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The emerging role of long non-coding RNA in gallbladder cancer pathogenesis

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ABSTRACT

Gallbladder cancer (GBC) is the most common and aggressive form of biliary tract carcinoma with an alarmingly low 5-year survival rate. Despite its high mortality rate, the underlying mechanisms of GBC pathogenesis are not completely understood. Recently, from a growing volume of literature, long non-coding RNAs (lncRNAs) have emerged as key regulators of gene expression and appear to play vital roles in many human cancers. To date, a number of lncRNAs have been implicated in GBC, but their potential roles in GBC have not been systematically examined. Thus, in this review, we critically discuss the emerging roles of lncRNAs in GBC, and the pathways involved. Specifically, we note that some lncRNAs show greater expression in T1 and T2 tumor stages compared to T3 and T4 tumor stages and that their dysregulation leads to alterations in cell cycle progression and can cause an increase in GBC cell proliferation or apoptosis. In addition, some lncRNAs control the epithelial-mesenchymal transition process, while others take part in the regulation of ERK/MAPK and Ras cancer-associated signaling pathways. We also present their potential utility in diagnosis, prognosis, and/or treatment of GBC. The overall goal of this review is to stimulate interest in the role of lncRNAs in GBC, which may open new avenues in the determination of GBC pathogenesis and may lead to the development of new preventive and therapeutic strategies for GBC.

Contents

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1. Introduction

Gallbladder cancer (GBC), an aggressive type of biliary tract carcinoma, is the fifth most common gastrointestinal malignancy worldwide [1]. Despite recent advances in the understanding and treatment of GBC, the prognosis is poor with a strikingly low 5-year survival rate. This is due in part to the advanced stages at which GBC is frequently diagnosed because patients in the early stages of GBC often exhibit no symptoms [2–4]. Therefore, a comprehensive approach is warranted to identify and validate novel tumor markers to improve the diagnosis, prognosis and treatment for GBC patients.

Based on results from the human genome project and next-generation sequencing technologies, it has been estimated that only ~1.2% of the mammalian genome encodes for proteins, while more than 90% of the genome is transcribed into tens of thousands of non-coding RNAs (ncRNA) [5,6]. A large number of ncRNAs such as microRNAs [generally between 22 and 25 nucleotides (nt)], snoRNAs, piwiRNAs (between 26 and 31 nt), and long non-coding RNAs (lncRNAs; which are often longer than 200 nt) have been found to play critical roles in cancer progression, development and metastasis [7–9]. Among these ncRNAs, lncRNAs show the most diversity and can be constitutively expressed throughout development in a time- and tissue-specific manner [10,11] with a median half-life of 3.5 h [12,13]. Owing to their significant involvement in many physiological as well as pathological processes, these lncRNA molecules are the subject of widespread research. In a previous review on lncRNAs and lung cancer [14], we provided an extensive description of lncRNA biogenesis and their involvement in the regulation of the cellular activities, which involves chromatin (epigenetic gene expression, gene silencing, gene imprinting, and chromatin modification), transcription (activation control) and post-transcriptional modifications (splicing, RNA copy number), translation, RNA interference, stress-regulation, and cell-cycle control. In the same review, their roles as signaling, guide, and scaffolding molecules, as well as miRNA "sponges" were also discussed [14]. Along with these properties, lncRNAs are also reported to be dysregulated in various cancers where they act as tumor suppressive or oncogenic molecules.

Recently, expression of several lncRNAs such as MEG3 (Maternally Expressed Gene 3) [15], H19 [16,17], MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) [18], HOTAIR (HOX Transcript Antisense RNA) [19], KIAA0125 [20], Linc-ITGB1 (Long intergenic non-coding RNA-ITGB1) [21], lncRNA-LET (Long non-coding. RNA-Low Expression in Tumor) [22], CCAT1 (Colon cancer associated-transcript 1) [23] and ANRIL (Antisense non-coding RNA in the INK4 locus) [15] have been implicated in various cancers, including GBC (Fig. 1A). Moreover, studies have indicated that these lncRNAs can play critical roles in modulating cell proliferation, migration, invasion, apoptosis (Fig. 1B), and "sponging" of miRNAs, all of which represent various hallmarks of cancer. However, the expression of lncRNAs and their implications for diagnosis and therapy of GBC remain obscure. In this review, we focus on lncRNAs involved in gallbladder pathogenesis (Table 1) and discuss the potential utility of lncRNAs as biomarkers (diagnostic and prognostic) and treatment strategies for GBC.

2. Involvement of long non-coding RNAs with gallbladder cancer

2.1. MEG3 (maternally expressed gene 3)

LncRNA *MEG3* is an imprinting gene located at the imprinted *DLK-MEG3* locus with a length of 1.6 kb and ten exons located on chromosome 14q32.3 [24] (Table 1). In humans, the *MEG3* gene is expressed ubiquitously in many cell types and plays an important role in development and growth [25]. It is found to regulate apoptosis and cell proliferation by increasing the expression of various tumor suppressor genes such as *p53, ARF, PTEN, BRCA1* [26,27]. It also interacts with VEGF pathway-related genes and suppresses tumor angiogenesis, invasion and metastasis [28], and has been reported to be down regulated in various cancers such as gliomas [29], gastric [30], hepatocellular [31,32] and bladder cancers [33].

Recently, Liu et al. (2016) demonstrated a substantial (~6.25-fold) reduction in the expression of *MEG3* in GBC tissues compared to normal tissue samples [15] (Table 1). A potential explanation for the low levels of expression of MEG3 in cancers, including GBC, may be due to the hypermethylation of MEG3 promoters [24]. In case of GBC, transfection of pcDNA-MEG3 plasmids in human GBC GBC-SD and QBC939 cell lines resulted in reduction in tumorigenic potential as assessed by cell growth in soft agar assays [15]. When five week-old male athymic BALB/c mice were injected with GBC transfected cells, smaller tumors resulted compared to cells treated with an empty vector [15]. At the molecular level, pcDNA-MEG3 plasmid transfection in GBC cells induced the accumulation of p53 protein while decreasing the expression of the *cyclin D1* gene [15] (Fig. 2). This growth inhibition may result because MEG3 can downregulate the expression of ubiquitin ligases and increase p53 levels, which activates various p53-dependent transcriptional activities to suppress tumorigenesis [34]. Results from flow cytometry experiments on these transfected cell lines showed an accumulation of cells at the G0/G1 phase compared to the S-phase of the cell cycle [15]. Furthermore, immunohistochemical analysis of mice injected with pcDNA-MEG3 transfected cells exhibited lower expression levels of ki-67 (an intracellular cell proliferation marker) and higher expression levels of Caspase-3, which implies that MEG3 also plays a vital role in the induction of apoptosis in GBC [15].

Collectively, evidence suggests that MEG3 may act as a tumor suppressor molecule in GBC that inhibits the growth of cancer cells by enhancing the expression of p53 and reducing the expression of cyclin D1 (Fig. 2). Thus, MEG3 may be explored as a potential therapeutic and biomarker for GBC, although further studies are needed to determine its clinical relevance.

2.2. H19

LncRNA *H19* is an imprinted gene and transcribed from the intergenic locus of the maternal allele. It spans ~2.3-kb on chromosome 11p15.5 (Table 1) and plays a vital role in mammalian development [16]. Several studies have demonstrated that H19 is

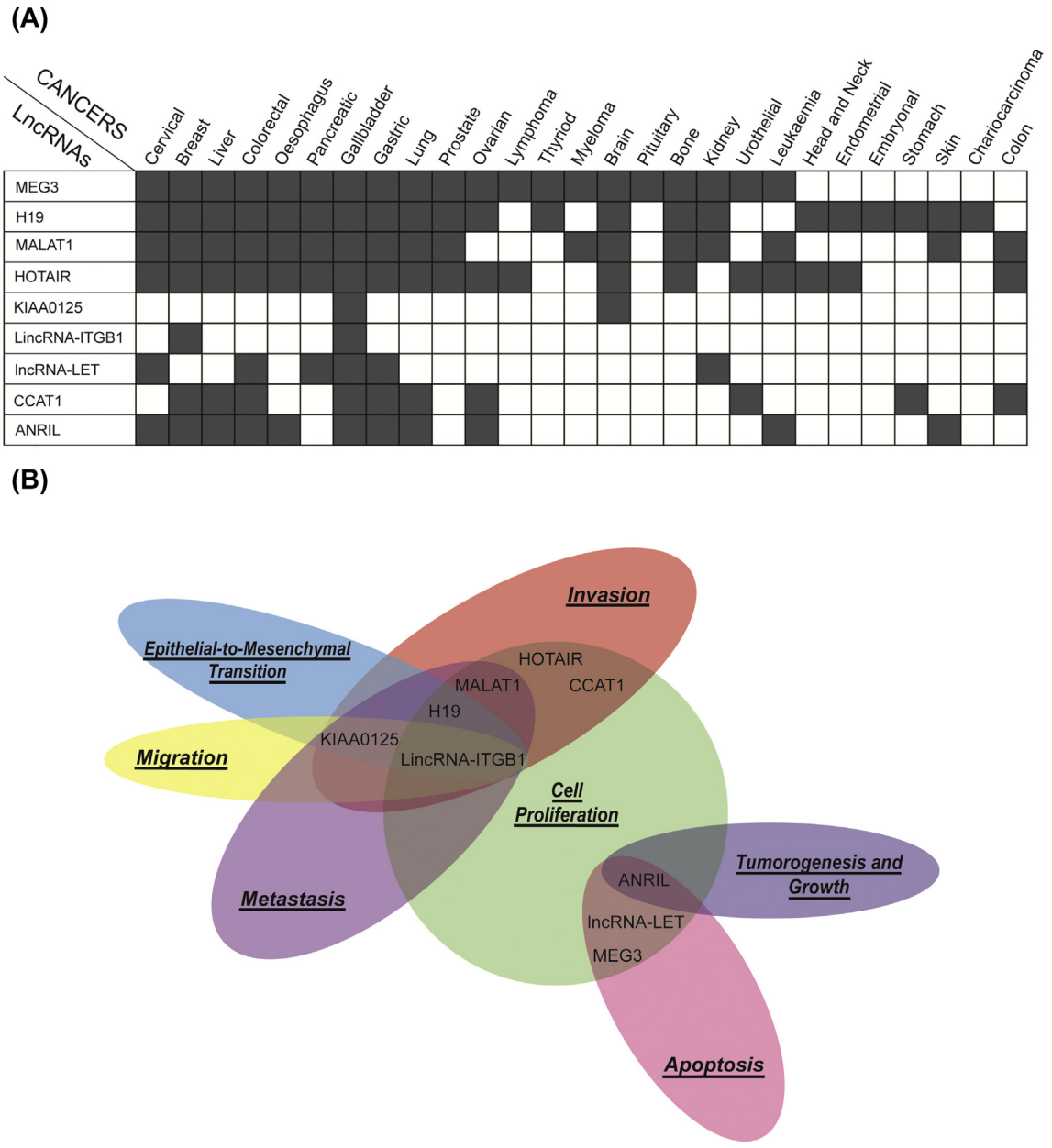

Fig. 1. LncRNAs and cancer. (A) Dysregulation of lncRNAs in various human cancer types. The shaded squares represent a relationship between the named lncRNA (on the left) and the cancer type (listed on top of the figure). (B) Schematic diagram of gallbladder cancer-associated lncRNAs modulating various hallmark events involved in cancer.

overexpressed in glioma [35], gastric [36], breast [37], ovarian [38] and lung cancers [14]. In lung cancer, it promotes cancer cell migration by regulating the expression of p53, c-Myc, and E2F transcription factors [14] while in colorectal cancer, it functions as a miRNA sponge [39]. Ectopic expression of H19 enhances the tumorigenicity of breast cancer cells [40], while its inactivation has been implicated in the development of Wilms' tumor [41].

Relevant to GBC, the expression of H19 was found to be significantly upregulated (~2.3-fold) (Table 1) in cancerous tissues compared to adjacent non-cancerous gallbladder tissue, and was positively correlated with tumor size, and decreased survival of GBC patients [16,17]. In a 4-week-old male athymic nude mice model of human GBC, knockdown of H19 caused a significant decrease in tumor size compared to those in the control group. In addition, it was observed that knockdown of H19 inhibited progression of GBC-NOZ cells into S-phase [16]. Further, ectopic expression of H19 led to decreased expression of E-cadherin (an epithelial marker and cell adhesion molecule), increased expression of Vimentin (a mesenchymal marker) and Twist1 [an Epethilial-to-mesenchymal transition (EMT) related transcription factor] in cell lines as well as in mice [17]. Cell invasion was enhanced in GBC-SD cells following H19 overexpression, whereas, GBC cell invasion potential was significantly decreased when H19 was depleted using an H19-siRNA approach [16]. In this study [16] the authors elegantly demonstrated that H19 positively regulates the expression of the *AKT2* gene and subsequent protein levels, while negatively regulating the levels of miR-194-5p (Fig. 2). In another set of experiments [16], the authors showed that ectopic expression of H19 in GBC-NOZ cells upregulated the mRNA and protein levels of AKT2, which was reversed by miRNA-194-5p mimics. These results suggest that H19 potentially regulates AKT2 by inhibiting the expression of miR-194-5p. AKT2 is a putative

Table 1
Gallbladder cancer-associated long non-coding RNAs, their reported biological functions and affected pathways.

S.No.	LncRNA	Genomic location	Mean fold-change in expression compared to controls	Property	Validation methods	Biological significance	Genes/Proteins/Pathways affected	References
1)	MEG3	14q32.2	↓6.25-fold	Tumor suppressor	qRT-PCR; Western Blot	Inhibits cell proliferation and induces apoptosis	↑p53 gene; ↑Caspase-3 and ↓Cyclin D	[15]
2)	H19	11p15.5	↑2.3-fold	Oncogenic	qRT-PCR; Western Blot	Promotes cell proliferation, EMT, invasion and metastasis	H19/miR-194-5p/AKT2 axis; ↓E-cadherin; ↑Vimentin and ↑Twist1	[16,17]
3)	MALAT1	11q13.1	↑3.0-fold	Oncogenic	qRT-PCR	Promotes cell proliferation, migration and metastasis	↑MMP 9; ↑MEK1/2, ERK1/2, MAPK, JNK1/2/3 in ERK/MAPK pathway	[18]
4)	HOTAIR	12q13.13	↑3.0-fold	Oncogenic	qRT-PCR; ChIP	Promotes cell proliferation and invasion	↓miRNA-130a	[19]
5)	KIAA0125	14q32.33	↑5.83-fold	Oncogenic	qRT-PCR	EMT, cell migration, invasion and metastasis	↑Vimentin and ↓β-Catenin	[20]
6)	Linc-ITGB1	10p11.2	↑29.0-fold	Oncogenic	qRT-PCR; Western Blot	Promotes cell proliferation, EMT, migration, invasion and metastasis	↑Vimentin; ↓β-Catenin; ↑TCF8 and ↑Slug	[21]
7)	LncRNA-LET	15q24.1	↓7.5-fold	TumTumor Tumor suppressor	qRT-PCR; Western Blot	Inhibits cell proliferation and induce apoptosis	HIF-1a/HDAC3/lncRNA-LET/NF-90 axis ↑p21; ↑caspase-3 and ↑Bax/Bcl-2	[22]
8)	CCAT1	8q24.21	↑1.5-fold	Oncogenic	qRT-PCR; Western Blot	Promotes cell proliferation and invasion	↓miRNA-218-5p; ↑Bmi1	[23]
9)	ANRIL	9p21.3	↑2.43-fold	Oncogenic	qRT-PCR; Western Blot	Promotes cell proliferation and inhibits apoptosis	↓p53; ↓Caspase-3; ↑Cyclin D; ↑Ki-67; modulates INK4b-ARFINK4a locus and ↓p15INK4b and ↓p16INK4a; RAS pathway	[15]

oncogene, which was shown to be overexpressed in many cancers, including GBC, whereas miR-194-5p acts as a tumor suppressor in several cancers [16].

These findings suggest that H19 might function as an oncogene in GBC, primarily by playing a regulatory role in cell proliferation and EMT in GBC cells via an H19/miR-194-5p/AKT2 axis regulatory network [16,17]. Thus, H19 may be considered as a potential prognostic biomarker, and/or a therapeutic target for the treatment of GBC in humans.

2.3. *MALAT1 (metastasis associated lung adenocarcinoma transcript 1)*

LncRNA MALAT1 was first reported to play a role in metastatic non-small cell lung carcinoma and is conserved across several species, which highlights its functional importance [42]. MALAT1 expression has been shown to be significantly upregulated in many human cancers such as breast, prostate, colon, cervical and liver [43–47]. It has been reported that MALAT1 promotes cellular motility, invasion, proliferation and radio-resistance by regulating the expression of genes involved in various cancer pathways [48].

Liu et al. [18] analyzed the expression patterns of MALAT1 in 20 GBC cancer tissue samples by qRT-PCR, and found it to be expressed at levels ~3-fold higher than its normal counterparts (Table 1). Similarly, in GBC cell lines (SGC-996 and NOZ) MALAT1 expression levels were higher compared to normal cells. Suppression of MALAT1 transcripts using an siRNA approach has been shown to decrease colony formation and proliferation of GBC cells compared to control cells. Furthermore, the depletion of MALAT1 in GBC cells increased the number of cells at the G2/M phase of the cell cycle. The role of MALAT1 in promoting GBC metastasis was assessed using a xenograft BALB/c nude mouse model of human GBC, and it was found that the growth of tumors from MALAT1-depleted xenografts was significantly inhibited compared with that of tumors derived from the mock-infected or control cells. At the molecular level, knockdown of MALAT1 reduced the expression of the matrix

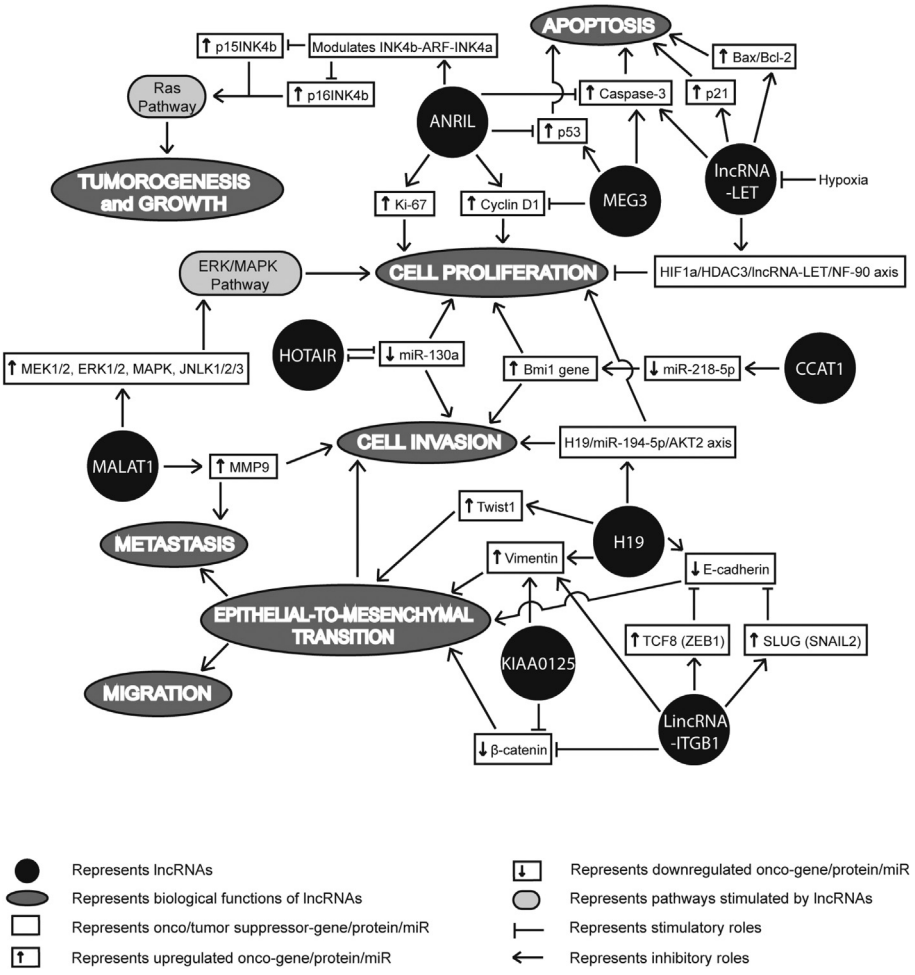

Fig. 2. Regulatory networks of various lncRNAs involved in the pathogenesis of gallbladder cancer. The schematic diagram depicts the putative cellular roles of lncRNAs in the upregulation (upward arrows) or downregulation (downward arrows) of gene products in various cellular events involved in gallbladder cancer pathogenesis.

metalloproteinase 9 (MMP-9) in GBC cells, an enzyme involved in the degradation of the extracellular matrix during cell division. Since MALAT1 has been implicated in several cancer types via regulation of various proteins/pathways such as NFkB, Smad2, Akt, ERK/MAPK, these cancer promoting pathways were analyzed in GBC knockdown cells. In this case, MALAT1 knockdown significantly reduced the levels of phosphorylated MEK1/2, ERK 1/2, MAPK, and JNK 1/2/3 proteins while no detectable changes were observed in their total levels. Thus, ERK/MAPK pathways may represent downstream targets of MALAT1 in promoting GBC (Fig. 2). These results reveal that MALAT1 upregulation in GBC plays a crucial role in tumor progression via regulating the expression of genes involved in cell invasion and proliferation processes. Thus, the inhibition of MALAT1 may provide a new approach for the treatment of GBC.

2.4. HOTAIR (HOX transcript antisense RNA)

LncRNA HOTAIR was one of the first lncRNAs found to have a fundamental role in cancer. It is located on the antisense strand of the *HOXC* gene locus on chromosome 12q13.13, and flanked by the *HOXC11* and *HOXC12* genes [49] (Table 1). It is known to interact with polycomb repressive complex 2 (PRC2), which induces histone H3 lysine-27 trimethylation at the *HOD* locus, which leads to transcriptional downregulation of several genes [50]. Elevated levels of HOTAIR have been associated with different tumor types, including lung [50], breast [51], gastric [52], bladder [53], and kidney [54]. In breast tumors, a striking 2000-fold increased expression over normal breast tissue has been observed. High levels of HOTAIR correlate with both metastasis and poor survival rates of GBC patients.

To determine the extent to which HOTAIR may play a role in GBC, Ma et al. [19], measured the expression patterns of HOTAIR in 65 GBC tissue samples by qRT-PCR. The results revealed that HOTAIR expression was significantly upregulated (~3-fold) in the tumor tissues compared to adjacent matched normal tissues (Table 1). Interestingly, HOTAIR was expressed at ~1.6-fold higher levels in stage T3+T4 tumors compared with tumors detected in the T1+T2 stage, implying a stage-dependent expression level of HOTAIR. Similarly, higher levels of HOTAIR expression were detected in GBC-SD cell lines compared to normal cell lines [55]. Molecular studies have revealed that HOTAIR expression is regulated by c-Myc via interaction with four putative c-Myc target response elements present in the upstream region of the *HOTAIR* gene [19]. Additional regulators of HOTAIR activity include miRNA-130a, a tumor suppressor miRNA, wherein HOTAIR and miRNA-130a form a reciprocal repression feedback loop. Ectopic expression of HOTAIR reduced the level of miRNA-130a, while miRNA-130a inhibition upregulated HOTAIR. This regulation is likely due to the binding of HOTAIR and miR-130a to the RISC complex, and it

has been hypothesized that HOTAIR may lead to the downregulation of miR-130a by increasing the methylation status of the miRNA-130a promoter [56]. Further, it was demonstrated that depletion of HOTAIR inhibited the invasion of GBC cells, while an miRNA-130a inhibitor reversed this decrease in invasiveness of GBC cells. Moreover, the depletion of HOTAIR resulted in the suppression of cell proliferation (Fig. 2). These composite results suggest that HOTAIR acts as an oncogenic lncRNA in GBC having a stage-specific expression, where it is regulated by both c-Myc and the tumor suppressor miRNA-130a [19]. Thus, HOTAIR may serve as a therapeutic target and as a potential diagnostic GBC biomarker.

2.5. KIAA0125

The *KIAA0125* gene is comprised of six exons and is located on chromosome 14q32.33 in human cells [57] (Table 1). A complete understanding of the potential functional roles of lncRNA KIAA0125 in human physiology and cancer is still lacking. However, a recent study showed that KIAA0125 plays an important role in neurogenesis, which may prevent the generation of dopaminergic neurons and induce astrocytosis.

Recently, Lv et al. [20], reported higher expression levels of the *KIAA0125* gene (~5.8-fold) (Table 1) in GBC metastatic-SD/M cell lines compared to non-metastatic GBC-SD cell lines. To investigate the regulatory role of the *KIAA0125* gene in malignant tumor progression, its expression was downregulated using a Lentivirus approach in GBC-SD/M cell lines. It was observed that downregulation of KIAA0125 significantly reduced invasion, migration, proliferation and colony formation compared to control cells. These results suggest that KIAA0125 plays a key role in regulating cell motility and migration. At the molecular level, the expression of EMT-associated genes was substantially altered in KIAA0125 knockdown cells. For example, KIAA0125 downregulation led to the reduction of the Vimentin and *N*-cadherin expression relative to control cells, which are important players in EMT. The reduction in Vimentin levels led to decreased cellular integrity, accelerated cell growth, and enhanced invasive potential of GBC cells [20]. Similarly, β-catenin is typically more abundant in epithelial-like cells, and loss of β-catenin predisposes cells to transition to a mesenchymal phenotype and hence, promotes cell migration. Therefore, the results described above suggest that KIAA0125 promotes GBC cell migration and invasion in part through the induction of Vimentin and suppression of β-catenin (Fig. 2). Because little has been reported on KIAA0125 in cancer, further investigation is warranted to clarify the mechanistic role(s) of KIAA0125 in GBC, as well as in other cancers.

2.6. Linc-ITGB1 (long intergenic non-coding RNA-ITGB1)

Linc-ITGB1 is a relatively newly discovered lncRNA and was first reported to play a role in GBC, though its function in tumorigenesis is still not clear. Recently its upregulation has been reported in both clinical breast cancer tissues and cultured breast cancer cell lines [58].

With respect to GBC, Linc-ITGB1 was found to be differentially expressed (~29-fold higher) (Table 1) in a pair of highly metastatic GBC-SD/M cell lines versus cell lines with lower metastatic potential GBC-SD cells [21], which suggests its involvement in GBC metastasis. Furthermore, the authors demonstrated a remarkable reduction in cancer cell proliferation, migration and invasion in the linc-ITGB1 GBC knockdown cells via MTT, wound healing and transwell assays [21]. Molecular studies revealed a decrease in Vimentin expression levels and an increase in β-catenin expression levels in GBC cells depleted for Linc-ITGB1 [21]. This was further confirmed through western blot analyses, demonstrating downregulation of Vimentin, Slug (also known as SNAIL2), and TCF8 (also known as ZEB1), and an upregulation of β-catenin in the Linc-ITGB1 knockdown cells, which indicated that EMT may be involved in Linc-ITGB1-induced GBC metastasis (Fig. 2) [21].

Taken together, the results described above suggest that Linc-ITGB1 promotes GBC invasion and metastasis perhaps via acceleration of the EMT process, and may present a potential therapeutic target for the treatment of GBC.

2.7. lncRNA-LET (long non-coding RNA-low expression in tumor)

lncRNA-LET, which is located on chromosome 15q24.1 in human cells, was first identified in hepatocellular carcinoma [59], (Table 1). It has since been reported to be downregulated in various cancers such as renal cell [60], gastric [61], cervical [62], colorectal, squamous-cell lung, and hepatocellular carcinomas [59].

Similarly, its expression in GBC was reported to be down-regulated (~7.5-fold) (Table 1) in 128 GBC tissues compared with the adjacent control non-tumor tissues, suggesting a tumor-suppressive role in GBC [22]. Low levels of lncRNA-LET have been found to be associated with a less differentiated histology, advanced tumor stage, nodal status, and clinical stage of GBC patients relative to a group of patients with high lncRNA-LET expression. However, no significant correlation was found between lncRNA-LET expression levels and GBC patient gender, age, or vessel invasion [22]. Moreover, the overall 5-year survival rates of low and high lncRNA-LET expression groups have been reported to be at ~38% and ~67%, respectively [22]. Based on Kaplan-Meier survival curve analysis and multivariate analysis, the low expression of lncRNA-LET was implicated as a significant predictor and an independent prognostic indicator for metastasis and death in GBC patients. Thus, the down-regulation of lncRNA-LET may have important roles in GBC progression and development.

Tumor hypoxia is known to be strongly associated with tumor propagation, malignant progression, and resistance to therapy [59]. Interestingly, Ma et al. (2015) [22] found that hypoxia correlated with decreased lncRNA-LET levels in GBC EZ-GB2 and SGC-996 cells. Moreover, they demonstrated that the invasive potential of GBC cells significantly decreased in cells overexpressing lncRNA-LET under hypoxia conditions, while the invasive potential of GBC cells increased in lncRNA-LET knockdown cells under hypoxic conditions. Similarly, it was found that ectopic expression of lncRNA-LET in GBC cells significantly decrease proliferating cell nuclear antigen (PCNA) which may lead to decreased cellular proliferation under hypoxia conditions [22]. The authors further showed that lncRNA-LET inhibited GBC cell proliferation by inducing a G0/G1 arrest under hypoxic conditions. At the molecular level, it was found that the overexpression of lncRNA-LET in GBC cells resulted in increased expression of p21 and Caspase-3, and increased the Bax/Bcl-2 ratio under hypoxic conditions, which suggests its role in promoting apoptosis in GBC [22]. It has been shown that the down-regulation of lncRNA-LET resulted in the stabilization of nuclear factor 90 protein (NF90) [59,63], which leads to hypoxia-induced cancer cell invasion, and nuclear lncRNA-LET regulated the HIF-1a/HDAC3/lncRNA-LET/NF-90 pathway in cell proliferation (Fig. 2).

Together, these findings indicate that lncRNA-LETmay serve as a potential therapeutic target and molecular biomarker for the prognosis of GBC.

2.8. CCAT1 (colon cancer associated-transcript 1)

LncRNA CCAT1 is an intergenic lncRNA, transcribed from chromosome 8q24.21 in human cells [64] (Table 1). As the name suggests, it was first found to be associated with colon cancer, and later

was implicated in other cancers as well [65]. In gastric cancer, its expression was reported to be significantly upregulated and controlled by the c-Myc transcription factor, resulting in the promotion of cell migration and proliferation [65].

Recently, Ma et al. (2015) demonstrated upregulation of CCAT1 (~1.5-fold) (Table 1) in 40 GBC tissues compared to paired normal tissues [23]. Furthermore, the expression of CCAT1 was even higher (~2.3-fold) in tumors extending beyond the gallbladder i. e, T3+T4 stage compared to T1+T2 stage cancer, suggesting a stage-dependent expression of CCAT1. Similarly, overexpression of CCAT1 was also found to be significantly associated with lymph node invasion and advanced node metastasis, but not with patient gender and age, highlighting its role in metastasis in GBC [23]. The authors further observed that ectopic expression of CCAT1 increased the transcript level of the polycomb group gene Bmi1 (B lymphoma Mo-MLV insertion region 1 homolog) in GBC-NOZ cells, while it decreased the expression level of miRNA-218-5p, suggesting that CCAT1 negatively modulates the expression of miRNA-218-5p [23]. miRNA-218-5p has been shown exhibit tumor-suppressive activity and to be downregulated in several carcinomas [66,67] including GBC [23]. miR-218-5p has also been found to regulate gene expression of Bmi1 [23], an oncogene known to enhance cell proliferation [68,69]. The authors speculated that CCAT1 upregulates Bmi1 by competitively 'sponging' the tumor suppressor miRNA-218-5p, as both shared the same miRNA-responsive element (MRE) in their sequences and displayed the same miRNA-218-5p-dependent regulation pattern [23] (Fig. 2). Thus, in the case of GBC, upregulation of CCAT1 promotes the proliferation and invasive capacity of the GBC cells [23] (Fig. 2). From the above results, we can conclude that CCAT1 may function as a part of the 'competitive endogenous RNA' (ceRNA) network to mediate its role in GBC manifestation, although a complete mechanistic understanding is still lacking.

2.9. ANRIL (antisense non-coding RNA in the INK4 locus)

LncRNA *ANRIL* was first identified in familial melanoma patients [70]. It is located on chromosome 9q21.3 (Table 1), is transcriptionally silenced or homozygously deleted in several human tumor types [70,71], and is dysregulated in a number of malignancies such as breast [72], gastric [73], lung, cervical [74] and bladder cancers [75].

Liu et al. (2016) [15] measured the expression level of ANRIL in 84 GBC patient tissues, and found that its expression was upregulated by ~2.4-fold when compared to surrounding normal tissues (Table 1). In addition, they found that patients with higher levels of ANRIL expression suffered lower overall survival rates, but its expression was not found to be associated with patient age, histological type, degree of differentiation, TNM staging, or lymph node metastasis [15]. Furthermore, the authors found that overexpression of ANRIL in GBC-SD cells significantly increased cellular proliferation and cell colony growth with more cells in S-phase compared to low-expressing cells [15]. Similarly, five week-old male athymic BALB/c mice injected with a pcDNA-ANRIL vector had significantly larger tumors than the empty vector control group [15]. Through flow cytometry assays, the authors found that cells overexpressing ANRIL accumulated in S-phase of the cell cycle and had a lower apoptosis rate than the control group. Furthermore by immunohistochemical analysis, it was found that mouse tissues overexpressing ANRIL showed an increase in ki-67, and a decrease in Caspase-3 protein, which suggests that ANRIL plays a role in suppressing apoptosis and increasing proliferation of GBC cells. Other groups have also reported similar findings, where higher expression levels of ANRIL promoted metastasis and reduced apoptosis in lung cancer cells [76]. Results from other studies have shown that ANRIL influenced carcinogenesis by modulating the INK4b-ARF-INK4a pathways, which inhibited the expression of *p15INK4b* and *p16INK4a* tumor suppressor genes (Fig. 2), and activated the Ras pathway to promote tumorigenesis [77,78].

Taken together, these results indicate that ANRIL functions as an oncogene in GBC and methods to downregulate its expression may be considered as a potential strategy for the inhibition of GBC progressio.

3. Conclusions and future perspectives

LncRNAs, in general, tend to harbor oncogenic properties (seven of nine lncRNAs presented herein have been found to stimulate oncogenesis) in human GBC. By understanding the roles of lncRNAs in cancer development and progression, strategies can be developed to prevent and/or treat GBC. For example, the downregulation of tumor suppressive lncRNAs (e.g. MEG3 and lncRNA-LET), and the upregulation of oncogenic lncRNAs (e.g. H19, ANRIL) stimulate a transition of cells from G0/G1 to S-phase; while upregulation of MALAT1 facilitates cells through the G2/M-phase of the cell cycle (Fig. 2), leading to an increase in GBC cell proliferation. In addition, upregulation of lncRNAs H19, HOTAIR and CCAT1 inhibits tumor-suppressive miRNAs, miR-194-5p, miR-130a and miR-218-5p respectively, and increases the expression of AKT2 and Bmi1 oncoproteins in GBC (Fig. 2). Their dysregulation also affects cell proliferation, invasion, migration and metastasis; for example, H19, KIAA0125 and Linc-ITGB1 regulate the EMT process while MALAT1 and ANRIL take part in regulating the ERK/MAPK and Ras cancer-signaling pathways (Fig. 2). Some lncRNAs, e.g., MEG3 and lncRNA-LET are also found to induce apoptosis and their upregulation inhibits GBC progression (Fig. 2).

From a clinical perspective, lncRNA dysregulation may provide a useful strategy to improve GBC patient outcome. Higher expression levels of HOTAIR, CCAT1 and lower expression levels of MEG3 and lncRNA-LET could be used for diagnostic and prognostic purposes. Further, the oncogenic (e.g. H19, MALAT1, Linc-ITGB1) and tumor suppressive (e.g. MEG3, lncRNA-LET) properties of lncRNAs could be exploited for therapeutic purposes by reversing their expression levels. In addition to this, HOTAIR and CCAT1 lncRNAs show differential expression patterns in T3+T4 and T1+T2 stage tumors, which broadens the utility of lncRNAs in stage-specific detection of GBC.

In light of the fact that asymptomatic GBC detection presents a major challenge for diagnosis and subsequent treatment (often due to advanced tumors by the time of diagnosis), efficient strategies are required to overcome such limitations. Recently, circulatory lncRNAs have been identified as biomarkers in body fluids such as blood plasma and urine for early detection of prostate and gastric cancers [79–81]. Therefore, measurements of circulatory lncRNAs may prove useful for early detection and subsequent monitoring of GBC patients.

In conclusion, we are hopeful that this review will stimulate an interest in further experimentation (both mechanistic and translational) to better understand the pathogenesis of GBC, which should lead to the development of improved strategies for the detection, prevention and/or treatment of GBC.

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