Gastroenterology and Hepatology From Bed to Bench.
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META-ANALYSIS

Meta-analysis of transcriptomics data identifies potential biomarkers and their associated regulatory networks in gallbladder cancer

Nidhi Singh[1], Rinku Sharma[2], Sujoy Bose[1]

[1] *Department of Biotechnology, Gauhati University, Guwahati, Assam, India*
[2] *Department of Life Sciences, Shiv Nadar University, Noida, Uttar Pradesh, India*

ABSTRACT

Aim: This study aimed to identify key genes, non-coding RNAs, and their possible regulatory interactions during gallbladder cancer (GBC).

Background: The early detection of GBC, i.e. before metastasis, is restricted by our limited knowledge of molecular markers and mechanism(s) involved during carcinogenesis. Therefore, identifying important disease-associated transcriptome-level alterations can be of clinical importance.

Methods: In this study, six NCBI-GEO microarray dataseries of GBC and control tissue samples were analyzed to identify differentially expressed genes (DEGs) and non-coding RNAs {microRNAs (DEmiRNAs) and long non-coding RNAs (DElncRNAs)} with a computational meta-analysis approach. A series of bioinformatic methods were applied to enrich functional pathways, create protein-protein interaction networks, identify hub genes, and screen potential targets of DEmiRNAs and DElncRNAs. Expression and interaction data were consolidated to reveal putative DElncRNAs:DEmiRNAs:DEGs interactions.

Results: In total, 351 DEGs (185 downregulated, 166 upregulated), 787 DEmiRNAs (299 downregulated, 488 upregulated), and 7436 DElncRNAs (3127 downregulated, 4309 upregulated) were identified. Eight genes (FGF, CDK1, RPN2, SEC61A1, SOX2, CALR, NGFR, and NCAM) were identified as hub genes. Genes associated with ubiquitin ligase activity, N-linked glycosylation, and blood coagulation were upregulated, while those for cell-cell adhesion, cell differentiation, and surface receptor-linked signaling were downregulated. DEGs-DEmiRNAs-DElncRNAs interaction network identified 46 DElncRNAs to be associated with 28 DEmiRNAs, consecutively regulating 27 DEGs. DEmiRNAs-hsa-miR-26b-5p and hsa-miR-335-5p; and DElnRNAs-LINC00657 and CTB-89H12.4 regulated the highest number of DEGs and DEmiRNAs, respectively.

Conclusion: The current study has identified meaningful transcriptome-level changes and gene-miRNA-lncRNA interactions during GBC and laid a platform for future studies on novel prognostic and diagnostic markers in GBC.

Keywords: Gallbladder cancer, Microarray, Transcriptome, Differentially expressed genes, Differentially expressed microRNAs, Differentially expressed long non-coding RNAs.

(Please cite as: **Singh N, Sharma R, Bose S. Meta-analysis of transcriptomics data identifies potential diagnostic biomarkers and their associated regulatory networks in gallbladder cancer. Gastroenterol Hepatol Bed Bench 2022;15(4):311-325. https://doi.org/10.22037/ghfbb.v15i4.2292).**

Introduction

Gallbladder cancer (GBC) is one of the most common biliary tract cancers and the fifth most common gastrointestinal cancer with a mean survival rate of less than 5% (1). Its distribution has ethnic and geographical variations, with inordinately high occurrence rates recorded in parts of South America, Southeast Asia, and Eastern Europe (2, 3). Due to the absence of early discernible symptoms, it is often detected during advanced stages, when surgical resection is the only curative option left, although only 10% of resection procedures are successful due to the aggressive and rapid progression of the cancer (4). A lack of GBC-specific markers and the absence of targeted therapy contribute to its late diagnosis and poor prognosis, often resulting in poor clinical outcomes. Therefore, an understanding of the molecular-level alterations during GBC conditions will be helpful in developing specific markers of diagnostic and prognostic value (5).

Received: 24 June 2022 *Accepted*: 21 September 2022
Reprint or Correspondence: **Nidhi Singh,** *Department of Biotechnology, Gauhati University, Guwahati – 781014, Assam, India.*
E-mail: nidhisingh866@gmail.com
ORCID ID: 0000-0002-9524-4299

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Although non-coding RNAs cannot code protein, they regulate gene expression at the transcriptional and post-transcriptional levels. microRNAs (miRNAs) are short non-coding RNAs which bind to the UTR regions of target mRNAs, thereby causing post-transcriptional silencing in almost 30% of the human proteins (6). On the other hand, long non-coding RNAs (lncRNAs) (<200 nucleotides) also regulate gene expression by acting as sponges for miRNAs, thus indirectly regulating mRNA expression (7). Accumulating evidence in recent years has indicated the tumor suppressive, or conversely, oncogenic potential of miRNAs and lncRNAs in carcinogenesis, with a few miRNAs and lncRNAs under investigation for diagnostic/prognostic/therapeutic targets for certain cancers (8). Molecular-level regulatory networks focusing on the interaction between coding genes and non-coding RNAs have recently gained attention in cancer studies, as these gene-miRNA-lncRNA interaction networks in cancer can clearly depict the loss of regulated homeostasis in biological processes during carcinogenesis (9). Deregulation in miRNA and lncRNA expression during GBC has also been implicated in a few studies, but the specific interactions have not been reported.

A meta-analysis-based approach combines

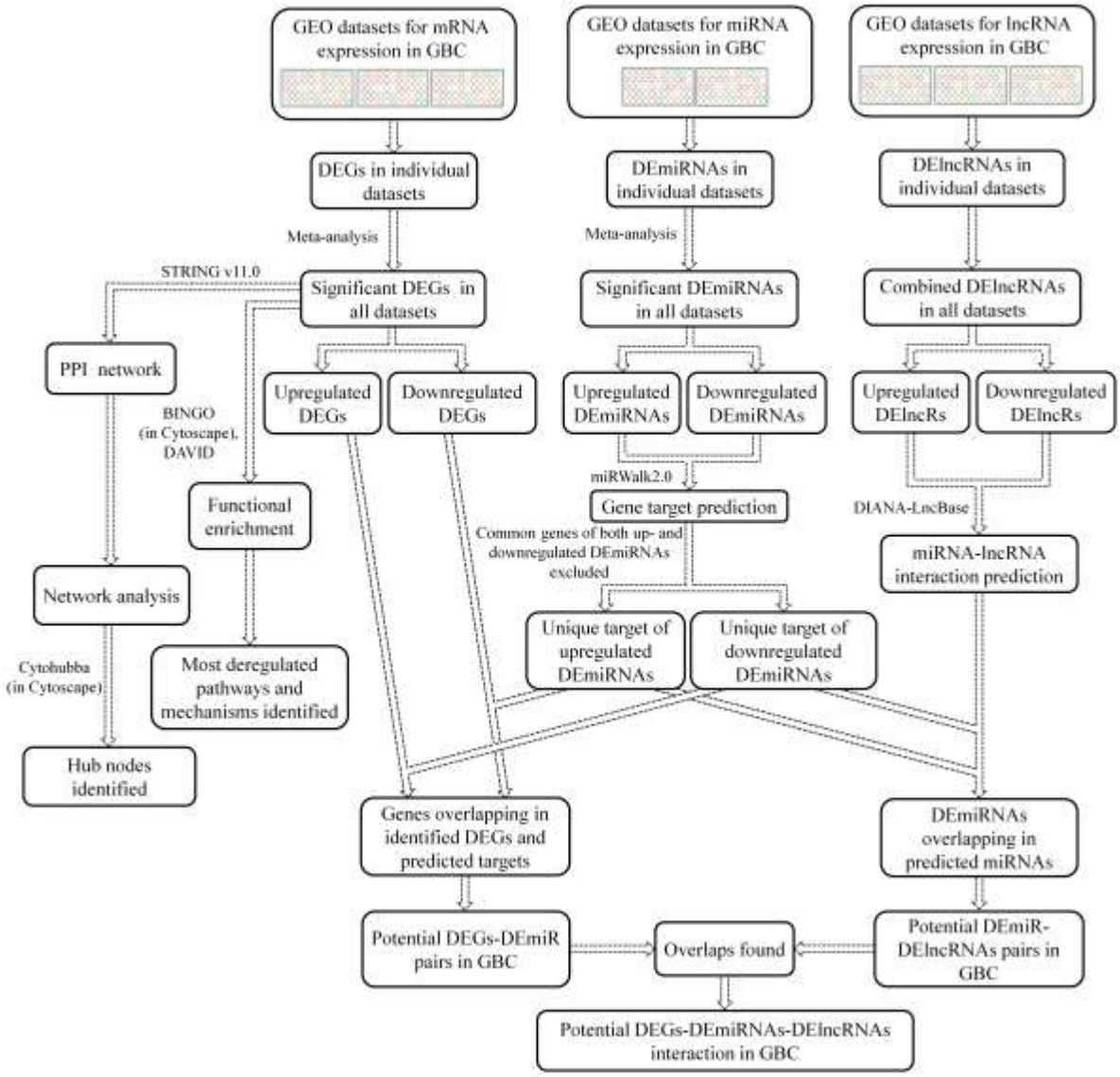

Figure 1. Diagrammatic representation of overview of methodology used in the study

expression profiles from multiple microarray studies and has been extensively utilized to identify genetic alterations and mechanisms associated with cancer (10). Multiple studies have utilized this approach to identify potential biomarkers, aberrant biological pathways, and/or gene-miRNA-lncRNA interactions in multiple cancers (11–14). However, similar meta-studies have never been performed on the gene, lncRNA, and miRNA expression profiles in GBC. With the recent availability of large-scale expression data, it is now possible to assemble a multi-level molecular regulatory network for the underlying gene-miRNA-lncRNA interactions in GBC.

With the objective of identifying potential biomarkers, associated altered pathways, and gene-non-coding RNA interactions in GBC, we analyzed NCBI-GEO microarray expression datasets to screen differentially expressed genes (DEGs) and differentially expressed non-coding RNAs (miRNAs and lncRNAs) in GBC patients, compared to controls. The identified DEGs were subjected to GO and KEGG pathway analysis to identify associated significantly altered pathways and mechanisms in GBC. A protein-protein-interaction network was created for the DEGs, and hub genes were identified from this network. Furthermore, we identified the differentially expressed miRNAs (DEmiRNAs) which could potentially regulate the DEGs as well as differentially expressed lncRNAs (DElncRNAs) associated with these DEmiRNAs. Finally, the expression data was integrated to obtain a gene-miRNA-lncRNA regulatory network, providing a coherent overview of the molecular-level regulatory mechanisms associated with GBC.

Methods

Figure 1 summarizes the workflow of the methodology used in the study.

Data collection, pre-processing, and screening of DEGs, DEmiRNAs, and DElncRNAs

The NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) was searched for publicly available expression microarray data on GBC using the keywords: “gallbladder cancer,” “gallbladder carcinoma,” “GBC,” “CAGB,” “cancer of gallbladder,” “carcinoma of gallbladder.” At the time of compilation of the manuscript, seven GBC related dataseries were found in the NCBI-GEO database having expression profile of genes, miRNAs, and/or lncRNAs on gallbladder cancer and liver metastatic gallbladder cancer along with control tissues. From within these dataseries, samples that included expression profiles of

Table 1. Details of the NCBI-GEO microarray datasets on GBC used in this study.

GEO accession	Experiment type	Contributor	Year	Sample size		platform
				Control	GBC	
GSE62335	mRNAs expression profiling Long noncoding RNAs expression profiling	Ma MZ, et al	2014	5	5	[HuGene-2_0-st] Affymetrix Human Gene 2.0 ST Array
GSE74048	mRNAs expression profiling Long noncoding RNAs expression profiling	Wang J and Liu H	2015	3	3	Agilent-067406 Human CBC lncRNA + mRNA microarray V4.0
GSE76633	Long noncoding RNAs expression profiling	Liu Y, et al	2016	9	9	Agilent-045142 Human LncRNA v4 4X180K
GSM3854440, GSM3854443, GSM3854446, GSM3854441, GSM3854444, GSM3854447 of GSE132223	mRNAs expression profiling	Liu Y and Li H	2019	3	3	Illumina HiSeq 2000 (Homo sapiens)
GSE104165	miRNA expression profiling	Roessler S, et al	2017	8	40	Agilent-046064 Unrestricted_Human_miRNA_V19.0_Microarray
GSE90001	miRNA expression profiling	Liu Y, et al	2016	4	4	Agilent-046064 Unrestricted_Human_miRNA_V19.0_Microarray

human GBC tissue samples and control tissues were included in the study (Table 1); dataseries performed on GBC cell lines or other organisms and/or whose raw cell intensity (CEL) files were not available were excluded. Six microarray expression dataseries of GBC that met the inclusion criteria were identified in the database.

The raw files (.CEL format) were downloaded from the database. The dataseries were normalized individually according to the platform used. For example, Affymetrix-based dataseries were normalized using GCRMA R package, whereas Illumina Hiseq-based dataseries were normalized using the limma package. After normalization and quality control, differentially expressed genes and non-coding RNAs of each dataseries were identified using limma. Probes with fold change >|1| ($p<0.05$) were considered differentially expressed. Probes not assigned to any genes or matching multiple annotations were not considered. If multiple probes were assigned to the same annotations, those with the lowest significant p-value were considered. Meta-analysis of all DEGs obtained from the dataseries was performed by merging the DEGs by the p-value combination technique using the fishercomb() and invnormcomb() functions of the metaRNASeq R package. Meta-analysis of DEmiRNAs obtained from the individual dataseries was performed using limma. Meta-analysis of identified DElncRNAs was not possible due to the lack of a common parameter, and therefore all DElncRNAs of the individual dataseries were combined.

Functional enrichment of identified DEGs

Gene ontology (GO) is a method for annotating genes by their functions at the molecular level,

Table 2. Top ten DEGS, DEmiRNAs, and DElnRNAs (as fold change, upregulated, and downregulated) in gallbladder cancer, ranked by p-values. The table shows top 10 most significantly upregulated and top 10 most significantly downregulated DEGs, DEmiRNAs, and DElncRNAs in GBC.

Upregulated	Fold change	p-value	Downregulated	Fold change	p-value
mRNAs					
CEMIP	25.51033	$2.25E^{-15}$	CHRDL1	-14.8119	2.62622E-21
MANF	3.530227	$2.16E^{-11}$	IPCEF1	-131.784	2.20047E-19
UAP1	57.96024	4.86E-11	TMEM132C	-64.4814	3.64091E-14
TMEM258	2.55462	8.3E-11	MT1A	-17.0527	8.93752E-14
HSD17B6	6.279835	9.62E-10	MGAM	-4.66978	2.37047E-12
MMP11	17.88872	1.38E-09	SHC3	-8.84884	7.59813E-12
PSAT1	6.256313	2.26E-09	ANGPTL7	-24.1559	8.98201E-10
OSTC	2.424898	4.21E-09	S100B	-10.8407	1.10399E-09
KIF26B	8.572264	4.5E-09	CADM2	-43.3201	1.72312E-09
SDF2L1	4.838387	9.64E-09	APC2	-38.7752	1.92319E-09
miRNAs					
hsa-miR-143-3p	3.931848753	4.34E-15	hsa-miR-4745-5p	-11.09759903	2.29E-11
hsa-miR-4734	3.911659969	4.50E-15	hsa-miR-144-5p	-3.047165571	2.54E-11
hsa-miR-9-5p	5.343422098	1.76E-14	hsa-miR-378h	-1.884246264	2.56E-11
hsa-miR-410	7.251264243	3.86E-14	hsa-miR-4271	-11.79238086	3.02E-11
hsa-miR-4725-5p	7.154180556	4.23E-14	hsa-miR-3131	-5.491897637	1.08E-10
hsa-miR-3202	4.095922645	1.26E-13	hsa-miR-195-5p	-4.719383486	1.48E-10
hsa-let-7f-1-3p	4.184298648	4.49E-13	hsa-miR-92a-3p	-2.577166124	1.91E-10
hsa-miR-4449	3.596988489	1.55E-11	hsa-miR-5708	-5.138975331	3.20E-10
hsa-miR-135a-3p	3.195508149	1.56E-11	hsa-miR-4731-3p	-8.256214283	3.30E-10
hsa-miR-1914-3p	3.158653464	1.64E-11	hsa-miR-4498	-7.493946518	3.51E-10
lnRNAs					
PYCR1	26.17287	6.97E-13	LOC388282	-6.453134074	3.18E-14
C1orf151-NBL1	19.29293	3.88E-12	ZCCHC16	-14.2214829	9.67E-14
MPZL1	5.314743	7.72E-12	PEBP1	-5.938094283	1.02E-12
PKM2	20.11221	9.57E-12	CYP2B6	-17.87659421	1.64E-12
TRIM59	19.83532	1.27E-11	GNMT	-111.4304721	2.18E-12
ELF4	6.062866	1.65E-11	SCP2	-17.3877578	3.52E-12
GSTP1	10.62949	1.87E-11	C1RL	-6.105036836	4.02E-12
ITPR3	20.25211	1.93E-11	CLEC4M	-30.06472797	4.18E-12
PFKP	18.50701	2.16E-11	MTMR14	-4.169863043	5.18E-12
OSBPL3	9.713559	2.99E-11	PLIN5	-12.04197398	7.47E-12

functional location in cells, and related biological processes (15). To determine the GO terms of significantly downregulated and upregulated DEGs, enrichment analysis was performed to assess overrepresented GO categories using Biological Network Gene Ontology (BiNGO) tool in Cytoscape (16, 17). Categories with false discovery rate (FDR) <0.05 ($p < 0.05$) were considered as significantly annotated.

Additionally, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed using the database for annotation, visualization, and integrated discovery (DAVID, https://david.ncifcrf.gov/) (18, 19). KEGG pathways with $p < 0.05$ were considered statistically significant for the analyzed category of genes.

Protein-protein interaction (PPI) network for DEGs, module analysis, and hub gene identification

Identified DEGs were queried in the STRING v11.0 database, and a protein-protein interaction network was created with an interaction score of medium confidence (score = 0.4). This network was imported and visualized in Cytoscape v3.7.1 (17). Potential hub nodes were determined using cytoHubba, employing a local metric (i.e. node degree) and global metric (i.e. closeness centrality, betweenness centrality, and stress centrality) approach (20). The overlapping genes in the top 20 nodes of the four parameters were selected as hub genes in GBC. Also, significant modules in the network were identified using the molecular complex detection clustering algorithm (MCODE) (21).

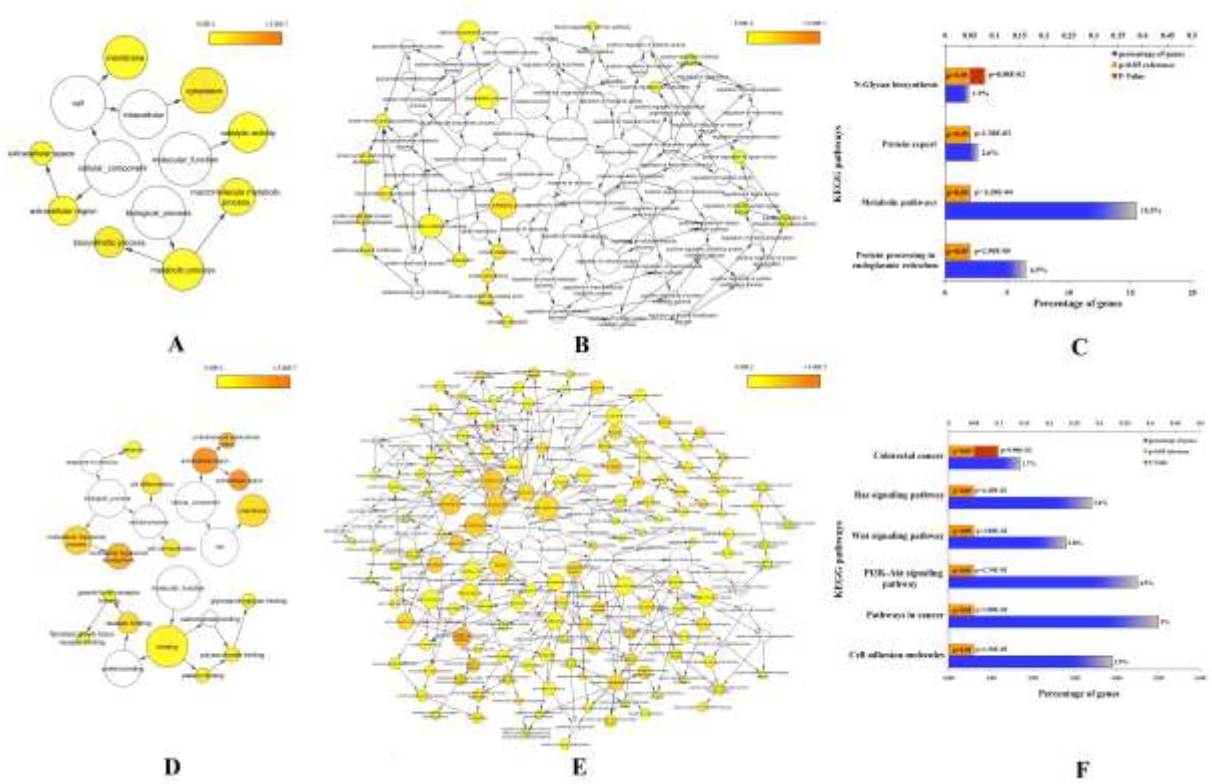

Figure 2. Gene ontology and KEGG pathway enrichment analysis of differentially expressed genes (DEGs) in gallbladder cancer (GBC). [2A, 2D], Biological Network Gene Ontology (BiNGO) tool in Cytoscape was used to perform gene ontology analysis which classified upregulated (2A) and downregulated (2B) genes in GBC into categories based on their molecular function, biological processes, and cellular location. Each node represents an enriched gene ontology (GO) term. Higher numbers of genes result in larger nodes in the network, and color intensity signifies statistical significance {node color ranges from white (not significantly over-represented genes) to yellow (p=0.01) to dark orange (p=0.05) magnitude smaller than 10-5)}; [2B, E], Detailed GO biological processes enriched in upregulated (2B) and downregulated DEGs (2E) in GBC; [2C,F], The top ten significantly enriched KEGG pathways of upregulated (2C) and downregulated DEGs (2F). KEGG pathway enrichment analysis of the DEGs was done by the DAVID (database for annotation, visualization, and integrated discovery) database, with $p<0.05$ considered as statistically significant.

MCODE identifies protein complexes by detecting heavily connected regions in the PPI network and has been reported to have higher precision than other module predictors (22). Significant modules in the PPI network were identified using MCODE, with node score cut-off = 0.3, K-Core = 4, and degree cutoff = 2, as reported previously (23–25). Finally, the functional and pathway enrichment of DEGs in each module was done using DAVID with a cut-off of $p < 0.05$.

Target prediction of identified DEmiRNAs

Target genes for the identified DEmiRNAs were predicted using miRWalk2.0, a database of predicted and experimentally validated miRNA-target interactions (26). We considered only the unique targets of upregulated and downregulated DEmiRNAs for further analysis, excluding genes targeted by both types of DEmiRNAs. The predicted targets were compared to the identified DEGs from our study, and overlapping genes with an opposite trend in expression compared to its associated DEmiRNA were selected; considered targets of upregulated miRNAs should be downregulated in expression and vice-versa.

Prediction of DEGs-DEmiRNAs-DElncRNAs regulatory interactions

miRNA-lncRNA interaction data was obtained from DIANA-LncBase Experimental v.2, a database containing low/high throughput, direct/indirect experimentally supported interactions (27). The DEmiRNAs identified by our study were mapped to the database to identify their corresponding regulatory lncRNAs. These lncRNAs were then mapped to the identified DElncRNAs from our study, and lncRNAs common to both sets, with an opposite trend in expression compared to that of the associated DEmiRNAs, were identified. The regulatory interaction network showing possible gene-miRNA-lncRNA interactions in GBC was created and visualized using Cytoscapev3.7.1.

Results

Identification of candidate DEGs involved in GBC

GSE62335 and GSE74048 contain expression data of genes and lncRNAs, six samples of GSE132223 contain expression data of genes, GSE104165 and GSE90001 contain expression data of miRNAs, and GSE76633 contains expression data of lncRNAs. A total of 64 GBC samples and 32 controls from multiple centers were included for this integrated analysis.

After meta-analyzing the dataseries for DEGs, 351 genes were identified as significantly differentially expressed in all three analyzed dataseries. Out of these, 185 DEGs were downregulated and 166 were upregulated in expression (supplementary material 1). Table 2 summarizes the top ten upregulated and downregulated DEGs identified in GBC compared to controls, ranked by their p-values. These 351 DEGs were considered as candidate genes for further analysis in the current study.

GO and KEGG pathway enrichment of identified DEGs

GO and KEGG pathway enrichment analyses were performed for the identified DEGs to define the associated deregulated functions and pathways in GBC. Upregulated DEGs were mostly involved in the molecular function of “catalytic activity” (39.74%) (Figure 2A), while downregulated DEGs were majorly involved in “binding” processes (90.79%) (Figure 2D), of which “glycosaminoglycan binding” (4.83%) and “fibroblast growth factor receptor binding” (2.76%) were the most enriched categories. Upregulated DEGs were mostly localized in the cytoplasm (41.82%), membrane (36.36%), and extracellular space (7.28%) (Figure 2A), while downregulated DEGs were localized mostly in membrane (50.26%), extracellular space (14.15%), and proteinaceous extra cellular matrix (7.33%) (Figure 2D). The upregulated DEGs were mostly enriched in the biological process of cellular biosynthetic processes (22.9%), positive regulation of ligase activity (3.82%), positive regulation of ubiquitin-protein ligase activity (3.82%), protein amino acid N-linked glycosylation (3.05%), and positive regulation of blood coagulation (2.29%) (Figure 2B), while downregulated DEGs were enriched in developmental processes (41.13%), signaling (37.59%), cell surface receptor linked signaling (26.24%), fibroblast growth factor receptor signaling (2.84%), positive regulation of peptidyl-tyrosine phosphorylation (2.84%), protein localization at cell surface (2.13%) and homotypic cell-cell adhesion (1.42%) (Figure 2E).

Overall, 43.2% of upregulated DEGS and 32.8% of downregulated DEGs were mapped to the KEGG database. The most significantly enriched pathway associated with upregulated DEGs was "protein processing in endoplasmic reticulum," while "cell adhesion molecules (CAMs)" was the most enriched category for downregulated DEGs. KEGG pathways with a significance level of $p \leq 0.05$ with percentage of detected genes are shown in Figure 2C, 2F.

Construction of PPI network, hub genes identification, and module analysis

A PPI network was created for the identified DEGs to infer possible interactions. A total of 349 DEGs were mapped to the network, resulting in a GBC-specific PPI network consisting of 349 nodes and 566 edges with a confidence score of more than 0.4 (Figure 3A, Supplementary material 2). The network had interactions with a PPI enrichment p-value of 2.22e-16, which is significantly higher than expected for a random set of proteins, implying that the proteins were partially biologically associated, suggesting the putative interactions to be meaningful. The degree of a node represents the number of interactions the node has with other nodes. The identified DEGs displayed a broad distribution of degrees, with highest and lowest degrees of 22 (FGF2) and 1 (multiple genes), respectively. The average degree value was 4, which indicates that the genes were functionally related and in a well-connected network.

The centrality parameter of a network indicates the importance of a node in holding the network together. A higher parameter value of a node signifies its higher relevance in connecting the network. Four parameters, viz., degree centrality, stress centrality, closeness centrality, and betweenness centrality, were computed for the network, and the top 20 nodes for each parameter were compared. The overlapping genes of these parameters were considered as hub nodes of the network. These included Fibroblast growth factor 2 (FGF2), Cyclin-dependent kinase 1 (CDK1), ribophorin II (RPN2), Protein transport protein Sec61 subunit

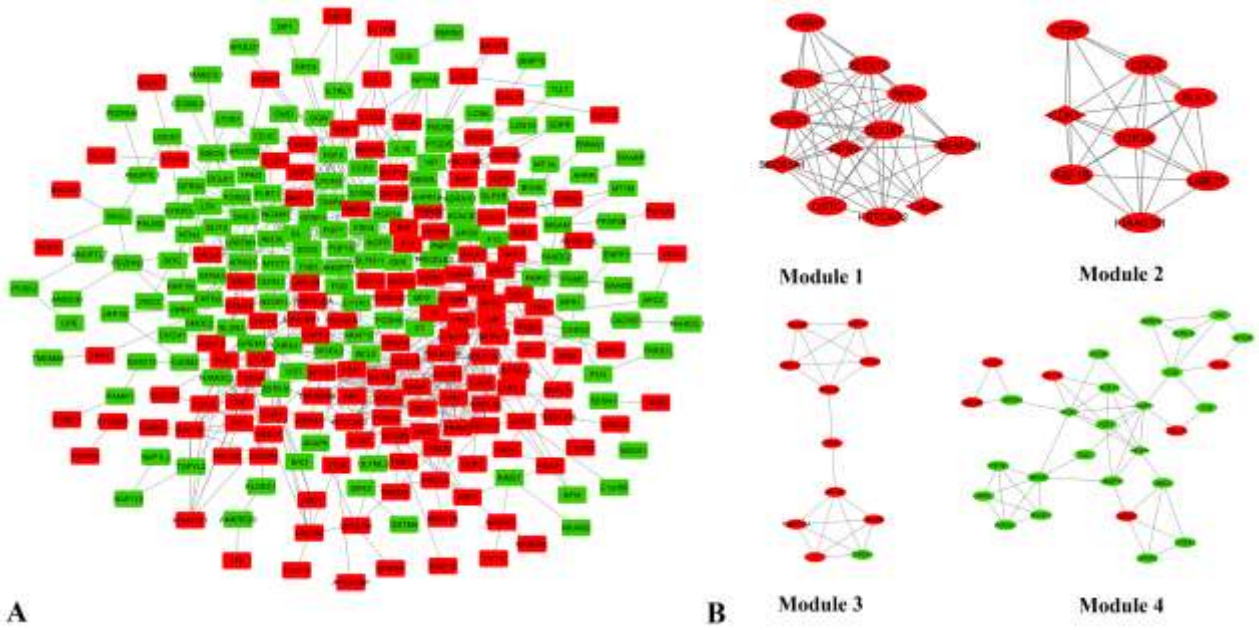

Figure 3. Protein-protein-interaction network of the differentially expressed genes (DEGs) and modular analysis. [A] The 351 differentially expressed genes identified in GBC were retrieved from the STRING v11.0 (Search Tool for the Retrieval of Interacting Genes/Proteins Interacting Genes) database, and the resulting protein-protein interaction (PPI) network was visualized in Cytoscape v3.7.1 with an interaction score of 0.4. Upregulated and downregulated genes are depicted in red and green colors in the PPI network, respectively. [B] Four significant modules were identified form the PPI network using MCODE clustering algorithm. Module 1 consisted of 12 nodes and 54 edges, module 2 consisted of 8 nodes and 26 edges, module 3 consisted of 11 nodes and 22 edges, and module 4 had 29 nodes and 57 edges. All genes in modules 1, 2, and 3 were upregulated, with the exception of CHRDL1 in module 3. Module 4 had a higher number of downregulated genes. Hub genes of the respective modules are represented as rhombuses.

alpha isoform 1 (SEC61A1), SRY (sex determining region Y)-Box 2 (SOX2), calreticulin (CALR), nerve growth factor receptor (NGFR), and neural cell adhesion molecule 1 (NCAM). FGF2, SOX2, NGFR, and NCAM were downregulated in expression, while CDK1, RPN2, SEC61A1, and CALR were upregulated.

In interaction networks, nodes that are highly connected (modules) are recognized as being involved in the same pathways. These highly connected modules are often of clinical importance, and their respective hub genes have been found to play key roles in multiple diseases, including cancer (28). We obtained four significant modules from the entire network using the MCODE clustering algorithm (Fig. 3B). Module 1 had a cluster density score of 9.818 with 12 nodes and 54 edges; module 2 had a score of 7.429 with 8 nodes and 26 edges; module 3 had a score of 4.400 with 11 nodes and 22 edges; and module 4 had a score of 4.071 with 29 nodes and 57 edges.

Furthermore, analysis revealed that the hub genes were distributed mostly in modules 1 (RPN2, SEC61A1, CALR) and 4 (FGF2, SOX2, NGFR), with

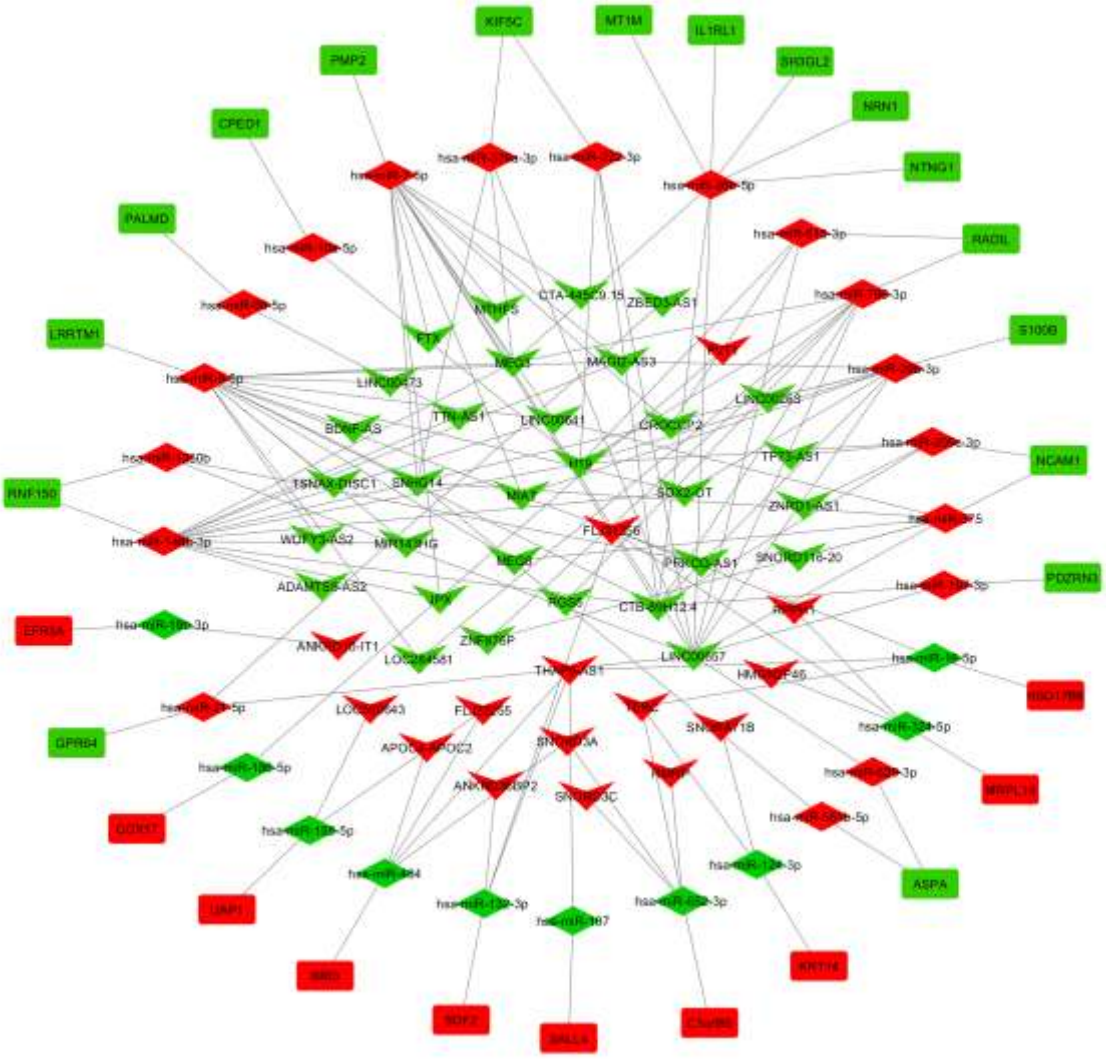

Figure 4. DEG-DEmiRNA-DElncRNA regulatory network in gallbladder cancer based on predicted and/or experimentally proven targets and their expression profile in GBC. Rectangular nodes represent differentially expressed genes (DEGs) in gallbladder cancer, rhombus nodes represent differentially expressed microRNAs (DEmiRNAs) associated with these DEGs, and triangle nodes represent differentially expressed long non-coding RNAs (DElncRNAs) associated with the DEmiRNAs. Red color nodes are upregulated, and green color nodes are downregulated in expression. A total of 46 DElncRNAs was found to be associated with 28 DEmiRNAs, which in turn could target 27 DEGs in GBC.

one hub gene in module 2 (CDK1) and none in module 3. KEGG pathway enrichment showed that genes in module 1 were significantly enriched in protein processing in endoplasmic reticulum, module 2 in cell cycle, and module 4 in Ras signaling pathway. No significant pathway was mapped to module 3. The four modules shared no pathways, indicating that they have relatively independent functions.

Identification of differentially expressed non-coding RNAs

To investigate the regulatory control of non-coding RNAs on the DEGs, we identified DEmiRNAs and DElncRNAs in GBC compared to controls. A total of 787 DEmiRNAs were identified, of which 299 were downregulated, while 488 had upregulated expression (Supplementary material 3). The most significantly upregulated DEmiRNA was hsa-miR-143-3p, and hsa-miR-4745-5p was the most downregulated.

A total of 7436 lncRNAs were found to be differentially expressed in GBC, of which 3127 were downregulated and 4309 were upregulated in expression (Supplementary material 4). PYCR1 and LOC388282 were the most significantly upregulated and downregulated DElncRNAs, respectively, in the current study. Table 2 summarizes the top ten upregulated and downregulated DEmiRNAs and DElncRNAs, ranked by their p-values.

Table 3. A DEG-DEmiRNA-DElncRNA interactions table showing regulatory DEmiRNAs in GBC which are associated with DEGs identified in GBC, and DElncRNAs associated with these DEmiRNAs.

DEGs	DEmiRNAs associated with DEG	DElnRNAs associated with DEmiRNAs
ASPA	hsa-miR-551b-5p	SNHG14
	hsa-miR-629-3p	LINC00657
BRI3	hsa-miR-484	APOC4-APOC2, SNORD3A, THAP9-AS1, FLJ32255
C1orf85	hsa-miR-652-3p	RMRP, SNORD3A, SNORD3C, TERC
COX17	hsa-miR-186-5p	PVT1
CPED1	hsa-miR-10a-5p	H19
EFR3A	hsa-miR-19b-3p	ANKRD10-IT1
GPR64	hsa-miR-21-5p	ZBED3-AS1, LINC00657
HSD17B8	hsa-miR-16-5p	THAP9-AS1, TERC, FLJ31356
IL1RL1	hsa-miR-26b-5p	LINC00657, CTB-89H12.4, MEG3
KIF5C	hsa-miR-222-3p	LINC00657, CTB-89H12.4, H19
	hsa-miR-376a-3p	SNHG14, LINC00657, MEG3
KRT14	hsa-miR-124-3p	TERC, SNORA71B
LRRTM1	hsa-miR-9-5p	MAGI2-AS3, PRKCQ-AS1, LOC284581, MEG8, LINC00641, MIR143HG, LINC00473, SNHG14, BDNF-AS, SOX2-OT, TSNAX-DISC1, CTB-89H12.4, WDFY3-AS2, MEG3
MRPL14	hsa-miR-324-5p	RPPH1, HMGN2P46, FLJ31356
MT1M	hsa-miR-26b-5p	LINC00657, CTB-89H12.4, MEG3
NCAM1	hsa-miR-200c-3p	TP73-AS1, CTB-89H12.4, ZNRD1-AS1, H19
	hsa-miR-375	MEG8, LINC00641, SNHG14, LINC00657, SNORD116-20, ZNF876P
NRN1	hsa-miR-26b-5p	LINC00657, CTB-89H12.4, MEG3
NTNG1	hsa-miR-26b-5p	LINC00657, CTB-89H12.4, MEG3
PALMD	hsa-miR-98-5p	TTN-AS1
PDZRN3	hsa-miR-197-3p	LINC00657, CTB-89H12.4
PMP2	hsa-miR-7-5p	MAGI2-AS3, MTHFS, LINC00641, FTX, MIAT, SNHG14, JPX, CROCCP2, LINC00657, CTA-445C9.15, CTB-89H12.4, MEG3
RADIL	hsa-miR-615-3p	CROCCP2, LINC00657, H19
	hsa-miR-766-3p	LOC284581, LINC00657, CTB-89H12.4, H19, RGS5, ZNF876P, MEG3
RNF150	hsa-miR-1260b	PRKCQ-AS1, LINC00657
	hsa-miR-148b-3p	MAGI2-AS3, TTN-AS1, ADAMTS9-AS2, LINC00641, MIR143HG, SNHG14, SOX2-OT, JPX, CTB-89H12.4, H19, MEG3
S100B	hsa-miR-29b-3p	MAGI2-AS3, LINC00263, MIAT, SNHG14, SOX2-OT, LINC00657, CTB-89H12.4, H19
SALL4	hsa-miR-107	THAP9-AS1
SDF2	hsa-miR-132-3p	THAP9-AS1, FLJ31356, ANKRD36BP2,
SH3GL2	hsa-miR-26b-5p	LINC00657, CTB-89H12.4, MEG3
UAP1	hsa-miR-155-5p	LOC550643, FLJ32255

Identification of DEmiRNAs-DEGs pairs

Target genes for the identified DEmiRNAs were predicted using miRWalk2.0. A total of 13,653 targets were predicted for the 735 DEmiRNAs, of which 9349 genes were possible targets of both upregulated and downregulated DEmiRNAs and, thus, were excluded from further analysis. A total of 2014 unique targets for upregulated DEmiRNAs and 2290 unique targets for downregulated DEmiRNAs were identified from the predicted targets.

The identified gene targets were mapped to the 351 DEGs previously identified in the current study. Because miRNAs inhibit the expression of their target genes, DEG-DEmiRNA pairs having opposite expression patterns were screened. We identified 20 upregulated DEGs that were targets of 18 downregulated DEmiRNAs, of which the highest number of DEGs were targeted by hsa-miR-26b-5p (5 DEGs). Moreover, 23 downregulated DEGs were found to be targets of 28 upregulated DEmiRNAs, of which hsa-miR-335-5p targeted the highest number of DEGs (6 DEGs) (Supplementary material 5).

Identification of DElncRNAs associated with the regulatory DEmiRNAs of DEGs

DIANA-LncBase Experimental v.2 database was used to identify target miRNAs for the 7497 DElncRNAs identified in the current study. A total of 111 DElncRNAs were mapped to the databases which were found to be targeting 723 miRNAs. Out of these723 miRNAs, 313 were found by the current study to be differentially expressed. Moreover, 92 DElncRNAs had an opposite trend in expression to their corresponding miRNAs, of which 63 were downregulated and 29 were upregulated in expression (Supplementary material 6). DEGs-DEmiRNA and DEmiRNAs-DElncRNA pairs, shown in supplementary materials 5 and 6, indicate that the identified DEGs were regulated by several non-coding RNAs. We further identified possible DEG-DEmiRNA-DElncRNA interactions based on expression status and predicted associations. LINC00657 and CTB-89H12.4 were associated with the highest number of DEmiRNAs (12 and 9, respectively), which in turn were targeting 10 and 9 DEGs, respectively. Overall, we found a total of 46 DElncRNAs to be associated with 28 DEmiRNAs, which in turn were targeting 27 DEGs in GBC (Figure 4, Table 3).

Discussion

Despite its aggressive nature and the lack of early detection techniques, GBC is still an orphan gastrointestinal cancer, and its underlying molecular mechanisms remain poorly understood. The current study used a meta-analysis-based bioinformatics approach to analyze microarray datasets and provide a statistically robust and clearer view of the gene, miRNA, and lncRNA expression profiles and their regulatory interdependence during GBC. We identified 351 DEGs in GBC compared to controls, of which 185 were downregulated and 166 were upregulated in expression. Recently, Ren et al. (2021) explored differentially expressed coding, non-coding, and circular RNAs in serum-derived exosomal samples of gallbladder cancer compared to xantho-granulomatous cholecystitis. They identified 1,940 mRNAs and 317 lncRNAs to be upregulated in plasma-derived exosomes of GBC (29). Among these, multiple genes such as FOS, SEC61G, CXCL5, FOXD3, and IRAK1 were also identified as differentially expressed in this study (29). The most significantly upregulated DEG in the current study, CEMIP, is a cell migration-inducing hyaluronan protein well known for promoting the motility, metastasis, and invasiveness of different cell types. It has been repeatedly associated with human carcinogenesis and was reported to be highly expressed in multiple cancers (30–34). It is has also been shown to have significantly improved diagnostic value compared with the traditional marker CA 19-9 alone in pancreatic cancer (35). The significantly higher expression of CEMIP observed in GBC suggests it plays an important role in GBC and can be more thoroughly investigated. The most significantly downregulated gene of the current study, CHRDL1, has been shown to be downregulated and associated with poor survival rate in gastric cancer and lung adenocarcinomas (36, 37). It is a secreted antagonist of bone morphogenetic protein (BMP), which inhibits BMP binding to its receptor (BMPR), thereby blocking a series of signaling responses and causing proliferation and invasiveness of cancer cells (38). However, to the best of our knowledge, the roles of CEMIP and

CHRDL1 have not been reported in GBC, and therefore, both of these genes could be possible novel targets for GBC diagnosis or prognosis. Apart from the most upregulated and downregulated genes, there is an interest in the role of hub genes, as their interactions with multiple nodes in the network indicate their potential for broad-spectrum effects during cancer (39). From the PPI network, we identified 8 hub genes (FGF2, CDK1, RPN2, SEC61A1, SOX2, CALR, NGFR, and NCAM), of which only SOX2, NCAM, CDK1, and CALR have been previously reported as involved in GBC (40). Although studies on the role of these genes are scarce, few genes have been recently reported. For example, deregulation in cell cycle is a primary and key step towards carcinogenesis, and CDK1 is a well-known cell cycle regulator whose upregulation has been reported in multiple cancers (41, 42). Although a few studies have reported deregulation in the cell cycle machinery in GBC, one recent study reported CDK1 activation via GPRIN1 as an important factor in promoting GBC progression (43). Similarly, SOX2, an undifferentiated cell marker, is responsible for maintaining stem cells in multiple cancers, and its deregulation, especially overexpression, has been reported to be oncogenic (44). However, few studies have reported the association between SOX2 downregulation and shorter survival time, poor prognosis, and less responsiveness to treatment in gastric cancer (45), and only one study has reported its potential role in GBC treatment (46). Recent reports have declared CALR to be an oncogene in GBC and that inhibiting CALR in GBC sensitizes the cancer to gemcitabine by inhibiting the PI3K/Akt pathway (47). Despite their established functions in other cancers, FGF2, RPN2, SEC61A1, and NGFR have not been reported in GBC to date. Considering that most of the hub genes were present in modules 1, 2, and 4 of the network, and that numerous studies have reported key roles of these genes in multiple cancers, we theorize that these genes can potentially play important roles in GBC carcinogenesis, and therefore, further investigation is warranted.

GO enrichment analysis revealed that the upregulated DEGs in the current study were associated with cytoplasmic proteins and involved in molecular functions of catalytic activity as well as biological processes of positively regulating ubiquitin-protein ligase activities, N-linked glycosylation (NLG) of amino acids, and blood coagulation. KEGG pathway analysis revealed the highest alterations in "protein processing in endoplasmic reticulum." The process of ubiquitylation-mediated protein degradation regulates the turnover and activity of many proteins, and thus has important implications in various cellular processes. As anticipated, ubiquitin ligases are frequently found to be upregulated in cancer (48, 49). The current results emphasize the oncogenic role of ubiquitination in GBC, as genes that positively regulate ubiquitin ligase activities are found to be upregulated. Furthermore, genes involved in promoting NLG were found to be upregulated in our study. Glycosylation is yet another neoplasia-linked, post-translational epigenetic modification which affects the stability, structure, folding patterns, and adhesion properties of a protein. Aberrant glycosylation of cancer-associated proteins may alter the biological properties of cancer cells, allowing them to metastasize. Previous studies have reported NLG inhibition as a possible strategy for cancer treatment (50); however, its role in GBC progression and initiation is less well understood, with only one report hinting at the role of O-glycosylation in GBC (51). Furthermore, in the current study, genes involved in the positive regulation of blood coagulation were found to be upregulated. High expression of thrombosis-initiating factor and its association with cancer aggressiveness and progression is well documented (52); in fact, thrombosis is often the first clinical presentation in many tumors (53). Notably, over 90% of cancer patients, including those with GBC, show aberrations in coagulation tests in vitro (54, 55).

The identified downregulated DEGs were part of membrane proteins, involved in binding-related molecular functions, especially glycosaminoglycan binding and fibroblast growth factor receptor binding. In addition, in the biological process category, downregulated DEGs were involved in developmental processes such as cell differentiation, signaling processes such as cell surface receptor linked signaling pathway and fibroblast growth factor receptor signaling pathway, localizing protein at cell surface, and homotypic cell-cell adhesion. KEGG pathway analysis revealed that downregulated genes were mostly part of "cell adhesion molecules (CAMs)." Cell-cell adhesion is an important biological process often found to be

deregulated and reduced in cancer, enabling the cell to invade and metastasize. Loss of cell-cell adhesiveness has been considered as a key morphological characteristic of malignant tumors (56), and studies have also reported the role of cell adhesion molecules in boosting the invasive capabilities of GBC cells (57). The observed downregulation of genes involved in cell-cell adhesion in the current study is consistent with previous reports and further highlights the importance of cell-cell adhesion in GBC. Glycosaminoglycan (GAG) chains are present in the surface of almost all cells, and their ability to bind different growth factors, cytokines, and enzymes has been implicated in cancer progression, invasion, and metastasis. Interestingly, GAGs have also been reported to be inhibitors of tumor progression depending on the type of interaction and cancer (58). On the other hand, fibroblast growth factor signaling regulates key biological processes involved with tissue homeostasis (59). The downregulation of genes involved in cell differentiation in the current study suggests the presence of undifferentiated tumor cells in GBC, which can greatly contribute to the aggressiveness of this cancer (60).

After analyzing the DEGs in GBC, we identified their associated, regulatory non-coding RNAs by integrating their expression profiles and predictions using bioinformatic tools. A total of 46 DEmiRNAs were identified which could target 43 DEGs. Eight of these DEmiRNAs (hsa-miR-155-5p, hsa-miR-200c-3p, hsa-miR-29b-3p, hsa-miR-30a-3p, hsa-miR-30e-3p, hsa-miR-324-5p, hsa-miR-335-5p, hsa-miR-98-5p) have been previously associated with poor survival rate and the aggressive nature of GBC (61, 62). The remaining 38 DEmiRNAs have not been reported in GBC, but all of them have been previously reported in cancers of multiple etiologies. Moreover, hsa-miR-26b-5p and hsa-miR-335-5p were found to be regulating the highest number of DEGs in the current study, suggesting their potential key regulatory roles during GBC. Both miRNAs have also been recently reported to be involved in circRNA-miRNA regulation in GBC (29).

A growing number of studies have suggested the involvement of lncRNAs in GBC carcinogenesis (63). Recently, two studies, those of Zang et al. (2018) and Kong et al. (2019), utilized GSE62335 and GSE76633 expression dataseries independently and identified 287 and 128 mRNA-lncRNA pairs in GBC, respectively (64, 65). We combined the three lncRNAs expression dataseries and cumulatively identified 7497 significant DElnRNAs in GBC. Few DElncRNAs identified in our study have been previously reported and experimentally validated in multiple cancers, including GBC. For instance, expression of CRNDE was significantly high in the current study, which is in accordance with previous reports of high expression in GBC (66). Similarly, MEG3 has been reported as a tumor suppressor in many cancers, including GBC (67), and was downregulated in the current study. We further combined the expression and prediction data to build the lncRNAs-miRNA-gene interaction network. Twenty-seven DEGs were found to be possibly regulated by 28 DEmiRNAs, which in turn were associated with 46 DElncRNAs. LINC000657 was found to target the highest number of DEmiRNAs and DEGs in the network. It was predicted to target hsa-miR-26b-5p, which in turn was targeting the highest number of DEGs. LINC000657 has been previously reported to be an inhibitor of hsa-miR-615-3p in esophageal squamous cell cancer (68). A few other interactions predicted in the network have also been previously reported in other cancers and validated, which indicates the reliability of the network. For example, the regulatory role of PVT1 in inhibiting hsa-miR-186-5p and promoting tumorigenesis has been previously reported in hepatocellular carcinoma (69). In our network, PVT1 was found to be a regulator of hsa-miR-186-5p, which in turn was targeting COX17. Furthermore, three lncRNAs of the network (H19, MEG3, and PVT1) have been previously reported in GBC (70). These were found to interact with 7, 5, and 1 DEmiRNAs, respectively, which in turn were targeting 6, 5, and 1 DEGs, respectively.

Conclusion

The current study identified 351 DEGs, 787 DEmiRNAs and 7436 DElncRNAs in GBC. We also found 10 GBC-specific DEGs, CEMIP, CHRDL1, FGF, CDK1, RPN2, SEC61A1, SOX2, CALR, NGFR, and NCAM, from the cumulative network and expression data. These could be the subjects of more in-depth studies to delineate their specific roles in GBC. We predicted DEG-DEmiRNA-DElncRNA interactions in GBC and found 27 DEGs to be regulated

by 28 DEmiRNAs, which were possible targets of 46 DElncRNAs. The regulatory interactions predicted in the network can be further explored to identify novel gene targets and to understand the mechanisms underlying GBC development. Multiple candidates and interactions identified in the current study have never been reported in GBC and warrant further studies. While the findings of the current study are predictive and require further validation, some of the identified candidates have been experimentally validated and reported to be crucial in carcinogenesis of other etiologies. This study improved our understanding of the human transcriptomic changes during GBC, reaffirmed previously reported findings, and identified new candidate genes and regulatory networks of prognostic and diagnostic value.

Acknowledgement

The first author, Nidhi Singh, acknowledges Department of Biotechnology (DBT), Government of India for providing supporting fellowship under DBT-JRF scheme.

Conflict of interests

The authors declare that they have no competing interests.

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