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RESEARCH

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Is cross-species horizontal gene transfer responsible for gallbladder carcinogenesis

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Abstract

Background Cross-species horizontal gene transfer (HGT) involves the transfer of genetic material between different species of organisms. In recent years, mounting evidence has emerged that cross-species HGT does take place and may play a role in the development and progression of diseases.

Methods Transcriptomic data obtained from patients with gallbladder cancer (GBC) was assessed for the differential expression of antisense RNAs (asRNAs). The Basic Local Alignment Search Tool (BLAST) was used for cross-species analysis with viral, bacterial, fungal, and ancient human genomes to elucidate the evolutionary cross species origins of these differential asRNAs. Functional enrichment analysis and text mining were conducted and a network of asRNAs targeting mRNAs was constructed to understand the function of differential asRNAs better.

Results A total of 17 differentially expressed antisense RNAs (asRNAs) were identified in gallbladder cancer tissue compared to that of normal gallbladder. BLAST analysis of 15 of these asRNAs (AFAP1-AS1, HMGA2-AS1, MNX1-AS1, SLC2A1-AS1, BBOX1-AS1, ELFN1-AS1, TRPM2-AS, DNAH17-AS1, DCST1-AS1, VPS9D1-AS1, MIR1-1HG-AS1, HAND2-AS1, PGM5P4-AS1, PGM5P3-AS1, and MAGI2-AS) showed varying degree of similarities with bacterial and viral genomes, except for UNC5B-AS1 and SOX21-AS1, which were conserved during evolution. Two of these 15 asRNAs, (VPS9D1-AS1 and SLC2A1-AS1) exhibited a high degree of similarity with viral genomes (Chikungunya virus, Human immunodeficiency virus 1, Stealth virus 1, and Zika virus) and bacterial genomes including (*Staphylococcus sp., Bradyrhizobium sp., Pasteurella multocida sp.,* and*, Klebsiella pneumoniae sp.*), indicating potential HGT during evolution.

Conclusion The results provide novel evidence supporting the hypothesis that differentially expressed asRNAs in GBC exhibit varying sequence similarity with bacterial, viral, and ancient human genomes, indicating a potential shared evolutionary origin. These non-coding genes are enriched with methylation and were found to be associated with cancer-related pathways, including the P53 and PI3K-AKT signaling pathways, suggesting their possible involvement in tumor development.

Keywords Cross-species horizontal gene transfer (CS-HGT), Antisense RNA (asRNAs), Gallbladder cancer, Transcriptomic profiling, asRNAs targeting mRNAs, Gene regulatory network

Introduction

Gallbladder cancer (GBC) is a highly lethal malignancy that is often diagnosed at advanced stages due to silent and asymptomatic nature of early disease [1]. Despite advances in diagnosis and treatment, the prognosis for patients with GBC remains poor, with a 5-year survival rate of less than 5% [2] . Diagnosis of GBC is often challenging, as it often presents with vague symptoms that can mimic those of other gastrointestinal disorders [3,

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4]. The biology of the disease is poorly understood with chronic inflammation, gallstones and bacterial infections like *Salmonella* being implicated in carcinogenesis along with a number of other carcinogens. There is very little information on molecular mechanisms and genomics of gallbladder cancer and the driver genes for gallbladder cancer are yet not identified [5–9].

Antisense RNA (asRNA) is a regulatory RNA molecule complementary to a target messenger RNA (mRNA) molecule and can interact with it to inhibit its expression [10, 11]. The asRNAs have emerged as a promising approach for cancer therapy due to their ability to inhibit the expression of specific target genes involved in tumor progression and metastasis [12]. Although the study of asRNAs is relatively recent, it has been suggested that the origins of asRNAs could be traced back to the early stages of life on Earth. The emergence of asRNA has played an essential role in the evolution of cellular life. In addition to its role in evolution, asRNAs have also been implicated in the horizontal transfer of genetic information between organisms.

Cross-species HGT refers to the transfer of genetic material between organisms of different species [13] . While HGT is a natural phenomenon that has played an essential role in the evolution of many species, it has also been implicated in the pathogenesis of cancer [14] . Specifically, the transfer of oncogenes, tumor suppressor genes [15], or other genes involved in cancer-related pathways from one organism to another through HGT can contribute to the development and progression of cancer [16, 17] . In recent years, several studies have explored the potential role of cross-species HGT in cancer, shedding light on a novel mechanism by which cancer can arise and spread.

Studies have suggested a potential association between GBC and bacterial infections, particularly those caused by *Salmonella typhi* [18, 19] and *Helicobacter pylori* [20] . While the exact mechanisms underlying this association remain unclear, several hypotheses have been proposed, including chronic inflammation, immune system activation, and bacterial carcinogenesis. Evidence suggests that asRNA has a deep evolutionary history that spans different domains of life. In bacteria, the first evidence of asRNA was discovered in the bacteriophage T4, which infects *Escherichia coli*. The phage produces an asRNA molecule that interacts with a target mRNA molecule to inhibit its expression [21] . This interaction was critical for regulating phage gene expression, indicating that antisense RNA has been present in bacteria for at least several billion years.

Viruses play a crucial role in the replication and pathogenesis of cancer, including HIV, hepatitis C, Hepatitis B, and Epstein Barr virus. Inhibition of the replication of HIV has been demonstrated by interaction of asRNA with the viral RNA genome and blocking its expression [22] . The mechanisms underlying the association between viral infections and GBC are not fully understood. However, viral infections leading to chronic inflammation, or suppression or alteration of human immune response can promote cancer development. Additionally, viruses may directly contribute to cancer development by altering the DNA of infected cells, leading to mutations or by blocking the cell-cycle check points.

Current evidence does not define the notion that viral or bacterial genes acquired through cross-species HGT alter human gene expression in cancer. Consequently, mechanisms underlying such phenomena remain unidentified. The research addresses this gap by demonstrating that non-conserved, putatively viral, or bacterial genes integrated into asRNAs via cross-species HGT may be involved in the regulation of coding genes, thus contributing to carcinogenic processes.

Materials & methods

Gallbladder tissue samples were collected from patients undergoing surgery or guided biopsy for gallbladder cancer after obtaining written informed consent and ethical committee approval. The samples were collected and subsequently stored at -80 °C until analysis.

RNA Isolation and cDNA Library preparation

The total RNA was extracted from tissue samples using TRIzol reagent (Thermo Fisher Scientific). RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer. For cDNA synthesis, we employed the NEBNext® RNA First Strand Synthesis Module and NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module according to the manufacturer's instructions. The resulting cDNA was purified using SPRIselect Beads or NEBNext Sample Purification Beads. We quantified the prepared libraries using the DNA 1000 Chip on an Agilent Bioanalyzer [23].

Identification of key ncRNAs by data processing

The cDNA samples obtained from 10 patients were pooled in equal amounts and subjected to sequencing using the Illumina Nova-seq 6000 platform (San Diego, California, USA) in the 125 bp paired-end mode. The resulting raw reads from the gallbladder samples were compared to those obtained from a control sample (SRA Database ID: ERX288537: HPA RNA-seq normal tissues gallbladder).

Based on a cut-off p-value score of less than 0.05 the differentially expressed asRNAs were identified based on the Log2 Fold change values the differentially expressed asRNAs were classified as downregulated or upregulated.

Gene enrichment analysis

The lncRNA annotation extractor and repository (Lantern) (https://sysbio.sitehost.iu.edu/lantern/) was used for the functional enrichment of long non-coding RNAs (lncRNAs). Lantern integrates a unique ontology annotation engine that provides precise annotations for lncRNAs. ENCORI (The Encyclopedia of RNA Interactomes) (https://starbase.sysu.edu.cn/) was used to predict the associated pathways, indicating possible interactions between them. Disease Ontology (DO) analysis was done using MalaCards (https://www.malacards.org/pages/info). Pathway analysis was performed A gene regulatory network was constructed to explore the asRNAs targeted mRNAs interaction by the NetworkAnalyst tool (http://www.networkanalyst.ca).

Phylogenetic analysis

To understand the relationship between different evolutionary transitions, exploring a fundamental framework for quantitatively analyzing the evolutionary changes, and similarities, or differences among taxa unrelated to one another. phylogenetic analysis was performed using Multiple sequence alignment (MSA) through ClaustalX (version 2.1) tool (http://www.ebi.ac.uk/tools/clustalw2). The results from MSA were used to create neighbor-joining phylogenetic tree by the MEGA11 tool (https://www.megasoftware.net/dload_mac_beta) with rectangular tree style with a distance scale of 0.1.

The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to identify regions of homology between genomic sequences. Specifically, pairwise alignment of the query sequence with database sequences was performed using the highly similar sequence (Mega blast) algorithm from different organisms, including bacteria (tax id: 2), viruses (tax id: 10,239), fungi (tax id: 4751), homo neanderthalensis (tax id: 62,331), and homo heidelbergensis (tax id: 1,425,170). The BLAST parameters used in this study included an Expect threshold of 0.05, a maximum target sequence of 50, a word size of 20, and match/mismatch scores of (1, -2). Then, the constructed BLAST tree was visualized with a maximum sequence difference cut-off of 0.75.

Results

Of the ten GBC patients enrolled in this study three were males and seven females. The mean age of the patients was 56 years (SD 14.68 months). Four patients had stage III and six patients had stage II GBC. A total of 891 asRNAs were identified as differentially expressed (DE-asRNAs) in cancer compared to normal gallbladder, of these 17 were found to be statistically significant and were subjected to further analysis (Table 1). Among the DE-asRNAs, 12 were up-regulated, (AFAP1-AS1, HMGA2-AS1, MNX1-AS1, UNC5B-AS1, SLC2A1-AS1, BBOX1-AS1, SOX21-AS1, ELFN1-AS1, TRPM2-AS, DNAH17-AS1, DCST1-AS1, and VPS9D1-AS1), while five were down-regulated, (MIR1-1HG-AS1, HAND2-AS1, PGM5P4-AS1, PGM5P3-AS1, and MAGI2-AS3).

The gene ontology analysis identified them to be involved in developmental processes, apoptosis, methylation, and cell proliferation (Supplementary Table 1).

Table 1 List of 17 significant asRNAs associated with GBC

S. No	Ensemble ID	log2 Fold Change	*p*-value	Gene Description & Gene Id	Regulation
1	ENSG00000272620	6.248241245	0.001089196	AFAP1 antisense RNA 1 (AFAP1-AS1)	Up-regulation
2	ENSG00000197301	6.074330706	0.002203224	HMGA2 antisense RNA 1 (HMGA2-AS1)	Up-regulation
3	ENSG00000174403	-7.111508315	0.004856029	MIR1-1HG antisense RNA 1 (MIR1-1HG-AS1)	Down-regulation
4	ENSG00000237125	-3.840870592	0.016730699	HAND2 antisense RNA 1 (HAND2-AS1)	Down-regulation
5	ENSG00000243479	5.955253617	0.01888703	MNX1 antisense RNA 1 (MNX1-AS1)	Up-regulation
6	ENSG00000237512	6.547595648	0.019144795	UNC5B antisense RNA 1 (UNC5B-AS1)	Up-regulation
7	ENSG00000227533	4.764087468	0.022706423	SLC2A1 antisense RNA 1 (SLC2A1-AS1)	Up-regulation
8	ENSG00000254560	5.547595648	0.031221006	BBOX1 antisense RNA 1 (BBOX1-AS1)	Up-regulation
9	ENSG00000227640	5.4247389	0.03581536	SOX21 antisense divergent transcript 1 (SOX21-AS1)	Up-regulation
10	ENSG00000277631	-5.196397213	0.039700928	PGM5P3 antisense RNA 1 (PGM5P3-AS1)	Down-regulation
11	ENSG00000234456	-3.19331782	0.042479642	MAGI2 antisense RNA 3 (MAGI2-AS3)	Down-regulation
12	ENSG00000231943	-5.266786541	0.044042244	PGM5P4 antisense RNA 1 (PGM5P4-AS1)	Down-regulation
13	ENSG00000236081	4.459431619	0.044372822	ELFN1 antisense RNA 1 (ELFN1-AS1)	Up-regulation
14	ENSG00000230061	5.218288023	0.04442787	TRPM2 antisense RNA (TRPM2-AS)	Up-regulation
15	ENSG00000267432	5.651704052	0.045472482	DNAH17 antisense RNA 1 (DNAH17-AS1)	Up-regulation
16	ENSG00000232093	5.614709844	0.046723983	DCST1 antisense RNA 1 (DCST1-AS1)	Up-regulation
17	ENSG00000261373	4.44946133	0.049887145	VPS9D1 antisense RNA 1 (VPS9D1-AS1)	Up-regulation

The pathway analysis revealed specific associations with different pathways for each asRNA (Supplementary Table 2). The HAND2-AS1, e.g., was linked with small-cell lung cancer and RNA degradation pathways, while MNX1-AS1 was enriched with WNT signaling, deadenylation-dependent mRNA decay, and the Eif4 pathway. UNC5B-AS1 was associated with pathways in cancer, basal cell carcinoma, and hedgehog signaling pathways. SLC2A1-AS1 was linked to RAP1 signaling and E2f-mediated regulation of DNA replication, while BBOX1-AS1 and VPS9D1-AS1 were associated with the hemostasis pathway. SOX21-AS1 was significantly involved in regulatory RNA pathways, the HIV-1 early elongation complex formation, RNA Pol III Transcription, and the influenza life cycle. Fourteen of the asRNAs (UNC5B-AS1, SLC2A1-AS1, BBOX1-AS1, SOX21-AS1, ELFN1-AS1, TRPM2-AS, DNAH17-AS1, DCST1-AS1, VPS9D1-AS1, MIR1-1HG-AS1, HAND2-AS1, PGM5P4-AS1, PGM5P3-AS1, and MAGI2-AS) are found to be associated with gallbladder cancer for the first time, while three have been previously reported (AFAP1-AS1, HMGA2-AS1, and MNX1-AS1) (Supplementary Table 3).

Phylogenetic analysis showed the similarities among significant DEasRNAs, as shown in Fig. 1. Five clusters were identified having 7, 5, 2, 2 As RNA, while TRPM2-AS was stand alone and was not part of any cluster. BLAST analysis revealed that 15 asRNA (AFAP1-AS1, HMGA2-AS1, MNX1-AS1, SLC2A1-AS1, BBOX1-AS1, ELFN1-AS1, TRPM2-AS, DNAH17-AS1, DCST1-AS1, VPS9D1-AS1, MIR1-1HG-AS1, HAND2-AS1, PGM5P4-AS1, PGM5P3-AS1, and MAGI2-AS) showed, variable degree of similarity with bacterial and viral genome except (UNC5B-AS1 and SOX21-AS1) as they were found to be fully conserved (Fig. 2 A to O). Two of these (VPS9D1-AS1 and SLC2A1-AS1) were found to have a high degree of similarity with the viral and bacterial genome. VPS9D1-AS1 showed similarity with human papilloma virus (HPV) type 16, Human immunodeficiency virus (HIV) *Klebsiella pneumonae*, and *Basillus paralichemiformis*. These results suggest that they were phylogenetically related to viral and bacterial genomes, suggesting horizontal gene transfer from both virus and bacteria at some point in evolution.

The blast analysis of these significant asRNAs failed to show any similarity with fungi. The overlaps were found with genome of Chikungunya virus, Human immunodeficiency virus 1, Stealth virus 1, *Staphylococcus aureus*, Zika virus, *Pasteurella multocida subsp., Klebsiella pneumoniae, Staphylococcus haemolyticus, Ralstonia solanacearum*, Human endogenous retrovirus, *Bacillus paralicheniformis*, and *Bradyrhizobium species*.

The query cover in BLAST with Chikungunya virus was 34%, with an E value of (9.00E-116), and a percentage identity of (91.96) with a sequence of VPS9D1. Additionally, the query cover in BLAST with Chikungunya virus

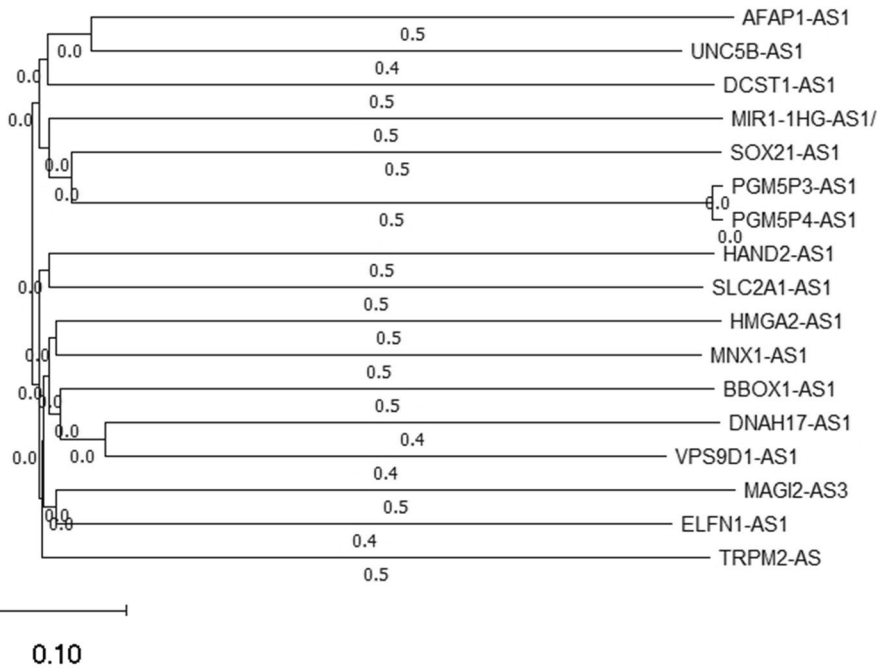

Fig. 1 Neighbour-joining phylogenetic tree of significant asRNAs showing 5 clusters

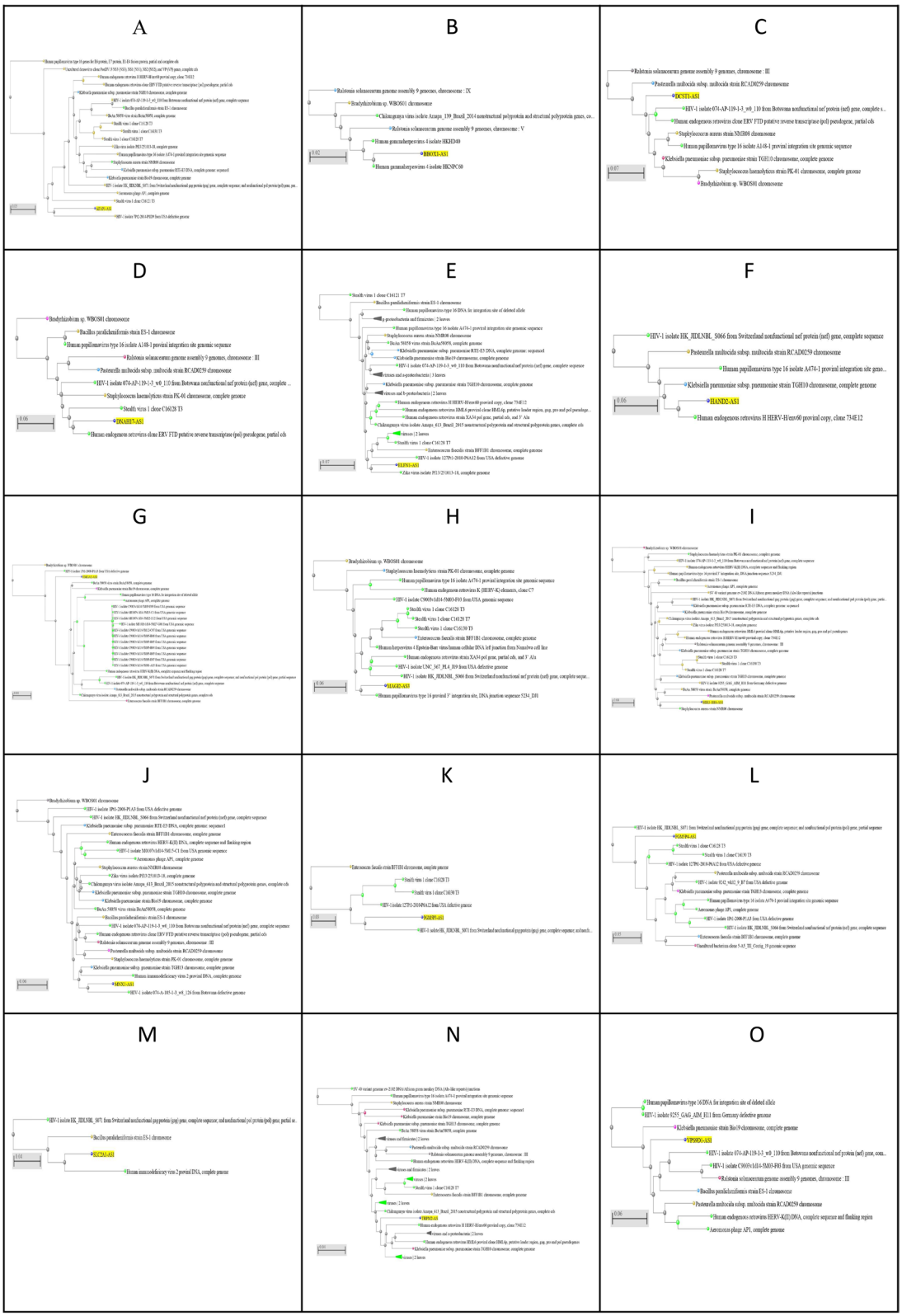

Fig. 2 Phylogenetic tree analysis showing ancestry with bacterial, viruses, fungi, *Homo neanderthalensis*, and *Homo heidelbergensis* DNA (**A-O**). Highlighted asRNAs indicate our DEGs, different colour nodes represent different species

was 19%, with an E value of (4.00E-115), and a percentage identity of (93.22) with a sequence of SLC2A1.

The 15 enriched DEasRNAs targeted mRNAs interaction showed that the targeted mRNAs were involved in the p53 signaling pathway, endometrial cancer, pathway in cancer, microRNA in cancer, and PI3K-AKT signaling pathway Supplementary Fig. 1. A gene regulatory network of asRNAs targeting mRNAs, revealing a unique mechanism of gene regulation that plays a critical role in cancer development that involved genes in PIK3CA-AKT and NOTCH signaling pathway among others (Fig. 3). A complex cross talk of the major pathways was prepared and is presented in Fig. 4. The interplay of downstream EGFR, and Wnt pathway with DNA repair genes appears to be the responsible for gallbladder carcinogenesis.

Discussion

Throughout our evolutionary history, cross-species HGT was a driving force of evolution that constantly reshaped our genomes [24] . It is responsible for the genetic flow between distantly related lineages which may facilitate the transition of host species by introducing new genes or altering existing genes, which leads to changes in cell behavior and potentially promotes tumor development [25]. This phenomenon is well-known among bacteria and viruses. However, it has recently been discovered among higher plants and animals including *Homo Sapiens*.

The findings of the functional enrichment analysis revealed that both the upregulated and downregulated asRNAs were enriched with the methylation and developmental processes.

Blast analysis of the identified asRNAs showed that they exhibit varying degrees of sequence similarity to several viral and bacterial species, including Chikungunya virus, Human immunodeficiency virus 1, Zika virus, Stealth virus 1, *Pasteurella multocida subsp., Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus haemolyticus, Ralstonia solanacearum,* Human endogenous retrovirus, *Bacillus paralicheniformis*, and *Bradyrhizobium species*.

Consequently, two asRNAs (VPS9D1 and SLC2A1-AS1) were identified, showing a high degree of similarity with various viral and bacterial species. In addition, 13 of them showed variable degrees of similarity. However, UNC5B-AS1 and SOX21-AS1 were conserved during evolution. Several studies have suggested that antisense RNAs may play a role in regulating viral and bacterial genes acquired through cross-species HGT. These results indicate that these asRNAs may have originated from or have been influenced by various sources, potentially including pathogenic organisms. Additionally, the observed sequence similarities may provide clues

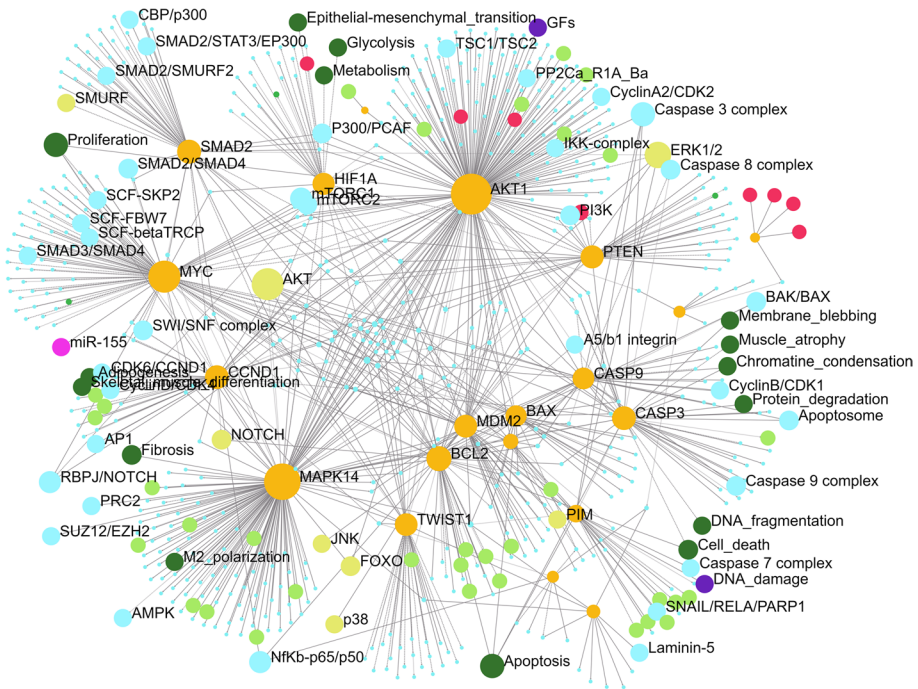

Fig. 3 asRNA targeted mRNA interaction network. Nodes in the network are represented by dots with various colors and shapes. The link between the two nodes is represented by the edge. Seeds (circle yellow), complex (Circle light blue), miRNA (circle dark pink), chemical (circle dark green), protein family (circle peach), small molecule (circle purple), stimulus (circle brown), phenotype (circle orange), and protein (circle pink)

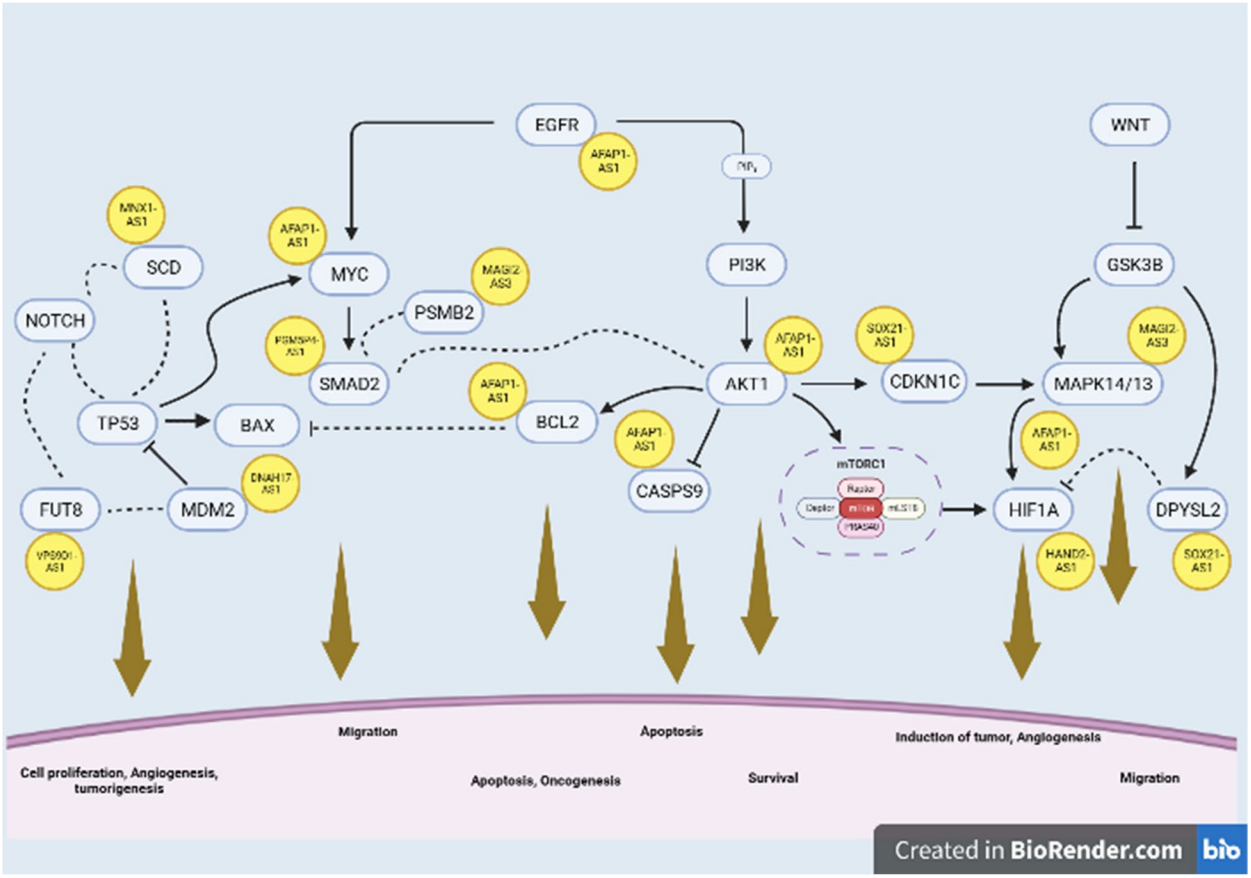

Fig. 4 Crosstalk between the significant asRNAs and the targeted mRNAs. Yellow color circle – Significant asRNAs, blue rectangular box—targeted mRNAs

as to the evolutionary history of these asRNAs and their potential roles in host–pathogen interactions.

Since long, bacteria have been thought to be associated with gallbladder cancers and various hypothesis have been proposed. *Helicobacter pylori* and other species of *Helicobacter* like *bilis* and *hepaticus* have been proposed to facilitate chronic inflammation, that promotes tumor growth [26]. The other hypothesis is that the mechanism of carcinogenesis is brought about by altering the gut microbiota, which may encourage the development of carcinogenic bacteria in the gallbladder [27]. Other bacteria like *Salmonella*, *Escherichia coli*, *Peptostreptococcus*, and *B fragilis* have been identified in gallbladder cancer and are implicated in development of GBC [28, 29].

Numerous viruses have been linked to GBC through both direct and indirect mechanisms involving diverse antisense RNAs (asRNAs) (Supplementary Table 4). Epstein-Barr virus is commonly associated with several other types of cancer, such as nasopharyngeal carcinoma [30], gastric cancer [31], and lymphomas [32]. Studies have found that EBV infection is associated with development of acalculous cholecystitis and a single case of EBV virus associated mixed lymphoepithelioma-like carcinoma and adenocarcinoma is reported [33]. EBV may contribute to the development of GBC by inducing chronic inflammation, promoting cell proliferation, and inhibiting apoptosis.

Similarly, previous studies also reported the association of the human papillomavirus (HPV) in the pathogenesis of GBC [34–36]. HPV is a well-known cause of cervical cancer [37] and is associated with other cancers, such as anal [38] and oropharyngeal cancer [39]. Chronic HBV and HCV infection can lead to chronic liver inflammation, which can, in turn, may increase the risk of developing gallbladder cancer.

Subsequent analysis of the top 15 enriched DE asRNAs revealed that they were associated with several key biological pathways, including the p53 signaling pathway, endometrial cancer pathway, pathway in cancer, miRNA in cancer, and PI3K-AKT signaling pathway. These findings suggest that asRNAs may exert their regulatory effects on gene expression by modulating these fundamental cellular pathways, providing new insights into the complex molecular mechanisms underlying cancer

development and progression. Additionally, our findings demonstrate crosstalk between involved coding genes, highlighting a novel and important aspect of gene regulation (Fig. 4).

The role of cross-species HGT in GBC development appears to be complex and multifactorial. However, it appears that the transfer of genetic material from bacteria, viruses, and fungi does occur, and this may contribute to the dysregulation of cellular processes. Whether this dysregulation requires presence of the bacteria or viral infection or can be triggered by some other mechanism like inflammation is still not clear. The evidence on the possible role of bacterial infection is in plenty, however, there are only handful of reports of virus associated gallbladder cancer, and their link needs to be further explored. However, the insights into the potential role of these asRNAs in the development of GBC suggests that they could be used as potential targets. The data, however, needs further validation.

Conclusion

Cross-species HGT of viral and bacterial genes during human evolution can impact cell proliferation by regulating antisense RNAs that in turn can regulate mRNA and the coding genes involved in the cell-cycle and DNA repair mechanism in gallbladder cancer. Although the mechanism of carcinogenesis is not fully understood and may involve the activation of signaling pathways in the host cells. Further studies are needed to elucidate the mechanisms by which viral and bacterial genes acquired through HGT regulate antisense RNA expression and to determine the impact of this process on gallbladder cancer progression and treatment. Understanding the role of HGT in human diseases may provide new avenues for developing novel therapeutics.

Abbreviations

GBC	Gallbladder cancer
HGT	horizontal gene transfer
asRNAs	Antisense RNAs
DE-asRNAs	Differentially expressed asRNAs
FEA	Functional enrichment analysis
GO	Gene ontology
Webgestalt	WEB-based gene set analysis toolkit
RNAInter	RNA interactome database
ENCORI	The Encyclopedia of RNA interactomes
MSA	Multiple sequence alignment
mRNA	Messenger RNA
BLAST	Basic local alignment search tool

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
MR: Data collection, analysis and manuscript preparation RD: Data acquisition, analysis, interpretation and manuscript preparation MP: Concept and design, interpretation and editing of the manuscript VKS: Manuscript review and editing All authors have read and approved the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The research was approved by the Institute Ethics committee (No.Dean/2018/EC/561) and written informed consent was obtained from all patients.

Competing interests
The authors declare no competing interests.

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