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Identifying Angpt-4 As A Potential Gene in Progression of Hepatocellular Carcinoma Using Bioinformatics Tools and Analysis

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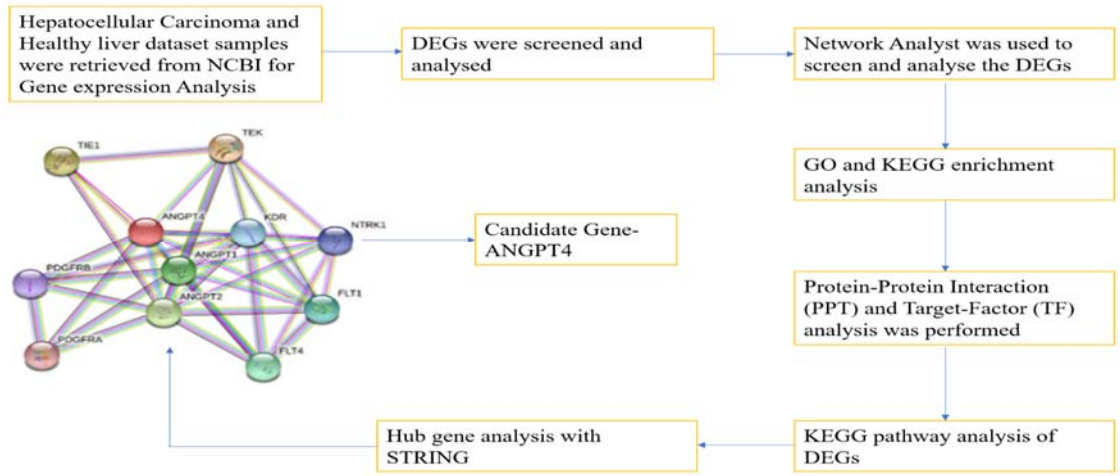
Graphical Abstract



Abstract

Hepatocellular carcinoma (HCC) is a highly lethal malignancy, and the mechanisms underlying its initiation, development, and progression remain poorly understood. To explore differentially expressed genes (DEGs) involved in HCC progression, we utilized the gene expression dataset GSE33006 from the GEO database on NCBI. This dataset includes expression profiles of 3 HCC tumor samples and 3 normal liver samples. To accomplish that, the following bioinformatics tools have been incorporated, and the downloaded data was analyzed with set-parameters Gene Ontology (GO) analysis, Protein-protein Interaction (PPI) network analysis, Target-factor (TF) gene Interactions, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway analysis; besides, STRING database was also used for enrichment and validation of genes. As a result, a total of 896 genes were seen as Up-regulated genes, and 368 genes were down-regulated. From the analysis among the Up-regulated genes, Angiopoietin 4 (ANGPT-4) was identified as one of the feasible candidate genes. The expression of ANGPT-4 shows results similar to past conducted research proving similarities between both data. This study provides insight into the prognosis of HCC and the possible gene ANGPT-4 along with the HIF-1α signaling pathway that is responsible for the progression of Hepatocellular carcinoma.

Keywords: Hepatocellular Carcinoma, Microarray data analysis, Differently expressed genes, Hub genes, Pathway analysis.

1. Introduction

Cancer is best characterized as an abnormal and uncontrolled growth of living cells that can originate in any organ or tissue of the body. These malignant cells have the potential to invade surrounding tissues and spread to other parts of the body through the bloodstream or lymphatic system, a process known as metastasis. (Mesleh., 2017). Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is a malignant tumor that develops in the liver. (McGlynn et al., 2021).

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The deaths related to Liver cancer account for about 830,000 worldwide and it is the third leading cause of death related to cancer, the first being lung and next colon and rectum cancers (Rumgay et al., 2022). The majority of liver cancer is due to the cause of Hepatocellular carcinoma, and minorly due to Cholangiocarcinoma (CAA) or also to the mixture of both CAA and HCC (Affo et al., 2017). Carcinogenic cell development involves, changes to gene expression, and usage of various regulatory pathways, therefore, the distinct etiology of HCC progression is vague (Yang WX et al., 2019). The diagnosis of HCC is strongly influenced by the stage of disease progression. At an early stage, curative therapeutic strategies such as surgical resection or liver transplantation are viable options, with reported 5-year survival rates approaching 70%. In contrast, patients diagnosed at an advanced stage are typically managed with palliative or systemic therapies, which offer limited benefits, with median survival generally less than 12 months (Singal et al., 2014); moreover, the majority of patients with early-stage HCC miss the appropriate treatment period as there are no early clinical symptoms shown by HCC (Zhang C et al., 2017). Normally, all HCCs emanate from chronic diseases such as NAFLD (non-alcoholic fatty liver disease) or hereditary hemochromatosis (HH), Chronic Hepatitis Virus (B & C), increase in corpulence, alcoholic liver disease (Justin Hartke, Matthew Johnson and Marwan Ghabril., 2017). Hepatocellular Carcinoma often originates from genetic mutations that modify the metabolic pathways consequently inducing the proliferation of cells (Chen X et al., 2020). Hepatitis C is a fatal virus as it is symptomless in the majority of patients and is prone to causing HCC and leading to death (Ringelhan et al., 2017). People infected with Hepatitis C have an increased risk of developing liver cirrhosis which is then followed up by fibrosis and thereby developing into HCC meantime (Sebastiani et al., 2014). Angiogenesis can be identified as a process where new leaky vessels develop from pre-existing blood vessels to transport oxygen and nutrients that are crucial and essential for HCC development. Angiogenesis is mainly initiated by various Vascular Endothelial Growth Factors (VEGFs) from cancerous cells (Dai et al., 2019; Jasani et al., 2024). The VEGF growth factor family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E; a majority of cancerous cells secretes VEGF-A and by interaction with tyrosine kinase receptors (RTK) namely VEGFR1 and VEGFR2 promotes angiogenesis, tumor cells over-expresses VEFG thereby promoting angiogenesis (Rafii et al., 2002). Increased VEGF levels in HCC have appeared to be in correspondence with the progression of angiogenesis by cancerous/tumor cells (Morse MA et al., 2018). In recent years, the usage of the bioinformatics-based approach has had a principal role in identifying and studying the expression levels of tumor genes and probing for further tumor key genes (Cao et al., 2023). Microarray technology is a well-advanced technique that has been widely used in the identification of abnormal genes and variations, and using this we have been able to identify the Differentially Expressed Genes (DEGs) and complex multiple pathways that were involved in the proliferation and progression of HCC. In this current study, we establish that HCC-associated DEGs when compared between cancerous and normal samples using the following techniques such as enrichment analysis, PPI and TF network analysis, and KEGG analysis to establish the pathway and genes associated with Hepatocellular carcinoma. As a result, a total of 896 genes were seen as Up-regulated genes, and 368 genes were down-regulated from this pool of genes; and Angiopoietin 4 was found as a significant candidate.

2. Materials and Methods

2.1. Microarray Data Acquisition

Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) (Edgar et al., 2002) database has a collective dataset of Microarray and Next-generation sequences. The hepatocellular carcinoma-associated microarray dataset with accession number GSE33006 (Huang et al., 2012) was downloaded from NCBI. The GSE33006 dataset altogether consists of 6 datasets, which include 3 normal liver datasets and 3 HCC-affected datasets that were obtained for our study. The dataset GSE33006 was established from GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array) (Chen et al., 2020). We have utilized GSE33006 dataset to analyze the genes.

2.2. Data Set normalization and analysis

The downloaded data were processed and analyzed with the help of the GSE33006 series matrix file, and the downloaded genes were checked for their gene symbol; a total of 45119 genes were obtained after pre-processing the datasets. To screen the DEGs, logFC (Fold change) ≥2 and logFC ≤2 were set as the benchmark criteria to discover desirable genes. After the filtration, a total of 2529 genes were obtained for logFC ≥2 and were termed Up-regulated genes, and for logFC ≤, 2 1087 genes were obtained and termed Down-regulated genes.

2.3. Differentially Expressed Gene's Enrichment Analysis

To identify potential DEGs, Network Analyst was utilized for this process and the gene's significance was analyzed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG), Reactome, Gene Ontology (GO) analysis that includes a biological process (BP), Molecular function (MF) and Cellular component (CC). To be statistically significant for enrichment, the threshold for the genes was adjusted to P-Value(using false discovery rate FDR) < 0.5 and log2 FC was adjusted to <1.0.

2.4. Protein-protein Interaction (PPI) network analysis

PPI Network analysis is an important and key step that helps in the identification of the key genes that were involved in the development of hepatocellular carcinoma. Network analyst integrated STRING interactome (Szklarczyk D et al., 2014) with a cut-off score of 900 was used for the construction and identification of the PPI network of DEGs. We used the Functional Explorer tool in the Network analyst to discover possible modules with DEGs.

2.5. Target-factor (TF) gene Interactions

Gene regulatory networks influenced TF-gene interaction was implemented to recognize DEGs in Network analyst, TF gene database combined with JASPAR (Khan A et al., 2018) was used to discover the Differently expressed genes.

3. Results

3.1. Identification of Differentially expressed genes (DEGs)

We processed our data GSE33006 from NCBI using Network analyst to obtain the required set of DEGs and by applying the cut-off criteria as P-value <0.5 and log2 FC <1.0, a total of 4167 Differentially Expressed Genes were identified, and out of this a total of 896 genes were identified as Up-regulated genes based on parameter logFC ≥2 and 368 genes were identified as Down-regulated genes on the parameter of logFC ≤2 from our bioinformatics analysis.

3.2. Analysis of DEGs

In the due process, we also obtained the significant genes from the Network analyst and we correlated the obtained significant genes with Up-regulated and Down-regulated DEGs that were identified based on the above-mentioned parameters. After the correlation, normal genes were eliminated leaving only the Up-regulated and Down-regulated genes along with their Gene Ontology (GO) terms. The Gene Ontology (GO) annotations encompass a variety of pathways, including key signaling pathways such as MAPK, FOXO, and HIF-1α. Additionally, GO terms cover several cancer types, including colorectal and lung cancers, as well as diseases like Fanconi anemia and chronic myeloid leukemia.

3.3. Gene Ontology Analysis & Enrichment

Gene Ontology analysis was done on the current dataset genes that include Up-regulated and Down-regulated genes, and gene ontology was performed to realize the features and biological implications of the targeted pathways. The gene ontology was categorized into three categories, which include Biological process (BP), Molecular function (MF), and Cellular component (CC). According to Network analysis, Viral reproductive process was significantly enriched in the Biological process, Enzyme binding was highly enriched in the Molecular function, and Cytosol was also highly enriched in the Cellular component. More comprehensive Gene Ontology enrichment analyses are shown in Figures 1 and 2. Further evaluation of HCC prognosis could be enhanced by examining the roles of other notable enriched terms and pathways involving differentially expressed genes

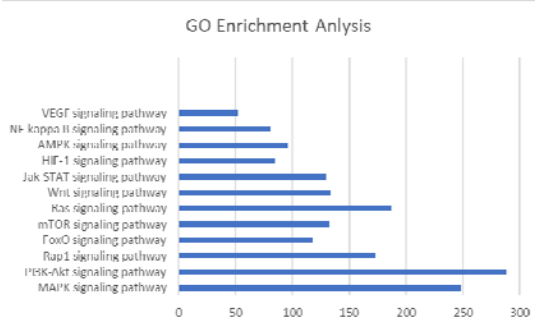

Figure 1. GO Enrichment analysis of top pathways related to our Differently expressed genes with logFC ≥2. It includes both Upregulated and Down-regulated genes.

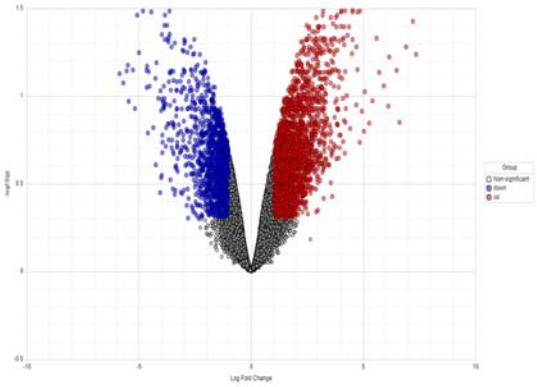

Figure 2. The Volcano plot. The log value fold change of significant genes of logFC ≥2 and logFC ≤2 were used as a parameter, here red colour denotes Up-regulated genes and blue colour denotes Down-regulated genes and the colourless genes are non-significant genes.

3.4. KEGG Pathway Analysis

We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) to analyze the presence of possible DEGs in a pathway, and the enrichment analysis revealed possible pathways related to our desired genes. The analysis revealed 5 possible pathways that show the presence of our shortlisted DEGs; they are the MAPK signaling pathway, Ras signaling pathway, Rap1 signaling pathway, HIF-1 signaling pathway, and PI3-Akt signaling pathway. Various DEGs that include both Up-regulated and Down-regulated genes were found in these pathways for their involvement in the possible regulation of causing Hepatocellular carcinoma. To find the competent pathway, we utilized PANTHER (http://www.pantherdb.org/) a classification system based on the DEGs and pathways as shown in Figures 3 and 4, PANTHER was used to verify the potential competent pathway from our desirable list, and the HIF-1 signaling pathway was found as a feasible candidate.

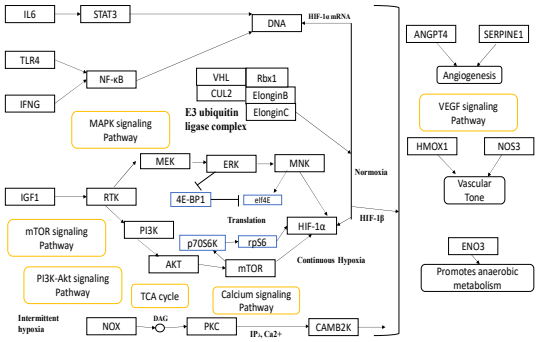

Figure 3. KEGG pathway of the HIF-1 signaling pathway. Note: Only the differently expressed genes are shown here.

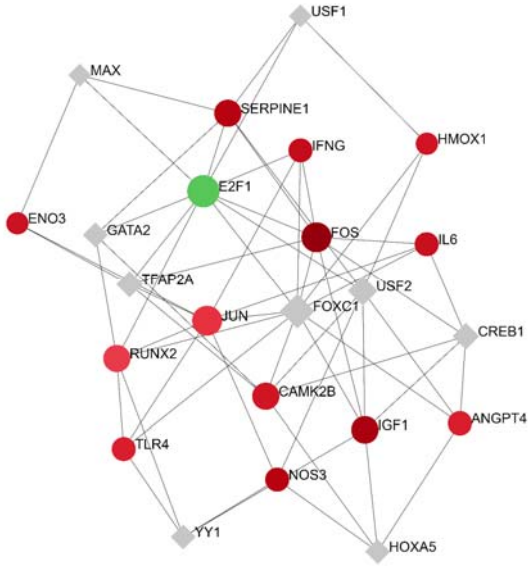

Figure 4. TF-gene interaction analysis of HIF-1 signaling pathway genes, the TF module was constructed using NetworkAnalyst and it consists of differently expressed genes of the HIF-1 signaling pathway.

3.5. PPI Network analysis and Identification of hub gene

To further evaluate the interaction among the identified DEGs and their pathways, a PPI network was analyzed to study the DEGs. The Protein-Protein Interaction (PPI) network for these DEGs consisting of 5548 nodes, 16221 edges, and 1627 seeds was constructed and analyzed by Network analyst based on the STRING database. Among all the Differentially expressed genes based on our research interest and compatibility of the genes concerning the prognosis of hepatocellular carcinoma, a few genes were shortlisted including LDHA, CAMK2B, PGK1, ANGPT4, IL6R, EPO, ENO2, ANGPT2, TEK, INS shown in Figure 5 and 6. Significant genes were shortlisted for further studies and also for their involvement in various pathways.

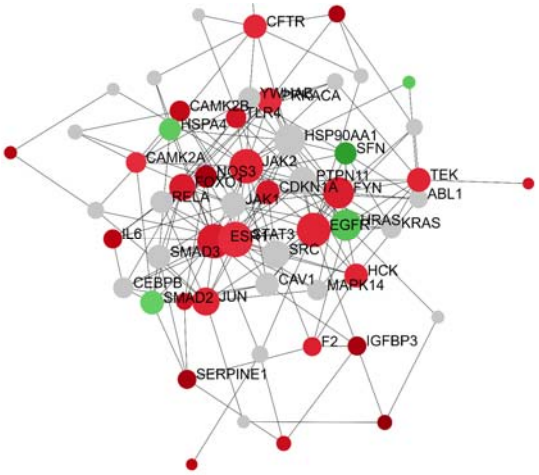

Figure 5. Protein-Protein Interaction analysis of Differently expressed genes at their transcriptional level and red colour denoted down-regulated genes and green colour denoted Up-regulated genes.

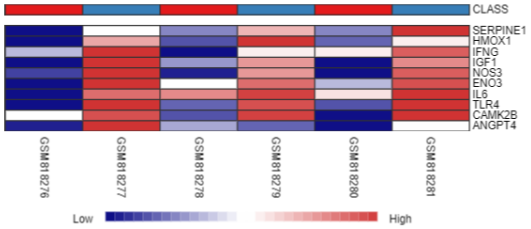

Figure 6. Heatmap of the genes that are differently expressed in the HIF-1 signaling pathway, is shown along with their expression levels in its dataset samples.

3.6. ANGPT4 -A candidate gene

We further studied the expression levels, prognosis, networking pathways, and expression levels of these mentioned genes in shortlisted pathways; we have used GENECARDS (https://www.genecards.org/) a database that provides all the necessary information regarding annotated, human genes and genomic biological data (Rappaport N et al., 2017) to probe the genes to find the suitable gene that directly or indirectly promotes Hepatocellular Carcinoma. We also used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to study the relevance of the gene and to study on HIF-1 signaling pathway and all the genes that were involved in this pathway. Based on our findings and evaluating it with necessary aspects, we found that the gene ANGPT-4 is a promising candidate here. We have also detected that the expression level of ANGPT-4 from our dataset samples is Up-regulated in Hepatocellular carcinoma according to our findings. To further understand the gene regulation of ANGPT-4, and its receptors, we used the STRING database (https://string-db.org/) to further evaluate the role of ANGPT-4 in its possible progression of Hepatocellular carcinoma. STRING analysis confirmed the expression of the candidate gene in the HIF-1 signaling pathway and also its interaction with Tie receptors, ANGPT-1, ANGPT-2, TEK et., To further validate the expression level of this gene, we used the Human Protein Atlas (https://www.proteinatlas.org/) to authenticate the expression levels in HCC (Liver cancer) and also with all

other cancer types. Therefore, with our findings, we can observe the expression of ANGPT-4 in HCC shown in Figure 7.

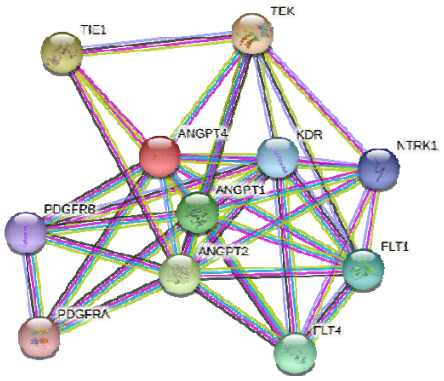

Figure 7. Sub-network analysis of genes and receptors connected with our candidate gene, the genes were analyzed and identified using String tools

4. Discussion

Bioinformatics is an essential tool that plays a critical role as an analyst in Oncogene study including diagnosis, and treatment as it assists in interpretation of the process of carcinogenesis by incorporating data at the genome level with various bioinformatics methods. Identification of prominent genes for HCC diagnosis and treatment is required, but studies related to gene expression levels are inadequate, therefore delaying the treatment process. In this study, Bioinformatics-based microarray analyses were analyzed to screen the desired key genes and their related pathway that directly or indirectly proliferate Hepatocellular Carcinoma. After analysis, 2,529 genes were up-regulated and 1,087 genes were Down-regulated; the Differently Expressed Genes of HCC datasets were screened based on fixed parameters, and this resulted in 896 up-regulated DEGs and 368 down-regulated DEGs were shortlisted along with the respective pathways from NCBI. The Bioinformatics analysis that was utilized to find out the DEGs are Gene Ontology enrichment analysis, KEGG pathway analysis, PPI network analysis, and TF network analysis to find out HCC-related genes and pathways. From Gene ontology enrichment, the desired results with the expected gene and pathway were proven and in KEGG the desirable pathway, the HIF-1 signaling pathway, was analyzed in correspondence with PPI and TF networks interactions, and the following genes were retrieved for further analysis of the following genes, SERPINE1, CAMK2B, HMOX1, ANGPT4, IFNG, IGF1, ENO3, NOS3, IL6, TLR4, ANGPT2, TEK, INS and it is observed from our data that all above-mentioned genes were down-regulated. Downregulation of ENO3 was also observed in HCC by Liu ZK (Liu ZK et al., 2019). SERPINE1 is known to cause carcinogenesis in our liver, and elevated levels of SERPINE1 are reported to be aggressive in tumors, thereby contributing towards angiogenesis stimulation, tumor proliferation, etc. Rosa et al., confirmed the significant increase in SERPINE1 expression promoting cancer progression in HCC, and through KEGG analysis we confirmed the presence of SERPINE1 in HIF-1 signaling pathway (Divella R et al., 2012; Divella R et al., 2001; Itoh T et al., 2000). The expression change of CAMK2B is yet to be found in HCC progression, but Kim JH et al. reported the Down-regulation of CAMK2B, and together with ARFGEF1, they work as biomarkers for the identification of breast cancer (Kim JH et al., 2011), and our data shows the down-regulation of CAMK2B and its presence in the HIF-1 signaling pathway. The downregulation of IGF1 was not only found in our data, but a study performed by Gao X et al. shows the down-regulation of the IGF1 gene and its significant role in HCC initiation and progression that proves our results (Gao X et al., 2018). The genes were co-related and analyzed with relatable pathways from KEGG and ANGPT4, ANGPT2, TEK, and INS were found to be present in the above-mentioned pathways. The analysis from ULCAN DATABASE, STRING DATABASE, HUMAN PROTEIN ATLAS, and GENECARDS DATABASE shows ANGPT4 is predicated to possibly induce HCC. Hepatocellular carcinoma is known to utilize a large number of blood vessels (hypervascularization) using the property of angiogenesis to indulge in an invasion, relapse, and metastasis of the disease (Poon RT et al., 2003); the angiogenic property is controlled based on the balance between anti-angiogenic inhibitors and pro-angiogenic activators and VEGF, FGF2, FGF4, HGF, IL8, Ephrin, CXCL12, ANGPT1, and ANGPT4; it is worth noting that the ligands for TIE family, TIE1 and TIE2 with receptor tyrosine kinases (TEK) are ANGPT1, ANGPT2, and ANGPT4, and they execute the role of HCC progression with interaction with the Tie receptors who are bound by tyrosine kinase receptors (Katoh Y et al., 2006). The angiopoietin/Tie2 system regulates the angiogenesis property, thereby contributing to the progression of HCC; however, the role of the angiopoietin/Tie2 system is ambiguous but recently it was reported that this system plays a crucial role in the initiation and progression of non-small cell lung carcinoma but their precise role in HCC needs further investigation (Zhang ZL et al., 2006). ANGPT4 was identified as a member of the angiopoietin family recently and is orthologous to ANGPT3 identified in mice (Valenzuela DM et al., 1999) but has a dissimilar protein structure, the study of ANGPT4 was derived from blood endothelial stem cells and vascularization properties thereby providing evidence that ANGPT4 expressed via autocrine signaling similar to ANGPT2 and stimulates responses to stimulus such as hypoxia (Lee HJ et al., 2004). ANGPT-4 firmly activates the TIE2 system and downstream signaling (Lund EL et al., 2004) and similar to ANGPT1, ANGPT4 is shown to promote and progress migration, survival, and vessel formation *in vitro,* and it is also reported that ANGPT-4 has initiated and promoted the growth of corneal angiogenesis in mice (Olsen MW., 2006). There is also strong evidence of ANGPT4's impact on breast cancer development and progression and evidence of suggestions for the potential for therapeutic targets (Yang H et al., 2021). ANGPT4 plays a key role in the growth and progression of human glioblastoma as it is up-regulated in it (Brunckhorst MK et al., 2006). The role of the Angpt-tie-2 system and ANGPT4 for initiation, cancer growth, and progression of ovarian cancer is significant from the findings where ANGPT4 is portrayed as a protumor (Brunckhorst MK et al., 2014). ANGPT4 was recorded to have swiftly activated the AKT and ERK-1,2 signaling pathways (Kesler CT et al., 2015). It is

reported that hypoxia-induced factors elevate the ANGPT4 level, thereby inducing angiogenesis in tumor areas (Lee HJ et al., 2004). ANGPT-4 is upregulated by HIF-1 and its pathway, therefore enhancing vessel formation (Andrikopoulou E et al., 2011). An important transcription factor that induces expression levels in ANGPT4 is the HIF-1α which provides an inter-cellular stabilization for growing tumors and it was proved that Hepatitis B virus X-protein on exposure to HIF-1α showed increased transcriptional levels and elevated protein levels thereby inducing angiogenesis (Morse MA et al., 2019). Jiang HY et al. reported higher expression levels of HMOX1 and SERPINE1 in hypoxia condition INHIF-1 signaling pathway that enhances the prognosis of HCC (Jiang HY et al., 2020), and the expression levels of HMOX1 was also reported by Wang Y et al. for its involvement in HCC progression (Wang Y et al., 2020). Freise C et al. reported on the activation of IGF1 that induces VEGF and HIF-1α to critically impart angiogenesis towards HCC growth (Freise C et al., 2011). The HIF-1 functions as a mediator for hypoxic regulation of ANGPT-2 and ANGPT-4 and plays a critical role in the formation of new vessels; under the influence of HIF-1, ANGPT-4 was shown to have improved vessel formation and controlled the permeability of endothelial layers (Yamakawa M et al., 2003). By substituting for ANGPT-1 as a TIE-2 receptor ANGPT-4 assist in angiogenesis in response to hypoxia and it was also confirmed that ANGPT-4 was up-regulated in normoxic conditions due to HIF-1 regulation and the molecular mechanism that is utilized by HIF-1 to upregulate ANFPT-4 levels is yet to be determined (Yamakawa M et al., 2004). By integrating our findings with clinical data from previous research studies and utilizing the KEGG pathway database, we identified similarities between our data and prior observations. This analysis revealed ANGPT-4 within the HIF-1α signaling pathway. Furthermore, our analysis of HCC tissues demonstrated upregulation of ANGPT-4 within the HIF-1 signaling pathway. Based on these findings, we hypothesize that ANGPT-4 may play a crucial role in HCC progression through the HIF-1α signaling pathway.

5. Conclusion

The acquired results from this study present ANGPT-4 as a key associated gene for the progression of Hepatocellular carcinoma, and using bioinformatics tools and various other databases we can detect ANGPT4. The expression levels related to ANGPT1 and ANGPT2 have been studied well for their involvement in HCC initiation, but in contrast the expression levels of ANGPT4 are yet to be explored in-depth for their involvement in cancer progression, including their underlying mechanism in various cancer types and HCC.

Acknowledgments

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Compliance with ethical standards

5.1. Conflict of Interests

The authors declare no conflict of interests.

5.2. Research involving animal/human participants

This article does not contain any studies with animal and human participants.

5.3. Informed Consent/Data Availability Statement

The data supporting the findings for this study are available with the corresponding author upon request.

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