Chapter VI

Understanding the rewiring patterns of transcriptional regulation in GBC pathogenesis and identifying the crucial transcription factors involved in GBC development.

6.1 Introduction

The human genome encodes over 2000 different transcription factors (TFs), many of which are expressed in a cell type-specific manner regulating the gene expression programs that are involved with the vast array of cellular processes [1]. TFs are proteins that bind DNA helix at specific regulatory sequences to regulate transcription either through a transactivation or trans-repression domain. The TFs are organized in different families reflecting homologies in their DNA-binding domains and, consequently, DNA-binding sequences [1-2]. Vaquerizas et al., (2009) identified 164 transcription factors (~12%) directly involved in about 277 diseases [3]. In cancer cells, the genes encoding TFs are often dysregulated or mutated which results in a gain or loss of function [4]. TF activity is also altered indirectly through non-coding DNA mutations that affect transcription factor binding [1]. For example; *TP*53 and *MYC*, which encode the TFs- p53 and c-Myc, are among the most commonly altered genes across all cancer types [5-6]. Furthermore, many oncogenic signal transduction cascades alter the function of downstream TFs to implement gene expression changes that drive cell transformation [2,4]. Many transcription factors have been identified to be oncogenic. The oncogenic TFs are either altered through fusion with other proteins or through deregulated expression.[7]. TFs play chief roles in many signaling pathways by regulating normal cellular processes, such as cell growth and proliferation, metabolism, apoptosis, immune responses, and differentiation [8]. Their activity is frequently deregulated in cancer. The activity of TFs is found to be frequently altered in cancer and therefore targeting TFs is a major focus of interest in cancer research.

TFs are known to be significantly associated with cancer hallmarks. It holds substantial importance in cancer, particularly in processes such as epithelial-mesenchymal transition (EMT), a critical driver of cancer progression that results in cancer metastasis [9]. TFs associated with the EMT function as master molecular switches in regulating gene expression and impacting therapy resistance by altering drug transporter expression [12]. Metastasis-associated TFs have been linked to clinicopathology and patient prognosis in GBC, influencing overall survival [11]. They regulate not only migration and invasion but also impact cancer stem-cell characteristics, block oncogene-induced senescence, and suppress the immune system, ultimately promoting tumor metastasis [10]. Understanding these TFs associated with cancer holds promise for targeted therapies to prevent and overcome resistance to cancer treatments.

Therefore, this chapter aims to identify the potential regulatory TFs and their related pathological pathways involved in GBC and GBC+GS pathogenesis using transcriptional regulatory network analysis. The methodology workflow for the identification of potential TFs in GBC pathogenesis is presented in **Figure 6.1**.

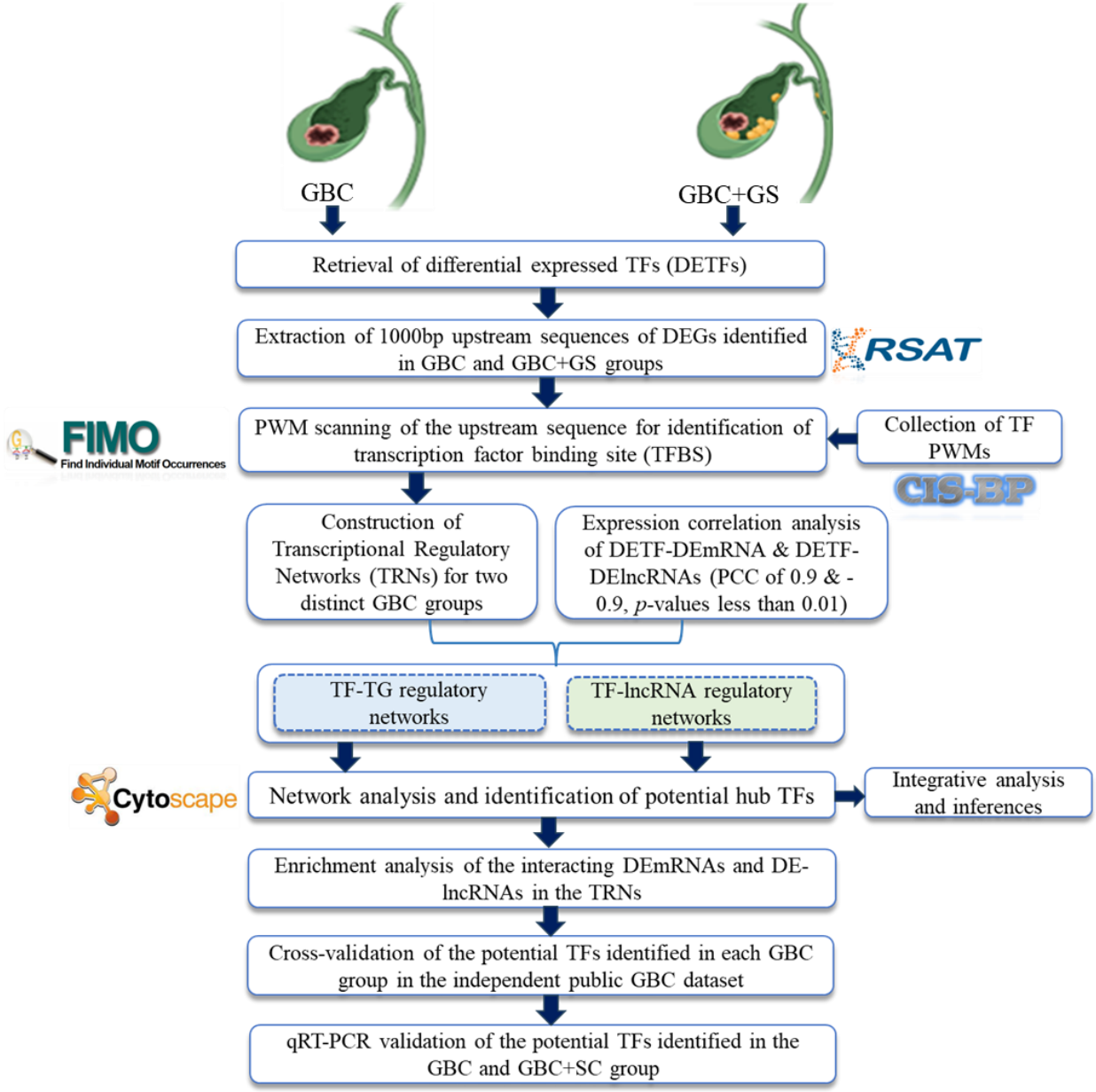


Figure 6.1: The overall schematic workflow of transcriptional regulatory network analysis and identification of potential TFs in GBC pathogenesis.

6.2 Results

6.2.1: Identification of differentially expressed TFs (DETFs)

DETFs in both the GBC and GBC+GS cases were identified by intersecting the entire set of TFs identified in humans with the significant DEGs identified in each GBC group. A total of 76 DETFs were identified in GBC cases, and 82 were identified in GBC+GS cases [**Figure 6.2 A**]. In both groups of GBC patients, higher counts of downregulated DETFs were identified as compared to upregulated ones. Moreover, the complete linkage hierarchical clustering analysis of DETFs revealed distinct expression patterns between the groups of GBC cases and control cases [**Figure 6.2 B**]. This indicates that the expression of TFs is dysregulated in GBC conditions, implying their significant involvement in the pathogenesis and development of GBC.

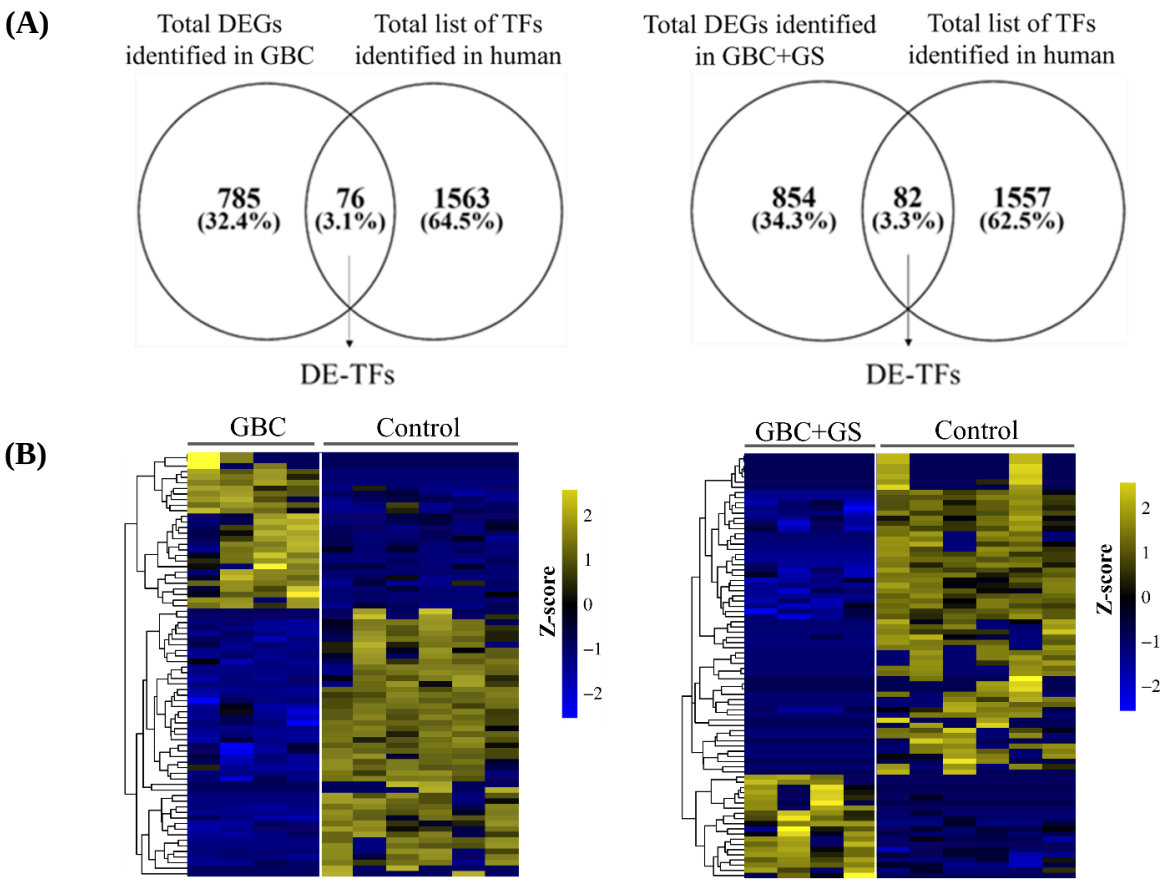


Figure 6.2: Identification of differentially expressed TFs. (A) Venn diagram representing the number of DETFs identified in GBC and GBC+GS cases respectively. **(B)** Expression heatmap of the DETFs identified in GBC and GBC+GS cases compared to controls. The heatmaps were plotted using the Zscore.

6.2.2 Functional pathway enrichment of the DETFs identified in GBC and GBC+GS groups

Pathway enrichment analysis was performed using the DETFs identified in each GBC group and the top five significantly enriched KEGG pathways and molecular signature hallmark pathways were identified. In the GBC group, the DETFs showed significant associations with cancer-related pathways, including Herpes simplex virus 1 infection, Kaposi sarcoma-associated herpes virus infection, breast cancer, and signaling pathways related to stem cell pluripotency. Signature hallmark pathways associated with DETFs in the GBC group primarily involves immune signaling pathways like TNF-alpha signaling, interferon alpha and gamma response, and Wnt-beta signaling, recognized as crucial pathways in cancer development [**Figure 6.3 A-B**]. In the GBC+GS group, the identified DETFs were found to be significantly enriched in pathways commonly implicated in cancer, such as transcriptional misregulation in cancer, cell cycle regulation, Epstein-Barr virus infection, and generalized pathways in cancer development. The significant hallmark pathways found to be associated with DETF in the GBC+GS groups are TNF-alpha signaling, UV response upregulation, hypoxia responses, and mechanisms regulating cholesterol homeostasis. Interestingly, the TNF-alpha signaling pathway and Herpes simplex virus 1 infection emerged as common and highly significant pathways in both the GBC and GBC+GS groups, demonstrating consistency in their association across two distinct GBC groups [**Figure 6.3 C-D**].

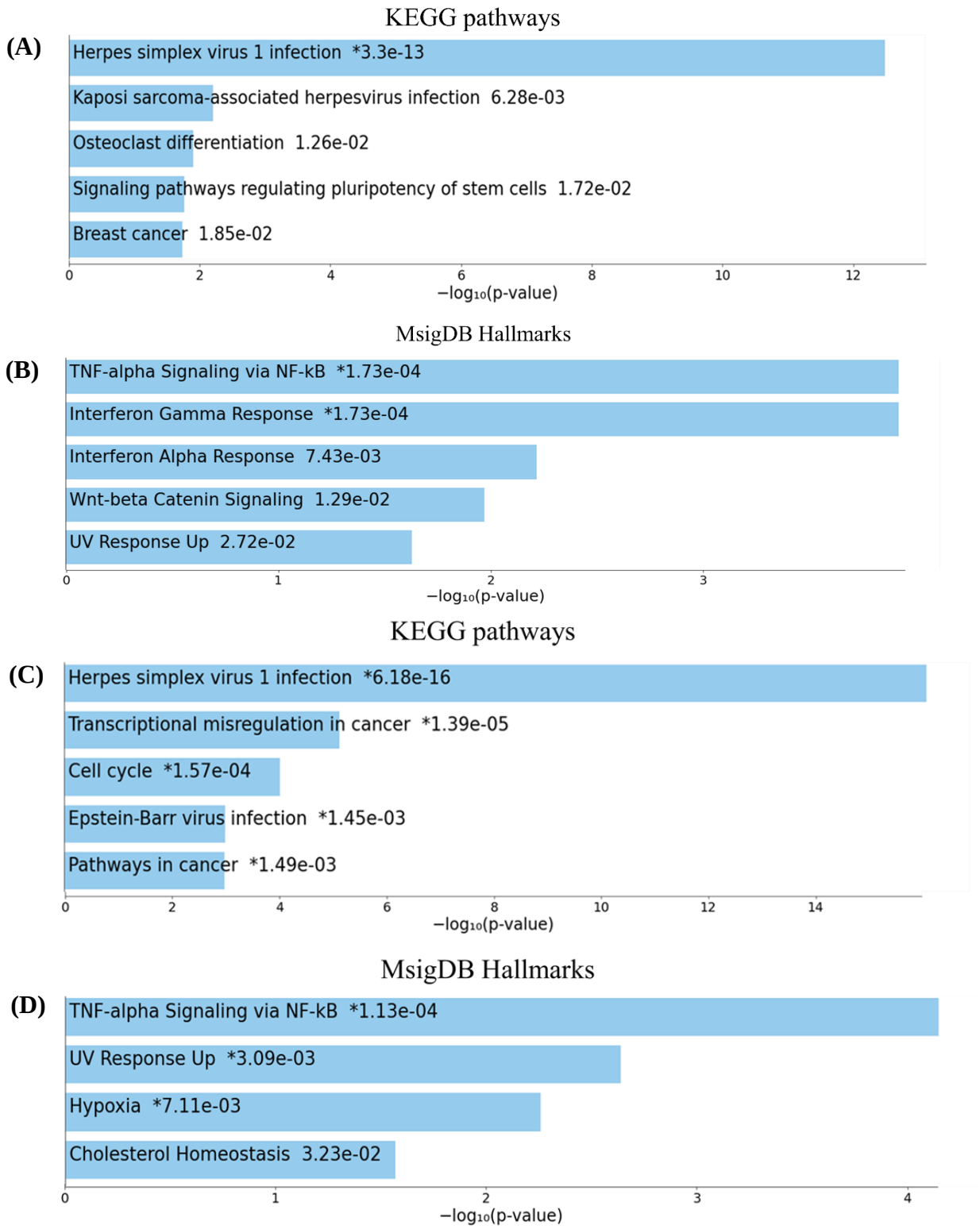


Figure 6.3: Identification of significant pathways involved with DETFs identified in GBC and GBC+GS groups. Bar plot showing the top five significantly enriched KEGG and hallmark pathways associated with DETFs identified in GBC **(A-B)** and GBC+GS **(C-D)** groups respectively. The X-axis and Y-axis represent the p-values and the significant KEGG and molecular signature hallmark pathways respectively.

6.2.3. Construction of Transcription Factor-Target Gene (TF-TG) and TF-lncRNA regulatory networks and identification of potential hub TFs in GBC and GBC+GS groups.

TF-TG and TF-lncRNA regulatory networks were constructed in GBC and GBC+GS groups using the DEGs (TG) and DElncRNAs identified in Chapter 4 (4.2.4) and Chapter 5 respectively. The TFs are considered as source nodes and their target genes and target lncRNAs as target nodes for constructing the transcriptional regulatory networks. The top ten highly interacted potential regulatory TFs were identified based on degree centrality measures from TF-TG and TF-lncRNA networks of the two GBC groups [**Figure 6.4**]. In the GBC group, out of ten hub TFs identified in the TF-TG and TF-lnc regulatory network, nine TFs- *LEF1*, *BACH1*, *KLF15*, *MZF1*, *ZFP82*, *SP3*, *NR2F1*, *IRF3*, and *ZNF880* were found to interact commonly with both DEGs and DElncRNAs. However, in the GBC+GS group, only 4 hub TFs- *ZNF580*, *TGIF1*, *MECOM*, and *MAFG* were found to be commonly interacting with both target genes and target lncRNAs.

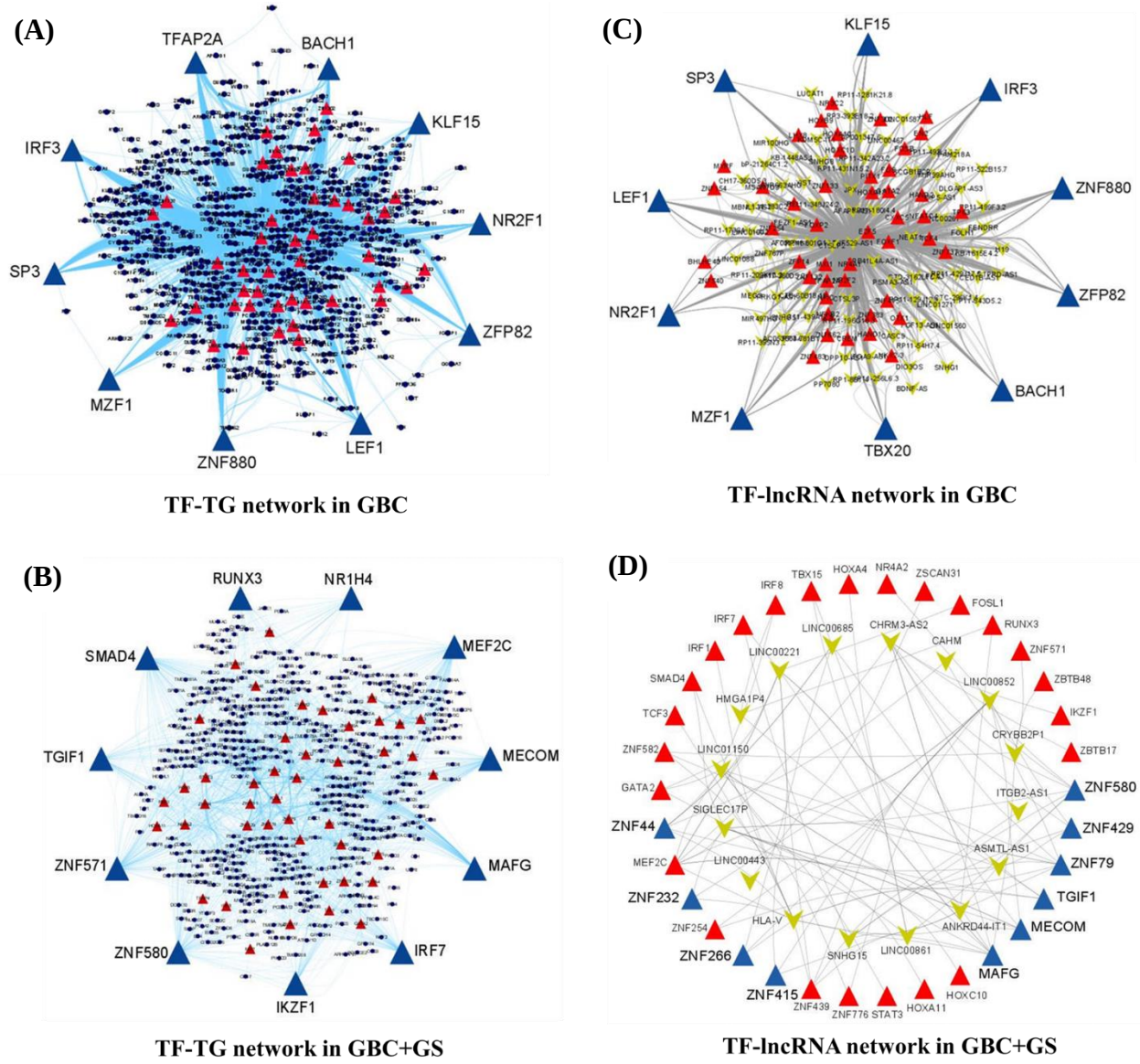


Figure 6.4: Transcriptional regulatory networks construction and identification of hub TFs. (A-B) Construction of TF-TG regulatory network and identification of hub TFs in GBC and GBC+GS group respectively. **(C-D)** Construction of TF-lncRNA regulatory network and identification of hub TFs in GBC and GBC+GS group respectively**.** The blue triangular nodes represent the hub TFs identified using the highest degree of centrality. The red triangles and V-shaped nodes represent the DETFs and DElncRNAs respectively.

The analysis of transcriptional regulatory networks in two distinct groups of GBC revealed that the interaction between TFs and their target DEGs and DElncRNAs were considerably more pronounced in the GBC group compared to the GBC+GS group. Specifically, within the GBC group, the DETFs exhibited a significantly higher level of

interaction, with 463 having the highest degree of interaction in the TF-TG network and 66 in the TF-lncRNA network [**Table 6.1**]. In contrast, the highest degree of hub DETFs in the TF-TG and TF-lncRNA networks within the GBC+GS group were 66 and 18, respectively [**Table 6.2**]. This discrepancy highlights the significant role played by TFs in the pathogenesis of GBC cases without gallstones.

Table 6.1: List of top ten hub DETFs identified from TF-TG and TF-lncRNA regulatory networks in the GBC group. The asterisk symbol indicates the shared DETFs identified in both TF-TG and TF-lncRNA networks.

Hub DETFs	Degree	Average path length	Topological coefficient
Hub DETFs identified from TF-TG regulatory network			
TFAP2A	416	2.155	0.488
**KLF15*	463	2.101	0.46
**NR2F1*	389	2.185	0.491
**ZFP82*	401	2.185	0.448
**LEF1*	415	2.185	0.502
**BACH1*	414	2.185	0.478
**SP3*	427	2.185	0.481
**IRF3*	409	2.185	0.484
**ZNF880*	265	2.185	0.46
**MZF1*	435	2.185	0.491
Hub DETFs identified from TF-lncRNA regulatory network			
**IRF3*	66	1.625	0.413
**KLF15*	63	1.683	0.402
**ZNF880*	62	1.698	0.417
**ZFP82*	59	1.727	0.423
**SP3*	58	1.757	0.4
**LEF1*	54	1.801	0.417
**NR2F1*	54	1.816	0.418
**MZF1*	53	1.83	0.425
TBX20	53	1.816	0.422
**BACH1*	51	1.845	0.422

Table 6.2: List of top ten hub DETFs identified from TF-TG and TF-lncRNA regulatory networks in the GBC+GS group. The asterisk symbol indicates the shared DETFs identified in both TF-TG and TF-lncRNA networks.

Hub DETFs	Degree	Average path length	Topological coefficient
Hub DETFs identified from TF-TG regulatory network			
ZNF571	101	2.236	0.036
**ZNF580*	99	2.454	0.044
**MAFG*	94	2.314	0.042
**MEF2C*	90	2.45	0.049
IRF7	67	2.52	0.048
**MECOM*	65	2.411	0.052
**TGIF1*	55	2.341	0.053
IKZF1	42	2.899	0.12
NR1H4	29	3.207	0.093
RUNX3	28	2.674	0.076
Hub DETFs identified from TF-lncRNA regulatory network			
**MEF2C*	18	2.063	0.275
ZNF79	15	2.148	0.299
**MAFG*	15	2.063	0.222
**ZNF580*	13	2.361	0.244
ZNF44	12	2.489	0.318
**MECOM*	11	2.319	0.346
ZNF429	7	2.829	0.39
**TGIF1*	6	2.659	0.437
ZNF415	6	2.787	0.5
ZNF266	6	2.744	0.491

6.2.4: Pathway enrichment analysis of the DEGs and DElncRNAs targeted by the hub TFs identified from the GBC and GBC+GS networks.

Analysis of the target DEGs targeted by hub TFs revealed involvement of specific biological pathways in the GBC and GBC+GS groups. In GBC, these targeted mRNAs are significantly linked to immune response pathways like IL-17 signaling, cytokine-cytokine receptor signaling, TNF signaling, and chemokines. The strong connection between these hub TFs and immune responses suggests their potential role in GBC progression, proposing a potential avenue for targeted immunotherapy [**Figure 6.5 A**]. In contrast, hub TFs identified in GBC+GS group are associated with diverse pathways, primarily metabolic pathways such as progesterone-mediated oocyte maturation, N-glycan biosynthesis, and lipid metabolism. Alongside metabolic pathways, these hub TFs are also involved in immune response processes

such as Human T-cell leukemia virus 1 infection and primary immunodeficiency [Figure 6.5B]. However, the lncRNAs targeted by hub TFs in both GBC and GBC+GS groups do not exhibit significant associations with pathways documented in the KEGG and Reactome databases.

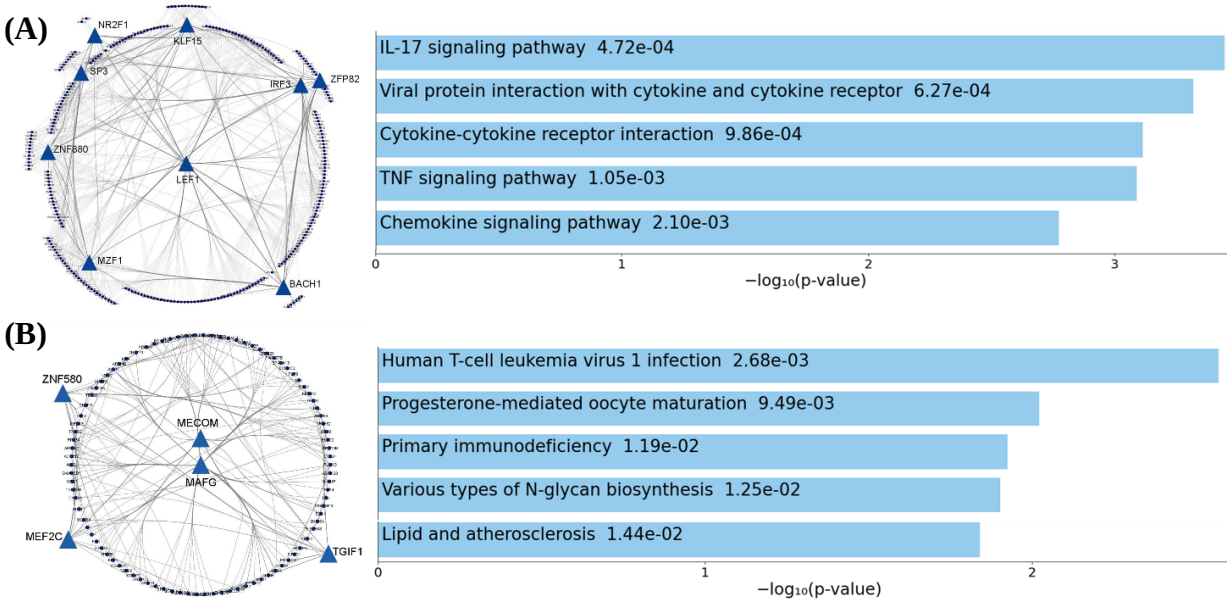


Figure 6.5: Pathway enrichment of the target genes identified in TF-TG regulatory networks. The bar plot (right panel) represents the top five identified significant (p-value < 0.05) KEGG pathways associated with hub DETFs identified from **(A)** GBC and **(B)** GBC+GS regulatory networks (left panel).

Furthermore, the analysis of the DElncRNAs in LncACTdb database identified to be interacting with the hub TFs in both the GBC and GBC+GS groups are involved in important cancer hallmark processes. In the GBC group, the majority of the DElncRNAs are associated with cell growth, apoptosis, EMT, and metastasis [**Figure 6.6 A**]. Whereas; the target DElncRNAs in GBC+GS groups are majorly associated with apoptosis and metastasis processes [**Figure 6.6 B**]. This suggests that the hub TFs regulate or interact with DElncRNAs linked to important hallmark biological processes. Therefore, dysregulations of the DElncRNAs by TFs may contribute to the onset and advancement of GBC.

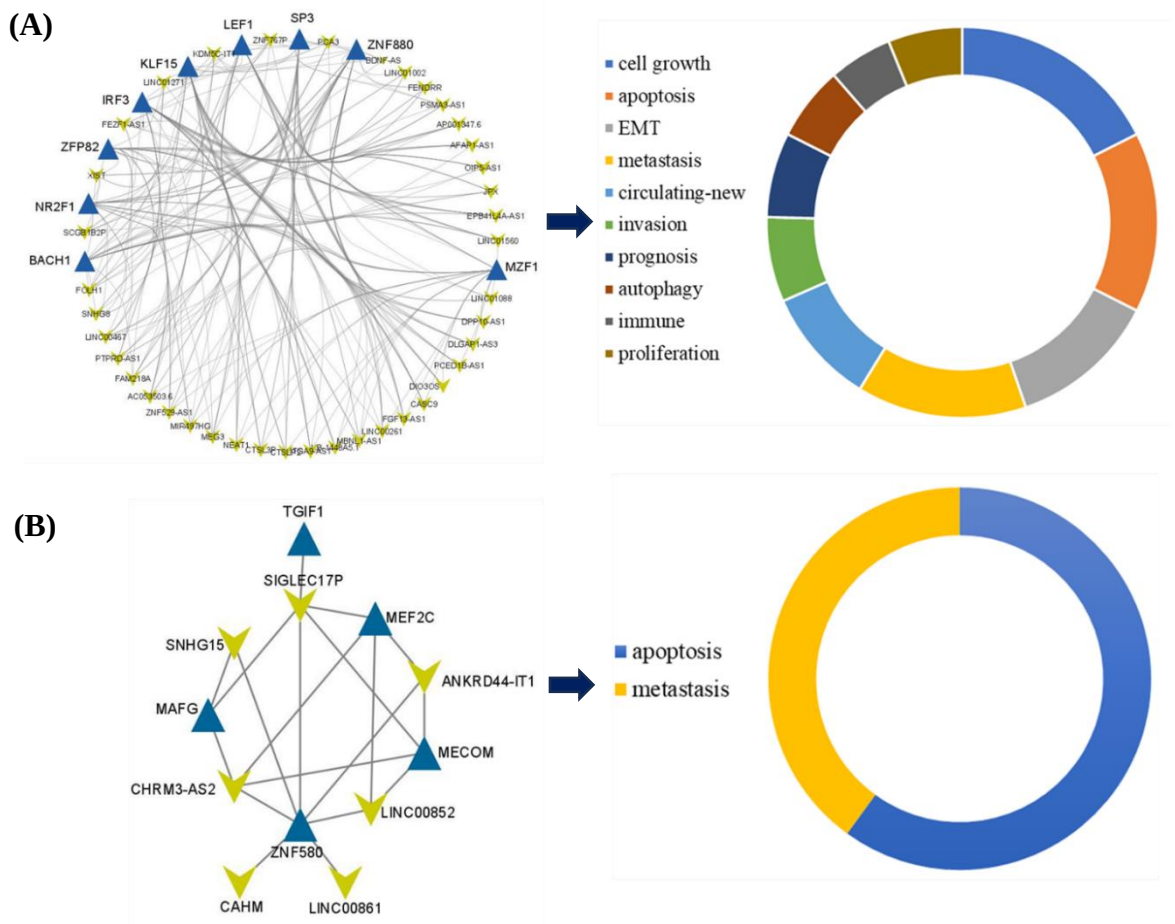


Figure 6.6: Enriched cancer hallmark processes associated with DElncRNAs identified in TF-lncRNA networks. The pie chart showing the enriched cancer hallmarks linked to DElncRNA targeted by hub TFs identified in the GBC **(A)** and GBC+GS **(B)** networks.

6.2.5: Cross-validation of the identified potential DETFs in independent publicly available GBC transcriptomic dataset and TCGA datasets of gastrointestinal cancers.

The public GBC transcriptome dataset (Accession ID: GSE139682) analyzed in the case 2 study in chapter 4 (as referred to in 4.2.2) has been further considered as an independent GBC dataset to cross-validate the DETFs identified from in-house generated GBC transcriptome dataset. Out of the 2980 significant DEGs identified in GBC as compared to adjacent normal, 164 DEGs code for transcription factors (TFs). Considering these DETFs, the transcriptional regulatory network was constructed [**Figure 6.7 A**]. Based on the degree centrality, the hub TFs were identified in GBC which includes *PAX6, KLF15, NR2F1, TFAP2C, FOXJ2*, and FLR.

Further, three distinct algorithms – 76 gene signatures (76GS), Kolmogorov Smirnov (KS) test, and the multinomial logistic regression (MLR) were employed to assess the extent of epithelial-mesenchymal transition (EMT) in transcriptomic data [**Figure 6.7 B**]. Higher scores from KS and MLR algorithms indicated a more mesenchymal state, whereas a high 76GS score pointed towards a more epithelial phenotype. The levels of *KLF15* and *NR2F1* were linked to a more mesenchymal state, showing a positive correlation with KS and MLR scores and a negative correlation with 76GS scores. Similarly, *FOXJ2* displayed similar trends, although without statistical significance. Whereas, *FLR*, *PAX6*, and *TFAP2C* were associated with an epithelial state, exhibiting a positive correlation with 76GS scores and a negative correlation with KS and MLR scores. This distinction suggested that the six identified hub TFs in GBC were differentially associated with epithelial and mesenchymal statuses. They appeared to form two distinct groups: one group promoting EMT, while the other set acted to inhibit EMT. The analysis of the transcriptional regulatory network revealed that *KLF15* emerged as a potential hub TF in both public and in-house GBC transcriptome datasets and is significantly associated with the EMT. This suggests that *KLF15* is a crucial TF involved in the pathogenesis and metastatic progression of GBC cases without gallstones.

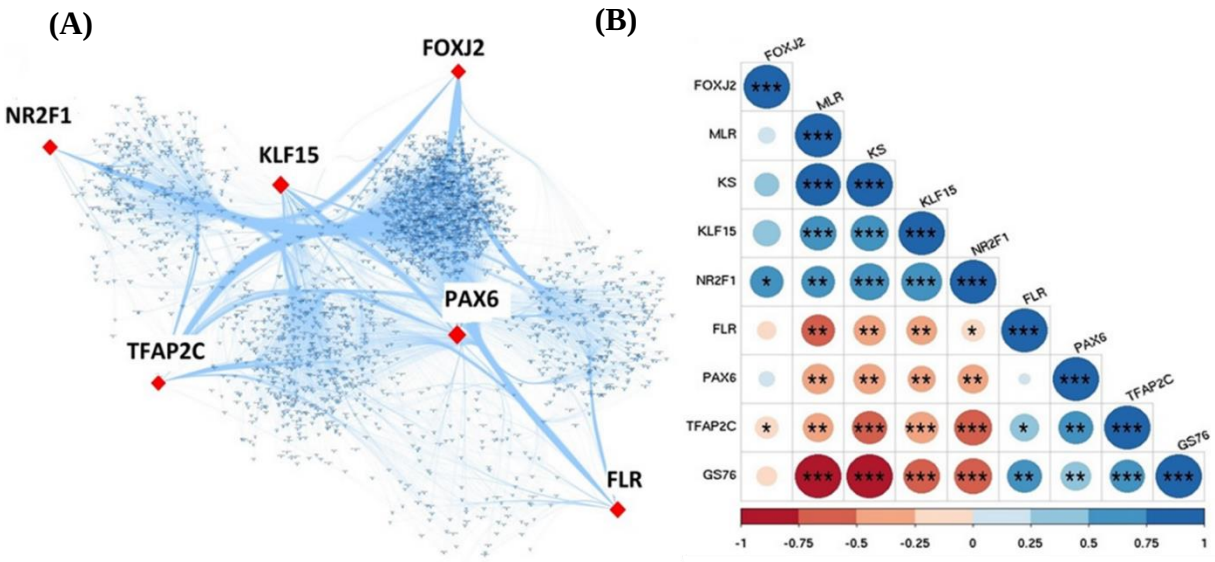
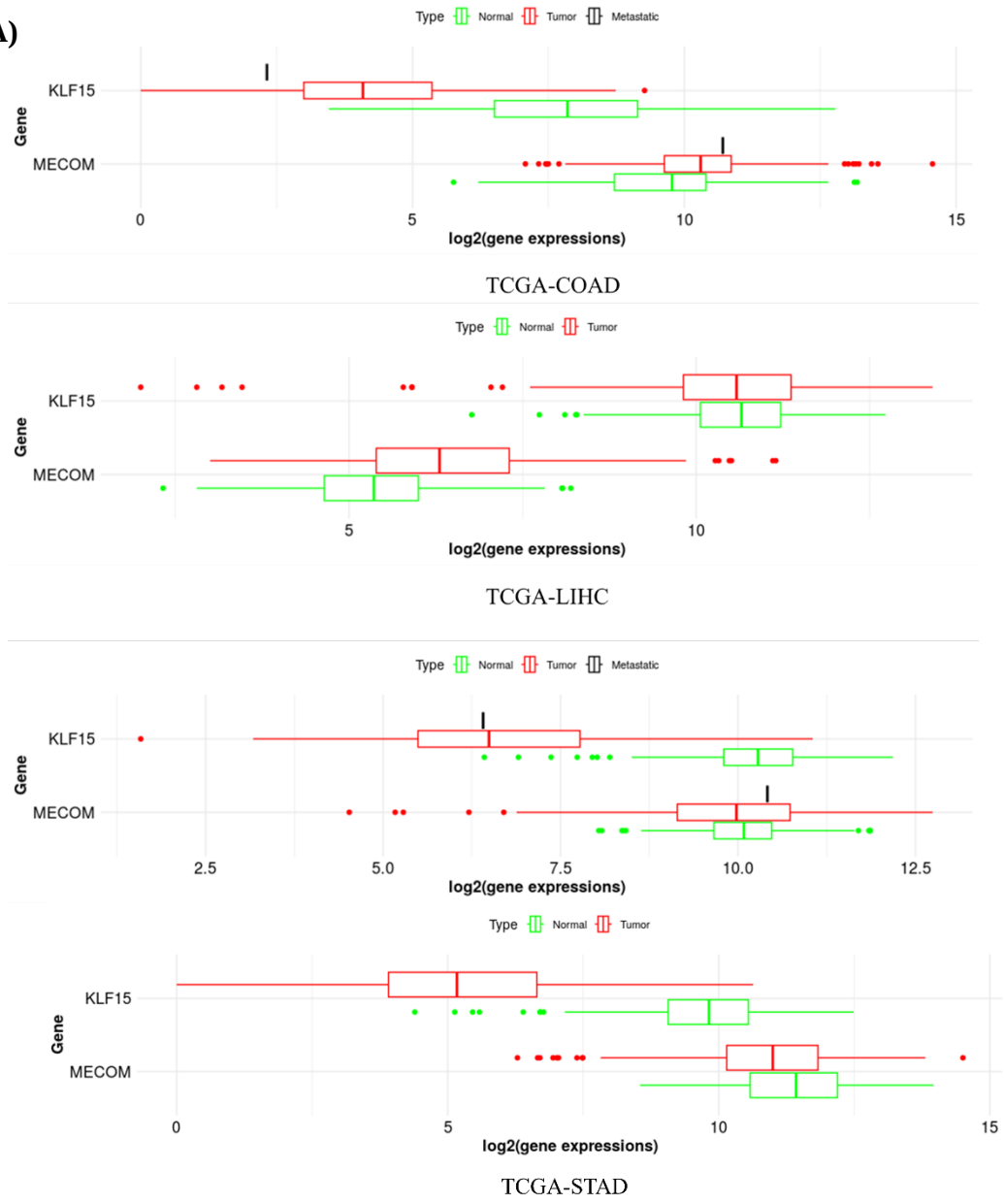


Figure 6.7: Construction of a transcriptional regulatory network of DETFs identified in GBC compared to normal. **(A)** Identification of hub TFs in GBC based on degree centrality. The red node represents the top hub TFs and the small blue nodes represent target genes. **(B)** Pairwise correlation of EMT score of hub TFs identified through TF-TG interactions. The significance of each hub TF is represented with a symbol- *p*-value < 0.001 (***); *p*-value < 0.01 (**) and *p*-value < 0.05 (*).

To validate the expression the identified hub TFs in other gastrointestinal cancers, the potentials TFs- KLF15 and MECOM were considered for further analysis. The expression of KLF15 and MECOM were evaluated in four TCGA datasets- TCGA-COAD, TCGA-LIHC, TCGA-PAAD, and TCGA-STAD [**Figure 6.8 A**] and it was found that the trend of the expression level of *KLF15* (downregulated) and *MECOM* (upregulated) is similar in GBC and other cancers of the gastrointestinal tract. Next, the mutational profile of *KLF15* and *MECOM*, identified from GBC and GBC+GS TRN analysis was further validated in TCGA datasets using the oncoprint tool. The analysis revealed that *KLF15* experiences mutations and deep deletions in a considerable portion of cancer samples, while *MECOM* primarily undergoes amplification [**Figure 6.8 B**].

(A)



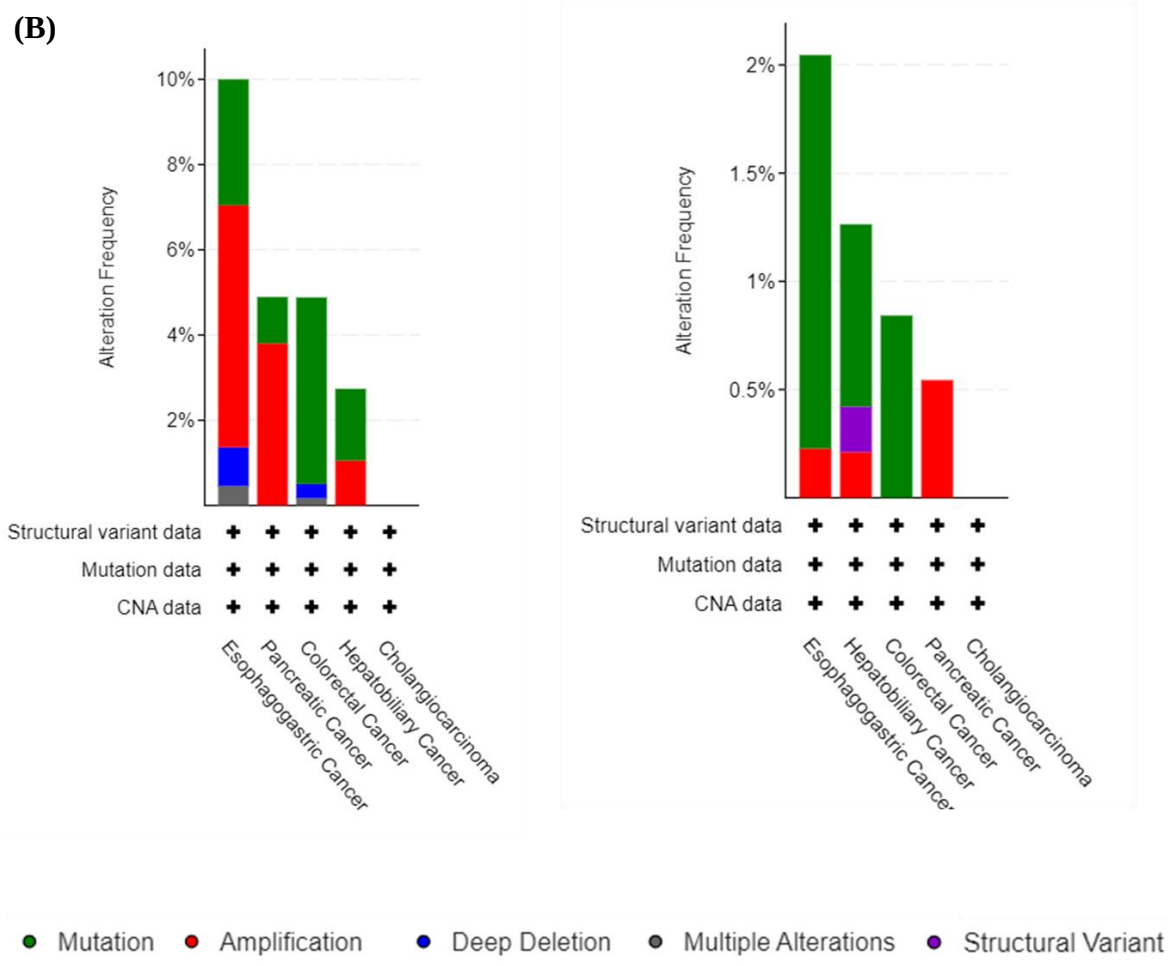


Figure 6.8: Validation of *KLF15* and *MECOM* expression in TCGA datasets. (A) Box plot showing the expression level (log2foldchange) of *KLF15* and *MECOM* in four different TCGA datasets of gastrointestinal cancers. **(B)** Bar plot representing the mutational profile of KLF15 and MECOM in five different cancers of the gastrointestinal tract.

6.2.6: Validation of the selected hub TFs through qRT-PCR

The expression of the hub TFs was further identified in GBC and GBC+GS groups and was validated through qRT-PCR. qRT-PCR analysis showed the expression of *KLF15* and *MECOM* identified in the GBC and GBC+GS group respectively correlated with the expression level identified through RNAseq data analysis. The expression of *KLF15* is significantly downregulated whereas, *MECOM* showed increased expression in GBC as compared to control. The Δ^{ct} and $2^{-\Delta\Delta ct}$ methods were used to analyze the qRT-PCR data, where Δ^{ct} and $2^{-\Delta\Delta ct}$ represent the sample's expression and relative expression of the target genes respectively [**Figure 6.9**].

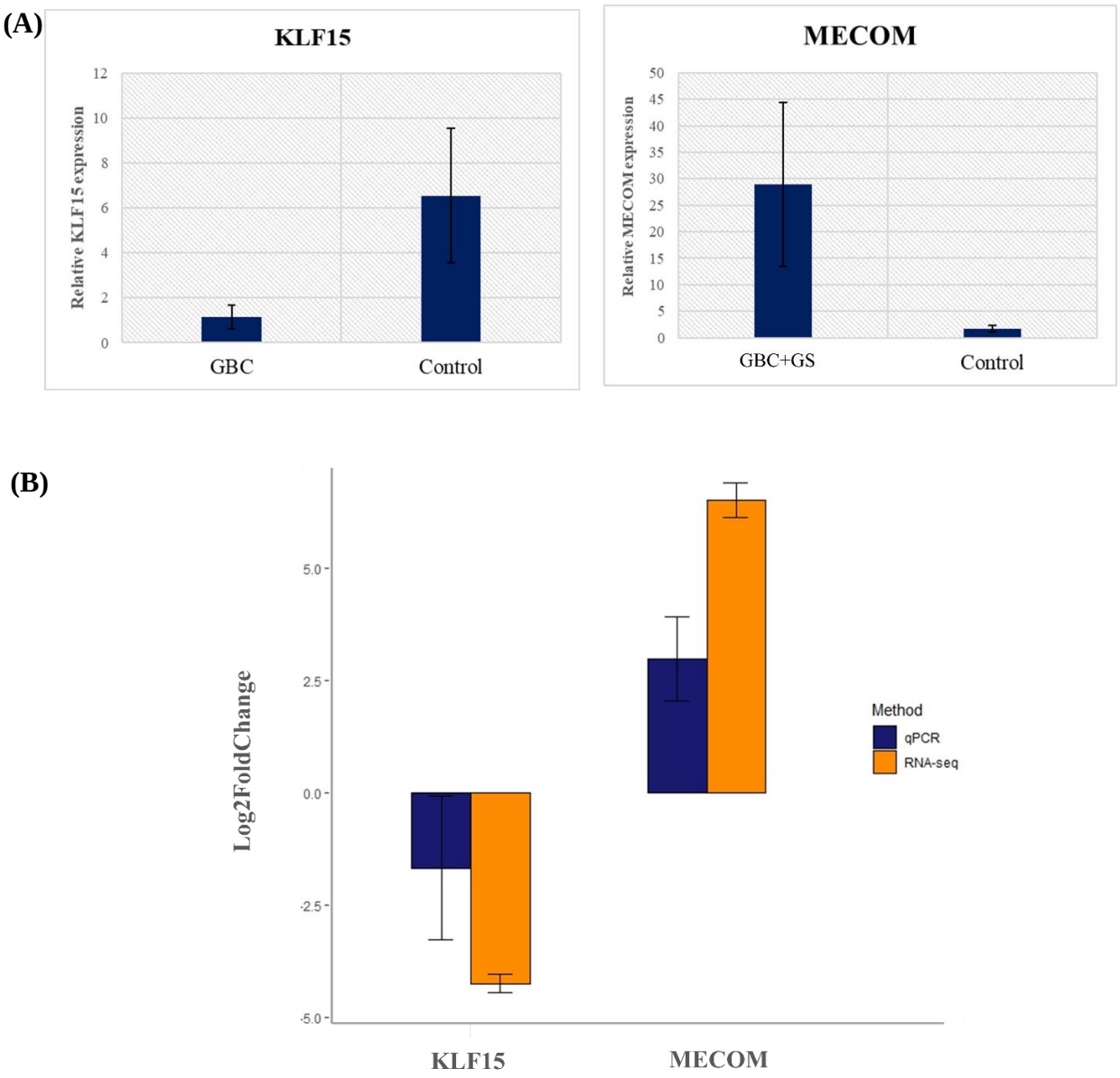


Figure 6.9: qRT-PCR validation of the hub lncRNAs identified in the GBC and GBC+GS group. (A) Bar plot representing the relative expression of *KLF15* and *MECOM* TFs identified using qRT-PCR data analysis in GBC and GBC+GS group compared to control. **(B)** Bar plot showing the gene expression level (log2FoldChange) of *KLF15* and *MECOM* identified through RNAseq and qRT-PCR.

6.3 Discussions

It is well-established that transcription factors serve as master regulators in both embryonic development and adult homeostasis. They are intricately regulated by cell signaling pathways through transient protein interactions and modifications [13-14]. TFs regulate diverse biological processes that are crucial for maintaining cellular homeostasis. They represent nearly 20% of identified oncogenes and hold promise as potential targets for cancer treatment [15-17]. TFs are known to interact with multiple regulatory domains of other TFs, ancillary factors, and chromatin regulators reversibly and dynamically to elicit a particular cellular response [18]. GBC patients have one of the worst survival outcomes, with 5-year survival rates ranging from 10 to 20 %. TFs play an important role in GBC metastasis and are reported to be significantly linked to the pathophysiology and prognosis of GBC, affecting the overall survival of GBC patients [11]. Therefore, there is an urgent need to develop novel and effective therapeutic regimens for GBC patients. Identification of potential TFs will unravel the intricate regulatory networks and mechanisms controlling gene expressions in GBC tumorigenesis and development.

This chapter investigates the potential DETFs identified in two GBC groups- GBC and GBC+GS through the construction of transcriptional regulatory networks (TRNs). TF-TG and TF-lncRNAs regulatory networks were constructed and the top ten highly interacting TFs were identified in GBC and GBC+GS groups based on degree centrality measure. The enrichment analysis of the DETFs revealed that the TNF-alpha signaling pathway and Herpes simplex virus 1 infection were significantly enriched as shared pathways linked to the identified TFs in both the GBC and GBC+GS groups. TNF-α, a potent cytokine, plays a crucial role in both innate and adaptive immunity. Its significant role in inflammation-related cancers is well-established. Emerging evidence suggests the association of TNF-alpha in the development and advancement of both experimental and human cancers, primarily through pathways that activate NF-κB and AP-1 transcription factor complexes within cells [19]. TNF-α signaling triggers various cellular responses such as inflammation, cell proliferation, and apoptosis [20-21] and acts as a master switch between inflammation and cancer. Another common pathway identified to be associated with DETFs in both GBC and GBC+GS groups is the herpes simplex 1 (HSV1) infection pathway. The association of *HSV1* infection with carcinogenesis has been long known. Herpesviruses such as human cytomegalovirus have been found to adversely influence surrounding cells, for example, by inhibiting innate immunity and lengthening

mitotic arrest [22]. HSV1 utilizes the host-cell Ras signaling pathway during infection and might contribute to increased susceptibility of cancer cells with an activated Ras signaling pathway to HSV infection [23].

Among the hub DETFs identified from transcriptional regulatory networks, hub DETFs -*KLF15* and *MECOM* identified in the GBC and GBC+GS groups respectively were considered as potential DETFs and were for further validated through qRT-PCR analysis. This is the first study that reported the association of *KLF15* and *MECOM* in GBC and GBC+GS pathogenesis respectively. Kruppel-like factors (KLFs) encompass a group of transcription factors characterized by three distinct C2H2-type zinc finger domains in their carboxy-terminal regions, critical for DNA binding and nuclear localization [24]. These KLFs regulate genes governing diverse biological functions like cell proliferation, differentiation, and apoptosis, impacting both normal homeostasis and pathological conditions [24-25]. KLFs demonstrate dual roles in cancer biology, acting as potential tumor suppressors by inhibiting cell proliferation, and migration, and prompting cell death, while some within this family promote oncogenesis. Their involvement in driving tumor progression is well-documented in cancer research [26-28]. Among these, *KLF15*, also known as kidney-enriched KLF (KKLF), exhibits widespread expression across tissues including the kidney, liver, heart, adipose, and skeletal muscle. Despite its prevalence in various tissues, its role in human cancers has been relatively understudied [29]. Recent in vitro investigations indicate that *KLF15* displays anti-proliferative effects on carcinoma cells found in the pancreas, endometrium, and breast [29-31]. A recent study revealed that in gastric cancer (GC) tissues, the expression levels of *KLF15* were significantly downregulated in GC tissues compared to adjacent normal tissues. Overexpression of *KLF15* was observed to inhibit cell proliferation by regulating CDKN1A/p21 and CDKN1C/p57 [32].

MECOM, the MDS1 and EVI1 complex locus protein identified in the GBC+GS group is a nuclear transcription factor with zinc finger properties. It plays a crucial role in various cellular pathways, such as cell cycle regulation, proliferation, and cell differentiation [33]. Initially, *MECOM* was identified as a proto-oncogene and has been associated with antiapoptotic effects by hindering JNK1-mediated c-Jun phosphorylation [34]. Its involvement in myeloproliferative neoplasms, particularly in leukemia, has been extensively studied for its oncogenic and prognostic implications [35]. However recent studies reported the involvement of *MECOM* in solid cancer progression, including breast cancer, ovarian cancer, and

glioblastoma multiforme [36-38]. Acting as a transcriptional factor, *MECOM* regulates the expression of several target genes by binding DNA through its zinc finger domain [39].

This study showcased that the two GBC subgroups were associated with distinct sets of transcription factors, which further highlights the molecular heterogeneity and complexity of GBC. Variations in the transcription regulatory networks between the two GBC subtypes can contribute to differences in tumor biology, disease progression, and response to treatment.

6.4 Summary

In the previous chapters, we have identified differentially regulated mRNAs and lncRNAs involved in GBC and GBC+GS groups. This chapter unravels potential transcriptional rewiring patterns in GBC with respect to gallstone status. *KLF15* and *MECOM* were identified as the crucial differentially regulated TFs in the GBC and GBC+GS groups that show strong interaction with both DEmRNAs and DElncRNAs. Therefore, targeting these transcriptions by modulating their activity or expression can offer new avenues for cancer treatment. Moreover, identifying these GBC subtype-specific transcription factors and their target genes can provide insights into potential therapeutic targets for each subtype. Targeting the unique regulatory mechanisms can contribute to personalized treatment strategies for GBC patients with and without gallstones.

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