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Identification of Novel Gene Expression Patterns and Pathways Involved with SOX17 Gene Dysregulation in Cholangiocyte and Cholangiocarcinoma

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Abstract

Aim: *To deduce the genes, pathways and molecular mechanism involved in SOX17 dysregulation observed in Cholangiocarcinoma.* ***Materials & methodology:*** *In this study we use bioinformatics tools to analyze the metaomics of the microarray data retrieved from GEO database (Gene Expression Omnibus, Accession Number: GSE77984). Raw data obtained from the GEO databases were analyzed by using the GEO2R web analyzer tool, which derived all the differentially expressed genes (DEGs) via by comparing the miRNA expression between NHC (Normal Healthy Cholangiocytes) and CAA (Cholangiocarcinoma Cells) at various regulation levels of SOX17 protein.* ***Results:*** *Among all the samples of SOX17 activity, a total of 24975 genes among 15453 significant genes were identified. From the total genes 24 common genes, 5 highly interacted DEGS, and pathways corresponding to gene networks interlinked to CAA were identified.* ***Conclusion:*** *Using computational biology we have identified five molecular biomarkers for CAA which might provide additional insights in diagnosis and prognosis of Cholangiocarcinoma.*

Key-words: Biomarker, Gene Expression Omnibus, Differentially Expressed Genes, STRING Analysis, Novel Molecular Mechanism, Molecular Biology, Genetic Analysis, Gene Expression.

1. Introduction

This study uses bioinformatics tools on genomic data derived from microarray databases which was analyzed to predict all the incorporating genes and pathways that were involved in the cause of CAA. Functional enrichment tools used to recognize molecular biomarkers for CAA will provide additional insight to identify the regulated genes in CAA and NHC (Wang et al. 2013).

Cholangiocarcinoma (CAA), a malignant tumor with heterogeneous groups that originate from the epithelial cells of biliary in liver or bile duct (Huang et al. 2016). It is characterized as the second most common type of hepato-biliary malignant carcinoma along with deficient diagnosis tools along with poor prognosis due to asymptomatic condition at early stages of carcinoma (Banales et al. 2016). Since the past three decades, many incidences of CAA are elevating in many countries along with mortality rates (Bridgewater et al. 2013). As per the RARECARE net project (2008), the incident cases of CAA were comparatively 40% higher in men compared to women (Zwan et al. 2012).Chronic inflammation and biliary duct cell injury are two main conditions for the cause of cholangiocarcinoma (Fava et al. 2007). During the inflammation mechanism, cytokines are released into the microenvironment of biliary duct which are responsible for carcinogenesis by imparting signals along with aberrations in the genetic makeup by mutation (Jaiswal et al. 2001). Abnormalities of epigenetic and genetic modulation by the mutation of certain genes is characterized as Cholangiocarcinoma (CAA).

Some gene promoters, such as the promoter of the SRY-related HMG-box 17 (SOX17) transcription factor, have been shown to be hypermethylated in CCA tumours (Kamachi and Kondoh 2013).The SOX17 gene regulates proliferation, cell cycle and angiogenesis during cancer progression(Goeppert et al. 2014) and also acts as Tumor suppressor gene(Du et al. 2009).. SOX gene family members play vital roles in tumorigenesis and metastasis (Jaiswal et al. 2001; Banales et al. 2020, 2016). SOX17 expression level can alter the development of biliary endoderm epithelium during embryogenesis by incorporating wnt/β-catenin as transcription factors (Li et al. 2015; Jia et al. 2010). Regulation at the SOX17 expression level can impact cell proliferation, morphogenesis (S. Higashiyama et al. 1992). The regulation of SOX17 gene can impact on other genes, these genes are associated by using microarray databases. By using Microarray dataset, the differentially expressed genes among the CAA and NHC cells along with range of expression level were predicted. Eventually by identifying the metabolomics of biological pathways and processes that are altered due SOX17 gene regulation in CAA and NHC. This data can be applied to predict the common genes and their shared pathways and molecular mechanisms involved, which helps to find possible markers along with feasible sites for targeting novel compounds as drugs.

Previously our team has a rich experience in working on various research projects across multiple disciplines (Sathish and Karthick 2020; Varghese, Ramesh, and Veeraiyan 2019; S. R. Samuel, Acharya, and Rao 2020; Venu, Raju, and Subramani 2019; M. S. Samuel et al. 2019; Venu, Subramani, and Raju 2019; Mehta et al. 2019; Sharma et al. 2019; Malli Sureshbabu et al. 2019; Krishnaswamy et al. 2020; Muthukrishnan et al. 2020; Gheena and Ezhilarasan 2019; Vignesh et al.

2019; Ke et al. 2019; Vijayakumar Jain et al. 2019; Jose, Ajitha, and Subbaiyan 2020). Now the growing trend in this area motivated us to pursue this project.

Bioinformatics models used in this study provide information regarding ways to diagnose and develop personalized medicine for CAA (Qian et al. 2018). Analysis of Microarray data of gene expression obtained from GEO database (NCBI) provided the genes and pathways that are differentially expressed in CAA and the direct effect of SOX17 dysregulation (Edgar, Domrachev, and Lash 2002). Functional enrichment tools used to recognize molecular biomarkers for CAA will provide additional insight to identify the regulated genes in CAA and NHC (Wang et al. 2013). This research work deals with the comparative analysis for existing gene expression microarray data regarding dysregulation of SOX17 gene in both NHC and CAA cells is present but the interaction of molecular mechanism and functional enrichment analysis of those genes involved were not analyzed. The aim of this study was to explore the DEGs along with molecular mechanisms by comparing the regulation of SOX17 gene in both the normal healthy Cholangiocytes and Cholangiocarcinoma cells by using microarray and to find possible markers for diagnosis CAA.

2. Materials and Methodology

2.1 Microarray Dataset

Gene expression profiling data was collected from the NCBI GEO database (Gene Expression Omnibus database), GSE77984 dataset. GSE77984 dataset contains 25 samples of data, includes the two cell types of *Homo sapiens* (Normal Health Cholangiocytes & Cholangiocarcinoma) which are treated with shRNA- SOX17 control and shRNA-SOX17 [Table. 1].

Table 1 - Gene expression Biochip Datasets of NHC and CAA from GEO Database ID: GSE77984

GROUP	No of Samples	Accession ID	Cell tittle	Cell type	Treatment type
G1	04	GSM2064133	N_shC-1	Normal human cholangiocytes	Lentivirus shRNA-control infected
		GSM2064134	N_shC-2	Normal human cholangiocytes	Lentivirus shRNA-control infected
		GSM2064135	N_shC-3	Normal human cholangiocytes	Lentivirus shRNA-control infected
		GSM2064136	N_shC-4	Normal human cholangiocytes	Lentivirus shRNA-control infected
		GSM2064137	N_shS-1	Normal human cholangiocytes	Lentivirus shRNA-SOX17 infected
		GSM2064138	N_shS-2	Normal human cholangiocytes	Lentivirus shRNA-SOX17 infected

G2	04	GSM2064139	N_shS-3	Normal human cholangiocytes	Lentivirus shRNA-SOX17 infected
		GSM2064140	N_shS-4		Lentivirus shRNA-SOX17 infected
G3	03	GSM2064144	E_pWPI-1	Cholangiocarcinoma cell	Lentivirus control-vector infected
		GSM2064145	E_pWPI-2	Cholangiocarcinoma cell	Lentivirus control-vector infected
		GSM2064146	E_pWPI-3	Cholangiocarcinoma cell	Lentivirus control-vector infected
G4	04	GSM2064147	E_SOX17-1	Cholangiocarcinoma cell	Lentivirus SOX17-vector infected
		GSM2064148	E_SOX17-2	Cholangiocarcinoma cell	Lentivirus SOX17-vector infected
		GSM2064149	E_SOX17-3	Cholangiocarcinoma cell	Lentivirus SOX17-vector infected
		GSM2064150	E_SOX17-4	Cholangiocarcinoma cell	Lentivirus SOX17-vector infected

The GE02R web tool was used to analyze the differentially expressed genes (DEGs) in the Biochips of the Microarray data. Among 25 samples biochip data, they are grouped into 4 types of selective datasets based on SOX17 regulation activity on NHC and CAA samples, NHC with overexpression of SOX17 (4 samples), NHC with downregulation of SOX17 (4samples), CAA with overexpression of SOX17 (3samples), CAA with downregulation of SOX17 (4samples).

2.2 Identification of Significant Genes

The significant genes were isolated from the DEGs which are obtained from the GEO2R analysis. Further analysis of GEO2R analysis, the significant genes were sorted on the basis of P-value which defines probability of expression. The genes whose P-values were greater than 0.05 were sorted as significant genes.

2.3 GENE-GENE Network Interaction (String)

The interaction between genes was identified by using the Search Tool for the Retrieval of Interacting Genes (STRING) database is an online tool (von Mering et al. 2003). A STRING tool was applied to evaluate a network of interlink interaction for the screened DEGs, with a combination score >0.99 as the threshold value (von Mering et al. 2003). Comparative protein -protein interactions provided by the STRING analysis tool are visualized and analyzed in Cytoscape Software [version 3.2.1], however it provides an easy way to interpret the connection between the proteins or genes (Mering et al. 2002).

2.4 Hub Genes Identification (MCODE)

The 'MCODE' plug-in of cytoscape was used to retrieve the interconnected regions or clusters from gene- genes or protein-protein interactions (PPI) shows number of highly small connected protein nodes (known as hubs) and many other imperfectly connected nodes and they are potentially interlinked with each other in functional analysis (Bader and Hogue 2003). The degree of interactions were statistically analyzed based on the combined algorithm used in the database (Chen et al. 2016).

2.5 Gene Enrichment Analysis

Gene functional analysis for the interlinked network of genes were performed by using the STRING analysis. String analysis provides a well known system of classification such as KEGG pathway (Table. 2), Gene Ontology, eventually it provides additional information regarding high-throughput text-mining as well as on a hierarchical clustering network associated with it (Szklarczyk et al. 2021).

Table 2 - Enriched Kyoto Encyclopedia of Genes and Genome (KEGG) Pathways of Common Genes ($p<0.05$).

Term	Description	Count	P-value
HSA0466	TNF Signaling pathway	4	0.00031
HSA04915	Estrogen Signaling Pathway	3	0.01031
HSA04210	Apoptosis	3	0.0103
HSA05166	HTLV-1 Infection	3	0.3101
HSA0900	TERpenoid Backbone Biosynthesis	2	0.103
HSA04924	Renin Secretion	2	0.0301

2.6 Refined Gene Enrichment Analysis (Funrich)

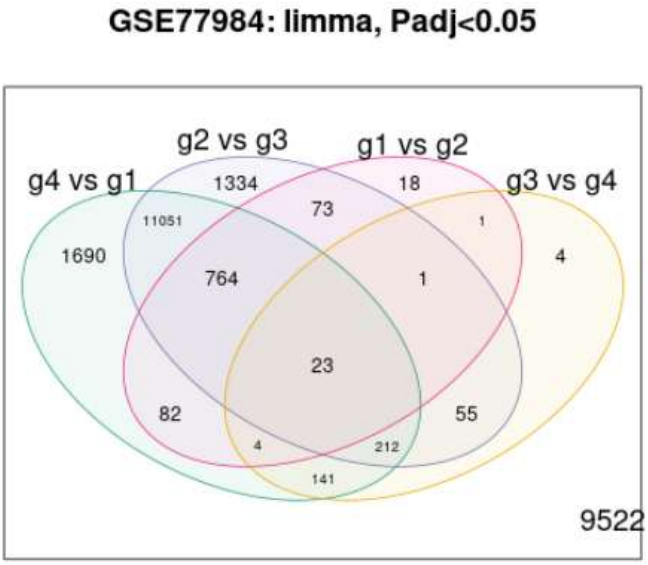
Functional enrichment and network analysis on DEGs that are involved in heterogeneous genomic and proteomic activities are analyzed by using the FunRich database (Szklarczyk et al. 2021; Fonseka et al. 2020). FunRich is an open-access tool that provides the user to download and analyse the data obtained the database and also for interpreting the functional enrichment analysis regarding heterogeneous data and provides the summary of gene ontology, site of expression, biological pathways, protein interactions, domains, or associated diseases, transcription factor, clinical phenotype and COSMIC (Pathan et al. 2017).

3. Results

3.1 Dataset

The microarray dataset of GSE77984 provided 6 datasets with 25 data samples which includes the two cell types i.e. Normal Health Cholangiocytes & Cholangiocarcinoma) which are treated with shRNA- SOX17 control and shRNA-SOX17. The GE02R web tool provided comparison data between all the samples data based on the level of expression and log Fc (Fold change) value shown in Fig. 1. Totally 18994 genes were expressed, only 23 common genes were identified and these further studied to develop the marker for diagnosis.

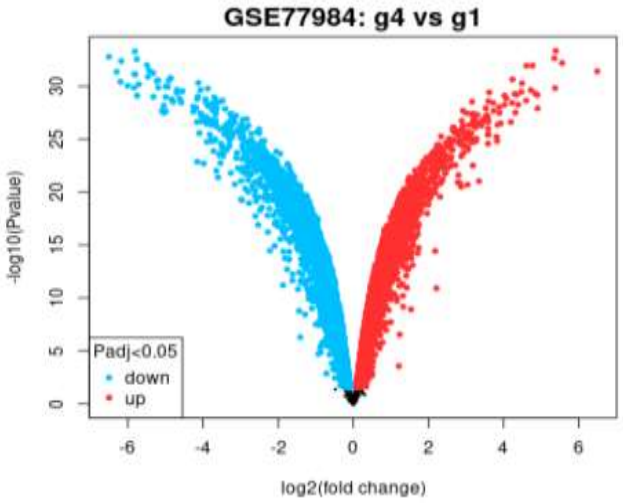
Fig. 1 - Venn Diagram representing the common DEGs between all the group Datasets among all the genes along with each other common genes. Totally 24975 genes among 15,453 significant genes were expressed and also 23 common genes were identified which were expressed among all the sample datasets. Whereas 18 genes are common in G1 and G2 datasets, 1334 common genes between G2 and G3, 4 common genes between G3 and G4, 1690 common genes among G4 and G1. Furtherly 11021 common genes between G1,G4 and G2 and G3; 73 common genes between G2,G3 and G1,G2; 1 common gene between G1, G2 and G3, G4; 55 common genes between G2,G3 and G3,G4; 212 common genes between G1,G4 and G3,G4 respectively.



3.2 Identification of Significant Genes

Further analysis of the GE02R web tool results, among totally 18994 DEGs are involved with 7042 significant genes are in the overexpression of SOX17 and 6156 significant genes are involved in the Downregulation of SOX17 in both NHC and CAA; 16118 significant genes in upregulation and downregulation of SOX17 in NHC and 16795 significant genes in upregulation and downregulation of SOX17 in CAA shown in Fig. 2.

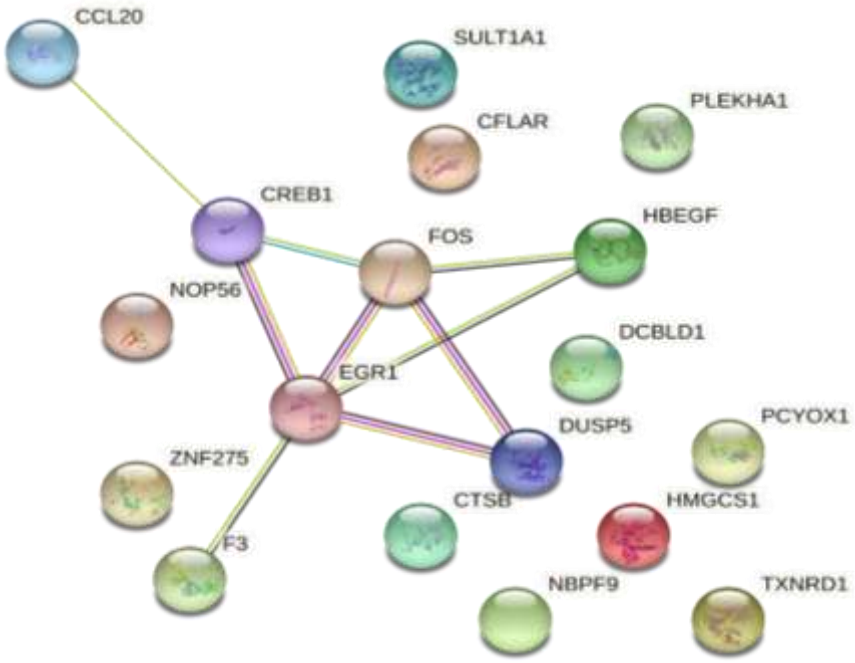
Fig. 2 - Volcano plot for differentially expressed genes (DEGS) in NHC and CAA cells from GSE77984 dataset. The x-axis represents Log2Fc, large magnitude fold change; Y-axis represents -log10 of a P-value, high statistical significance. Each dot represents a single gene. The dots above red and blue color represent DEGS whose LogFC >1and P-value<0.05.



3.3 Gene-Gene Interaction

The common genes derived from the previous tool were used as an input for STRING analysis. A total of 9 interactions with 19 interlinked nodes between the genes were obtained shown in Fig. 3.

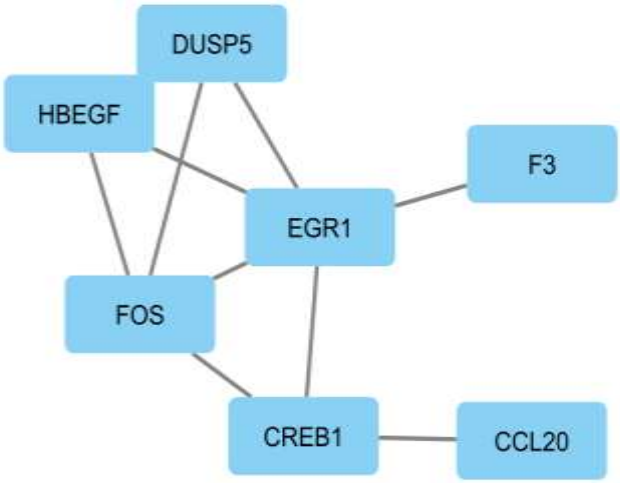
Fig. 3 - Gene-Gene interaction analysis of common genes, STRING database. FOS, EGR1, DUSp5 genes were interacted with 3 nodes interlinked with each other along interaction with CREB1 genes along with HBEGF gene interlinked with FOS andEGR1 genes with same internodes



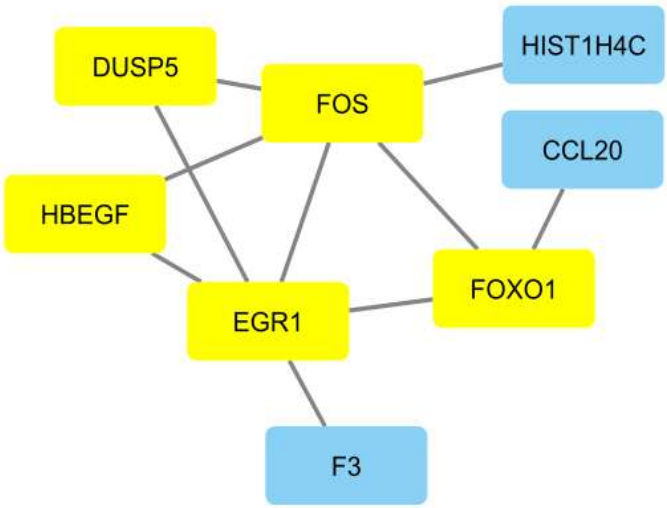
3.4 MCODE Analysis

The Cytoscape analysis provided a PPI network of genes which are interlinked among each other and included 7 nodes and 9 interactions [Fig. 4 (a)], The pugin MCODE provided 5 nodes and 7 edges with combined score >0.99. The interconnected regions or clusters from gene- genes or protein-protein interactions (PPI) have a small number of highly connected protein nodes (known as hubs) DUSP5; FOS, HBEGF, EGR1, FOXO1 [Fig. 4(b)].

Fig. 4 - Constructed Gene-Gene interaction network of COMMON genes, Nodes represent protein, edges represent interactions between two Genes. (a) Cytoscape analysis (b) MCODE analysis representing the highly interconnected genes which are represented as clusters highlighted in Yellow color DUSP5; FOS, HBEGF, EGR1, FOXO1.



(a)



(b)

3.5 Gene Enrichment Analysis

STRING analysis enhanced functional enrichment related to GO and KEGG analysis, a total of 90 and 20 terms were associated with common genes respectively. The significantly enriched terms were represented in Table 3 & 4. The top 2 significantly enriched KEGG terms were as follows : TNF signaling pathway, Estrogen signaling pathway. Following the GO analysis, the top 5 significantly enriched GO pathways as follows: primary metabolic process, organic substance metabolic process, cellular response to stimulus, regulation of primary metabolic process.

Table 3 - Gene Ontology (GO) Functional Enrichment Analysis of Common Genes ($p<0.01$).

GO-term	Description	Count	P-value
GO:0044238	Primary metabolic process	16	0.0163
GO:0071704	Organic substance metabolic process	16	0.0209
GO:0051716	Cellular response to stimulus	14	0.0104
GO:0070887	Cellular response to chemical stimulus	14	0.00008
GO:0080090	Regulation of primary metabolic process	13	0.03
GO:0007275	Multicellular organism development	12	0.0267
GO:0009888	Tissue development	11	0.0033
GO:0071310	Cellular response to organic substance	9	0.0064
GO:0051173	Positive regulation of nitrogen compound metabolic process	9	0.0209

Table 4 - Gene Enrichment Analysis of Common Genes Using Fun Rich Analysis

Analysis type	Activity	No of common gene involved	Genes
Site of expression	Liver	17	DUSP5; CCL20; FOS; SOX17; PCYOX1; FOXO1; EGR1; NOP56; CFLAR; F3; HMGCS1; HIST1H4C; HBEGF; ZNF275; TXNRD1; CTSB; SULT1A1;
Molecular function	Transcription factor activity	4	FOS; SOX17; FOXO1; EGR1;
Biological process	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	7	FOS; SOX17; FOXO1; EGR1; NOP56; HIST1H4C; ZNF275;
Cellular component	Nucleus	11	DUSP5; FOS; SOX17; FOXO1; EGR1; CFLAR; HIST1H4C; HBEGF; ZNF275; TXNRD1; CTSB;
Biological pathway	Plasma membrane estrogen receptor signaling	6	DUSP5; FOS; FOXO1; EGR1; CFLAR; HBEGF;
Transcription factor	SP1	8	DUSP5; SOX17; FOXO1; EGR1; NOP56; F3; HMGCS1; ZNF275;
Protein domain	signal peptide	6	CCL20; PCYOX1; F3; HBEGF; ZNF275; CTSB;

3.6 Refined Gene Enrichment Analysis

Analysis of Functional enrichment by using FUNRICH enhancement revealed the functional role and their pathways that were statistically significant, mostly these DEGs were found to be accumulated in the nucleus of the liver, arteries, Colon, prostate, lymph node and involved in Transcription factor activity, positive regulation of glomerular mesangial cell proliferation, Signal transduction, Energy pathways, Metabolism and Regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolism. The DEGs were mainly enriched in biological pathways such as Plasma membrane estrogen receptor signaling, ErbB receptor signaling network, V PAR1-mediated thrombin signaling events, EGF, and VEGFR signaling network, Calcineurin-regulated NFAT-dependent transcription in lymphocytes, Regulation of CDC42 activity, and signaling events. The high number of common gene activities were shown in Table 4.

4. Discussion

In this study raw data (GSE77984) were analyzed by using the GEO2R web analyzer tool, which predicted all the differentially expressed genes (DEGs) via comparison of miRNA expression between NHC and CAA cells at various regulation levels of SOX17 protein. A total of 15453 significantly differentially expressed genes (SG) along with 23 Common genes were identified from GEO2R analysis. Subsequently, we interpreted 9 interlinked genes by string analysis tool, besides 5 highly interacted genes were identified by using the MCODE tool. Hub nodes have more complex interactions compared with other genes, thus indicating that they have important roles in the underlying mechanisms of disease. Therefore, identification of hub genes may facilitate the development of effective therapeutic approaches for the treatment of patients. The highly interlinked genes which were obtained from the MCODE analysis are DUSP5, FOS, EGR, FOXO, HB-EGF were also known as Hub genes in the PPI network.

The Go (Gene Ontology) analysis of these helps to interpret the regulation activity of cells. The *DUSP5 gene* which involves in signal transduction, encodes a Mitogen-activated protein Kinase Phosphatase (MKP) (Yu et al. 2013). FOS interlinked the complex with the JUN/AP-1 transcription factor (Bossis et al. 2005), important role in signal transduction, cell proliferation and differentiation (Sharrocks 2010). EGR 1 regulates the transcription of numerous target genes, cell differentiation (Shimizu et al. 1992), and thereby plays an important role in regulating the response to growth factors (Hu et al. 2010), DNA damage, and ischemia (Parra and Ferreira 2010). FOXO involves cell

proliferation transcription factors (Brent, Anand, and Marmorstein 2008; Yamagata et al. 2008), regulates post-translational modification (Brent, Anand, and Marmorstein 2008). HB-EGF gene mainly involves the growth mediating factor (Elenius 1997), cell proliferation. All these are commonly involved in cell signal transduction, proliferation, transcription mechanisms (S. Higashiyama et al. 1992).

The network of interlinked genes obtained from the String and Cytoscape analysis have predicted that these are commonly involved in cell signal transduction, transcription mechanisms. Yet some studies predicted the connection of these genes to other diseases/disorders. The phosphorylation of FOXO gene conserved signaling for CDK1 cells for Promoting neuronal cell death reported by Zengqiang Yuan (Yuan et al. 2008). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) acts as the diphtheria toxin (DT) receptor reported by T Mitamura (Shigeki Higashiyama et al. 1995). However, the functionality of these is available individually, but the association of these genes activity was not available in the existing literature.

Our institution is passionate about high quality evidence based research and has excelled in various fields ((Vijayashree Priyadharsini 2019; Ezhilarasan, Apoorva, and Ashok Vardhan 2019; Ramesh et al. 2018; Mathew et al. 2020; Sridharan et al. 2019; Pc, Marimuthu, and Devadoss 2018; Ramadurai et al. 2019). We hope this study adds to this rich legacy.

The major limitation of our study is that these genes and pathways need to be confirmed by wet lab techniques such as western blot and RT-PCR before they can be clinically applied. Our study provides in-silico evidence for designing the novel marker and lead compound for diagnosis and treatment of CAA by targeting the potential Hub genes, including DUSP5, FOS, EGR, FOXO, HB-EGF genes. Our future scope would be to confirm these genes using wet lab techniques and to apply them in a animal model to confirm our results.

5. Conclusion

Cholangiocarcinoma (CAA), is a biliary malignant type of cancer that is caused by epigenetic and genetic modulation due to inflammatory reactions that involve cell injury of epithelial cells in the biliary duct. Hypermethylation of SOX17 promoters can result in cancer. In this study, we compared the NHC and CAA cells which are regulated by the activity of the SOX17 gene. 23 genes were commonly involved and the functional inter relationship among all common genes shows that almost 17 genes were present in the nucleus of liver, MCODE analysis provided the 5 highly interacted DEGS (HUB genes), which are mostly involved in Transcription factor activity. This study provides

a path for discovering the novel gene expression markers as well as novel treatment targets by designing the pot for the Cholangiocarcinoma may potentially use an identified Hub. However, further studies are needed for further verification of this Hypothesis.

Declarations

Conflict of Interest

The authors of this paper declare no conflict of interest.

Author Contribution

Author LR was involved in data collection, data analysis, manuscript writing. Author MD was involved in conceptualization, guidance and critical review of manuscript.

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