are expressed at the same time, and (e) intergenic, when located in a region not affected by other coding sequences [24]. From a strictly functional perspective, Isin and Dalay classified lncRNAs in three categories: (a) the lncRNAs guides which can bind and guide cellular proteins toward their target, (b) the lncRNAs scaffolds which can bind effector molecules and initiate the formation specific molecular complexes, and (c) the lncRNAs which can bind proteins or RNA molecules and thus prevent these from exerting their function (we could call them "inhibitors") [7].

The lncRNAs are implicated in a wide range of pre- and posttranscriptional functions, including nuclear architecture and import, immunity, imprinting, epigenetic regulations, cellular trafficking, splicing, precursors of smaller RNAs, and pluripotency of the embryonic stem cells. LncRNAs can regulate gene expression at different levels including chromatin modifications, transcription, splicing, translation, posttranscriptional regulation, processing of small RNAs, as well as several other functions [7]. They can affect and regulate the cell cycle and proliferation, differentiation and apoptosis and are involved in cancer development, maintenance, and progression [25]. Indeed, recent articles evidenced that approximately 18% of the total human lncRNAs are associated with several types of tumors [26]. The role of lncRNAs in gallbladder cancer has been investigated only in very recent years. Data about the roles of eleven lncRNAs have been published in the last three years; among them, eight have been demonstrated to be oncogenic and three onco-suppressors (**Table 2**).

3.1. Onco-suppressor lncRNAs

Three different lncRNAs have been found to display an onco-supressive role in gallbladder cancer (**Table 2**): GCASPC, LET, and MEG. In a study published in 2016, Ma et al. used RT-PCR to measure GCASPC levels in tissues from 42 gallbladder cancer patients, and the levels of GCASPC were further confirmed in a separate cohort of 89 gallbladder cancer patients [27]. Its levels were significantly lower in neoplastic than adjacent nontumor tissues and were associated with tumor size, stage, and prognosis. GCASPC overexpression suppressed cell proliferation *in vitro* and *in vivo*, whereas its silencing had opposite effects. The authors also identified pyruvate carboxylase as an RNA-binding protein associated to GCASPC. Because GCASPC is a target of miR-17-3p, they evidenced that both miR-17-3p and GCASPC downregulated pyruvate carboxylase level and activity. The authors defined this way a novel mechanism of lncRNA-regulated cell proliferation in gallbladder cancer, creating a new basis for understanding its pathophysiology [27].

LncRNA	Main effect	Interactions	References
AFAP-AS1	Oncogenic	MET proteins	[30]
ANRIL	Oncogenic	p53, p15INK4b, p16INK4a, cell cycle and apoptosis proteins	[29]
CCAT1	Oncogenic	miRNA 218-5p, Bmi1	[14]
GCASPC	Onco-suppressor	miRNA 17-3p, pyruvate carboxylase	[27]
H19	Oncogenic	miRNA 194-5p, AKT2, MET proteins	[31, 32]
HOTAIR	Oncogenic	miRNA 130a	[13]
ITGB1	Oncogenic	B-catenin, TCF8, MET proteins	[34]
KIAA0125	Oncogenic	B-catenin, MET proteins	[33]
LET	Onco-suppressor	p21, Bax/Bcl-2, apoptosis proteins	[28]
MALAT1	Oncogenic	ERK/MAPK	[35]
MEG3	Onco-suppressor	p53, cell cycle and apoptosis proteins	[29]

Table 2. The main lncRNAs studied in relation to their role in gallbladder cancer pathophysiology.

The same research group in a previous study evidenced that low levels of the lncRNA LET were associated with a less differentiated histology, advanced nodal status, and tumor stage, in relation to GBC patients with high LET expression [28]. Moreover, the overall 5-year survival rates of low and high LET expression groups was approximately 38 and 67%, respectively, with the low expression of this specific lncRNA being a significant predictor of metastasis and death in GBC patients [28]. Interestingly, the authors evidenced also that hypoxia correlated with decreased lncRNA LET levels in GBC EZ-GB2 and SGC-996 cells. They demonstrated that the invasive potential of GBC cells significantly decreased in cells overexpressing LET under hypoxia, while the invasive potential of GBC cells enhanced in LET knockdown cells under hypoxic conditions. They also showed that under hypoxic conditions, LET inhibited GBC cell proliferation by inducing a G0/G1 arrest, further confirming the tight connection between hypoxia and LET effects [28].

Regarding MEG3, Liu et al. demonstrated an approximately 6.25-fold reduction in its expression in GBC tissues compared to normal tissue samples [29]. The transfection of pcDNA-MEG3 plasmids in human GBC GBC-SD and QBC939 cell lines resulted in reduced tumorigenic potential. When 5-week-old male athymic BALB/c mice were injected with GBC transfected cells, smaller tumors resulted compared to those treated with an empty vector. pcDNA-MEG3 plasmid transfection in GBC cells induced the accumulation of p53 protein and reduction of the cyclin D1 gene expression. These transfected cell lines showed an accumulation of cells at the G0/G1 phase, lower expression levels of ki-67, and higher expression levels of Caspase-3, which implies that MEG3 also plays a vital role in the induction of apoptosis in GBC [29].

3.2. Oncogenic lncRNAs

Eight different lncRNAs showed oncogenic potential in GBC (**Table 2**): AFAP1-AS1, ANRIL, CCAT1, H19, HOTAIR, ITGB1, KIAA0125, and MALAT-1. In a recent study, Ma et al. analyzed the lncRNA AFAP1-AS1 expression by RT-PCR in 40 gallbladder cancer tissues and adjacent normal tissues [30]. The authors evidenced that the expression of lncRNA AFAP1-AS1 was significantly elevated in GBC tissues and GBC cell lines. In addition, its expression levels were significantly associated with tumor sizes and prognosis. Knockdown of AFAP1-AS1 suppressed cell growth and invasion in NOZ and GBC-SD cells. Furthermore, they found that knockdown of AFAP1-AS1 in GBC cells inhibited EMT by downregulating the transcription factor Twist1 and Vimentin and upregulated the E-cadherin [30].

The role of lncRNA ANRIL in the pathogenesis of GBC was studied by Liu et al. together with that of MEG3 mentioned before [29]. In that study, GBC tissues and adjacent normal samples were collected from 84 patients, and empty vector and pcDNA-ANRIL vectors were transfected into GBC-SD and QBC939 cells. The expression of ANRIL was significantly higher in GBC and pcDNA-ANRIL-transfected cells in comparison to controls, and it was associated with prognosis. Even if mice injected with pcDNA-ANRIL showed contrasting results, the authors concluded that ANRIL can improve the proliferation of gallbladder cells and inhibit apoptosis [29].

Recently, Ma et al. demonstrated an approximately 1.5-fold upregulation of CCAT1 in 40 GBC tissues compared to paired normal tissues [14]. The expression of CCAT1 was higher in tumors extending beyond the gallbladder, with a stage-dependent pattern of expression. Similarly, overexpression of CCAT1 was found to be significantly associated with lymph node invasion and advanced node metastasis, highlighting its role in metastasis in GBC. As we mentioned before, the authors further observed that ectopic expression of CCAT1 increased the transcript level of Bmi1 in GBC-NOZ cells, while it decreased the expression level of miRNA-218-5p which has a tumor suppressive activity in several carcinomas and regulates the Bmi1 gene expression. They advocate that CCAT1 up-regulates Bmi1 by competitively 'sponging' the tumor suppressor miRNA-218-5p, as both shared the same miRNA responsive element in their sequences and displayed the same miRNA-218-5p-dependent regulation pattern [14].

Similar evidences were found about the lncRNA H19, which was found to be significantly upregulated in GBC tissues compared to adjacent noncancerous tissue and was positively correlated with tumor size and decreased survival of GBC patients [31, 32]. Its oncogenic role was further experimentally confirmed in a 4-week-old male athymic nude mice model of human GBC. In addition, the ectopic expression of H19 led to decreased expression of E-cadherin, and increased the expression of Vimentin and Twist1, in cell lines as well as in mice [32]. Interestingly, it was found that H19 positively regulates the expression of the AKT2 gene (a putative oncogene) while reduces the levels of miR-194-5p, which demonstrated tumor-suppressive activity in several cancers [31].

Moreover, the levels of lncRNA HOTAIR were significantly higher in 65 GBC tissues, especially in those in higher pathological stage, in a recent report [13]. At a molecular level, HOTAIR expression was shown to be regulated by c-Myc [13]. As we mentioned before, regulators of HOTAIR activity include miRNA-130a, a tumor suppressor miRNA, and this reflects the complexity of the regulatory networks in gallbladder cancer, which include several types

of ncRNAs and coding genes. Ectopic expression of HOTAIR reduced the level of miRNA-130a, while miRNA-130a inhibition upregulated HOTAIR. Furthermore, it was demonstrated that depletion of HOTAIR inhibited the invasion of GBC cells, while a miRNA-130a inhibitor reversed this decrease in invasiveness of GBC cells. Moreover, the depletion of HOTAIR resulted in the suppression of cell proliferation [13].

Both the lncRNAs ITGB1 and KIAA0125 were found to be overexpressed in GBC tissues, and both of them influence the GBC cell migration and invasion, in part through the alteration of Vimentin and β-catenin levels [33, 34]. Also, MALAT1 was significantly upregulated in GBC tissues compared with corresponding noncancerous tissues [35]. Knockdown of MALAT1 in GBC cell lines (SGC-996 and NOZ) significantly inhibited the proliferation and metastasis of the GBC cells both *in vitro* and *in vivo* (xenograft BALB/c nude mouse model of human GBC). Furthermore, the ERK/MAPK pathway was found to be inactivated in the GBC cell lines after MALAT1 knockdown, as it significantly reduced the levels of phosphorylated MEK1/2, ERK 1/2, MAPK, and JNK 1/2/3 proteins, with no changes in their total levels. This suggests that MALAT1 acts as an oncogenic lncRNA that promotes proliferation and metastasis of GBC and activates the ERK/MAPK pathway [35].

4. Future perspectives

As we mentioned before, the number of the noncoding RNAs of the human genome, both miRNAs and lncRNAs or other species, is enormous, as is the number of their possible interactions with a myriad of biological networks in healthy and neoplastic tissues. This reflects how little we know about them, and the huge scientific efforts which should be made in the future in order to better understand their pathophysiological roles and use them as diagnostic or prognostic markers, as well as targets for effective specific therapies. This would be particularly desirable in malignancies such as GBC, characterized by an aggressive clinical behavior and poor prognosis.

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