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ATF1 Regulating Signaling Pathways between Nasopharyngeal Carcinoma Cells and Immortalized Epithelial Cells

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ABSTRACT This study aims to provide a comprehensive pathway regulated by ATF1 and screen for differences between cancer and normal cells. Genome-wide mRNA expression microarrays were performed using RNA isolated from ATF1 over-expressing CNE1 and NP69 cells. The ATF1 regulating expression pattern and signaling was investigated by GO annotation, KEGG pathway and PPI network. A total of 1651 genes were found to be expressed differentially between CNE1-ATF1 and CNE1-control cells, 4304 genes were shown to be expressed differentially between NP69-ATF1 and NP69-control cells. In the GO annotation, KEGG analysis and PPI networks, pathways associated with cancer, apoptosis and cell proliferation exhibited the most significant correlation with ATF1 modulation in both cells. Different pathways were suggested as immune response in CNE1 cells and metabolism in NP69 cells. Taken together, the data of this study provide a comprehensive signaling pathway regulated by ATF1 and figure out the differences between cancer and normal cells.

INTRODUCTION

Activating Transcription Factor 1 (ATF1) belongs to an ATF/CREB subfamily of basic leucine zipper transcription factor that mediate transcription responses to a variety of extracellular signals and participate in cellular transformation and survival (Mayr and Montminy 2001). Through the regulation of different transcription targets, ATF1 is involved in promoting gene expression programs which are connected with cell activities such as survival and growth. ATF1 is found to be a transcriptional repressor of the gene ferritin H by an antioxidative-response component and might be involved in iron homeostasis (Iwasaki et al. 2007). ATF1 is indicated to induce heme oxygenase-1 expression through heme and promote adaptation of macrophages to intraplaque hemorrhage (Boyle et al. 2012). GSS and GCLM, two pivotal enzymes in the GSH biosynthetic pathway, have been found to be direct transcription targets of ATF1 and CREB1. By suppressing the levels of GCLM and GSS, ATF1 and CREB1 could impair the biosynthesis of GSH and the cell ability to detoxify ROS, thus enhancing the susceptibility of cells to oxidant stress (Zhao et al. 2021). Down-regulation of ATF1 could lead to early neuroectoderm differentiation by up-regulating SOX2 expression in human embryonic stem cells (Yang et al. 2019b).

ATF1 plays the role of oncogene in cancers. ATF1 has a significant effect on cell proliferation, which may be associated with the occurrence and development of tumors (Takeda et al. 1995; Tian et al. 2019). ATF1 is suggested to enhance migration and invasion by regulating EGFR and MMP-2 in lung cancer cells (Cui et al. 2019). LncRNA GHET1 could promote cell tumorigenesis of hepatocellular carcinoma by modulating ATF1 (Ding et al. 2017). The researchers' former investigation suggests that ATF1 is modulated by prolyl isomerase Pin1 and promotes tumorigenesis of nasopharyngeal carcinoma (Huang et al. 2016). ATF1 is a potential molecular biomarker for tumor diagnosis or prognosis, as well as a molecular target for tumor therapy (Leckey et al. 2021; Shi et al. 2017; Yang et al. 2019a; Yang et al. 2019b). ATF1 is found to be a

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potential diagnostic marker of cervical cancer (Shi et al. 2017). Abolition of aspirin-induced ATF1/CREB phosphorylation is suggested to make hepatocellular carcinoma sensitive to aspirin (Zhang et al. 2021).

However, current studies on ATF1 mainly focus on its regulatory factors or pathways separately. Few analyses have been made on the networks or related pathways regulated by ATF1 and downstream molecules. Since ATF1 could modulate the expression level of multiple genes, it seems biased to analyze only one regulator or one pathway. The synergistic impact of ATF1 on cell processes should be analyzed. In this investigation, genome-wide mRNA expression profiling and comprehensive pathways analysis were performed using ATF1 over-expressing CNE1 cells and NP69 cells, which are human cells of nasopharyngeal carcinoma and of immortalized nasopharyngeal epithelium respectively, to provide a comprehensive network regulated by ATF1 and to investigate the different pathways between normal and cancer cells.

Objectives

The aim of this research is to provide a comprehensive pathway regulated by ATF1 and to screen the differences between normal and cancer cells.

METHODOLOGY

Cell Culture and Transfection

CNE1 and NP69 were provided by Dr. Binbin Li (Guangdong Medical University, China). CNE1 a highly differentiated cell line of human nasopharyngeal carcinoma, was cultivated using 1640 medium with 10 percent fetal bovine serum. NP69 a human cell line of immortalized nasopharyngeal epithelium, was cultivated using Keratinocyte-SFM (K-SFM) medium. CNE1 or NP69 cells stably expressing ATF1 were established as the researchers' previously report (Huang et al. 2016). CNE1-ATF1 was designated as the CNE1 cells stably expressing ATF1, and CNE1-control as the negative control. NP69-ATF1 was the NP69 cells stably expressing ATF1, while NP69-control as the negative control. All cells were cultivated using an incubator with 5 percent CO_2 at 37°C.

RNA Extraction and Purification

Cells (5×10^5) were inoculated in a 60 mm culture dish for 2 days, and then RNA was isolated. TRIzol Reagent (Life technologies, US) was applied to extract total RNA from cells and RIN values were determined by an Agilent Bioanalyzer 2100 (Agilent technologies, US).

Total RNA Amplification and Labeling, Microarray Experiment and Data Acquisition were carried out by Shanghai Biotechnology. Low Input Quick Amp Labeling Kit, One-Color (Agilent technologies, US) was used to amplify and label RNA. RNeasy mini kit (QIAGEN, Germany) was applied to purify labeled cRNA. Gene Expression Hybridization Kit (Agilent technologies, US) was applied to hybridize each slide with 1.65μg Cy3-labeled cRNA using Hybridization Oven (Agilent technologies, US). After hybridization for 17 hours, each slide was washed in staining dishes (Thermo Shandon, US) using Gene Expression Wash Buffer Kit (Agilent technologies, US). Agilent Microarray Scanner (Agilent technologies, US) was applied to scan each slide using default settings. Feature Extraction software 10.7 (Agilent technologies, US) was utilized for data extraction. The Quantile method was applied to normalize the original data using Gene Spring Software 11.0 (Agilent technologies, US). The datasets could be accessed in https://www.ncbi.nlm.nih.gov/, GSE188471, an online repository.

Data Processing and Pathway Analysis

Differentially expressed genes were determined when the fold change of the signal is greater than 2. R packages "pheatmap" and "VennDiagram" was applied to display the differentially expressed genes (DEGs). Enrichment pathway analysis was performed to further reveal the potential biological pathways in which the DEGs might be involved. The researchers utilized R package "org.Hs.eg.db" for gene annotation and searched enriched gene ontology (GO) entries using "ggplot2", "enrichplot" and "cluster Profiler". The researchers described the enrichment results from the aspect of cellular components (CC), biological processes (BP) and molecular functions (MF). In addition, the researchers further identified the Kyoto Encyclopedia of Genes

and Genomes (KEGG) hub path, and the results of p.adjust < 0.05 were selected for analysis and visualization.

Construction of PPI Network

PPI networks were constructed using genes with fold change over 5. STRING database (https://version-11-0b.string-db.org/) was applied to construct the PPI network of protein encoded by the target gene. The researchers defined the required confidence level greater than 0.7 as the threshold and downloaded the tsv format file. Cytoscape 3.8.2 was used to visualize the PPI network. Moreover, Cytohubba, a cellular landscape plug-in, was used to calculate the topological characteristics of PPI networks and another Cytoscape plugin, MCODE, was used to calculate K score and filter out subnetworks. The researchers used Reactome pathway analysis to explain the detailed functions provided by String database.

RESULTS

Differential Expression Patterns Regulated by ATF1 between CNE1 and NP69

Genes change with over two-fold were regarded as differential expression. The data indicated that there were 1651 genes differentially expressed between CNE1-ATF1 and CNE1-control cells, among which 799 genes were found to be increased and 852 genes were shown to be decreased. When comparing NP69-ATF1 to NP69-control cells, 4304 genes were found to be expressed differentially, among which 2071 genes were shown to be increased and 2233 genes were found to be decreased. Moreover, the researchers compared the differential gene expression regulated by ATF1 between CNE1 and NP69 cells (Fig. 1 A). Among the 799 up-regulated differentially expressed genes between CNE1-ATF1 and CNE1-control cells (CNE1-DEGs), only 100 genes were found to be increased, 136 genes were shown to be decreased, and 563 genes were unchanged when comparing NP69-ATF1 to NP69-control cells. Among the 852 down-regulated CNE1-DEGs, only 86 genes were shown to be increased, 172 genes were decreased, and 594 genes were unchanged when comparing NP69-ATF1 to NP69-control cells. The researchers selected genes with fold change over 20 to plot the expression pattern between cells (Fig. 1 B, C). A total of 63 genes showed more than 20-fold change between CNE1-ATF1 and CNE1-control cells, of which 28 genes were found to be increased and 35 genes decreased. Genes HHLA1, SELENBP1 and FAM24B were the most up-regulated, while LOC100132354, XLOC_004051 and CRYBA4 were the most down-regulated. However, there were no significant changes of HHLA1, SELENBP1, FAM24B, LOC100132354, XLOC_004051 and CRYBA4 between NP69-ATF1 and NP69-control cells. A total of 88 genes showed more than 20-fold change between NP69-ATF1 and NP69-control cells, with 17 genes shown to be increased and 71 genes decreased. Genes PCSK9, XLOC_013986 and DNMT3L had the largest up-regulation, while XLOC_011183, AFF3 and CA2 had the largest down-regulation. However, when comparing CNE1-ATF1 to CNE1-control cells, PCSK9, XLOC_013986, AFF3 and CA2 were stably expressed, while DNMT3L and XLOC_011183 were down-regulated.

KEGG Analysis

To account for each available pathway containing DEGs, KEGG pathway analysis was conducted (Fig. 2 A, B). Twenty-five pathways were enriched by CNE1-DEGs. Among them, the five pathways that were most significantly enriched included “Viral protein interaction with cytokine and cytokine receptor”, “Cytokine-cytokine receptor interaction”, “Malaria”, “PI3K-Akt signaling pathway” and “Transcriptional misregulation in cancer”. A total of 12 pathways were enriched by NP69-DEGs. Among them, the five most significant pathways were “AGE-RAGE signaling pathway in diabetic complications”, “Steroid biosynthesis”, “PPAR signaling pathway”, “Amoebiasis” and “Basal cell carcinoma”.

GO Annotation and Pathway Analysis

GO annotations were used to identify functional related genes and to render a complete picture of gene profile. According to the order of the ontological system, it is mainly divided into three kinds of processes: Biological Process (BP), Cel-

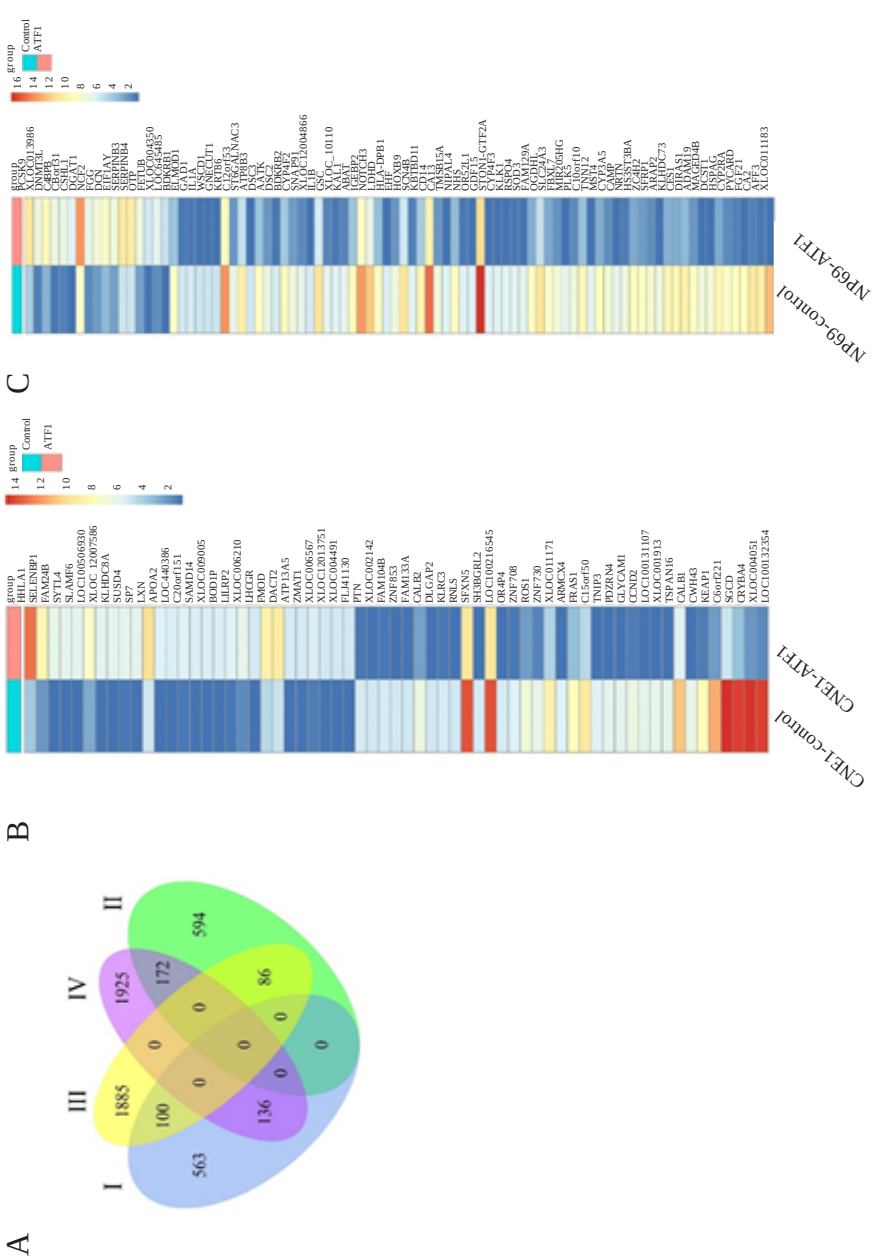

Fig. 1. (A)Venn diagram showing the overlap between CNE1-DEGs and NP69-DEGs
(I) Up-regulated differentially expressed genes between CNE1-ATF1 and CNE1-control cells (CNE1-DEGs)
(II) Down-regulated CNE1-DEGs
(III) Up-regulated differentially expressed genes between NP69-ATF1 and NP69-control cells (NP69-DEGs)
(IV) Down-regulated NP69-DEGs
(B) Heatmap of genes with fold change > 20 showed the expression pattern of differentially expressed genes in CNE1-ATF1 and CNE1-control cells (CNE1-DEGs).
(C) Heatmap of genes with fold change > 20 showed the expression pattern of differentially expressed genes in NP69-ATF1 and NP69-control cells (CNE1-DEGs)
Source: Author

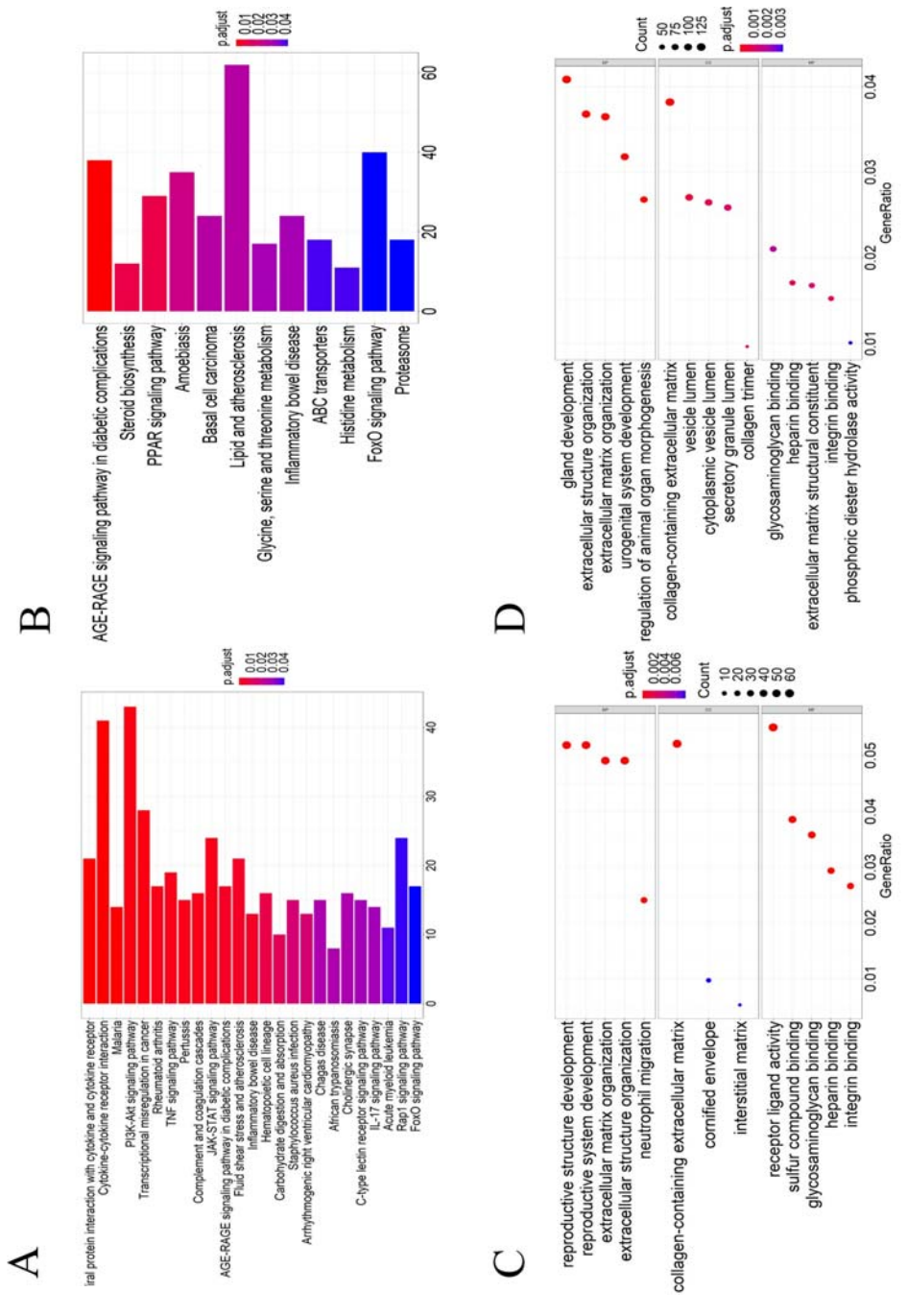

Fig. 2. KEGG pathway enrichment and GO enrichment
(A) The KEGG pathway enriched by CNE1-DEGs
(B) The KEGG pathway enriched by NP69-DEGs
(C) The GO terms enriched by CNE1-DEGs
(D) The GO terms enriched by NP69-DEGs
Source: Author

lular Components (CC), and Molecular Function (MF).

The results of GO terms analysis showed (Fig. 2 C,D), among the items enriched by CNE1-DEGs, the top 3 items enriched by BP group included "neutrophil migration", "extracellular matrix organization" and "extracellular structure organization"; The terms of significant enrichment in CC group were "collagen-containing extracellular matrix", "cornified envelope" and "interstitial matrix"; and the top 3 enriched items of MF group were "glycosaminoglycan binding", "sulfur compound binding" and "heparin binding". Among the items enriched by NP69-DEGs, the top 3 enriched terms in BP group included "GO:0048732-gland development", "GO:2000027-regulation of animal organ morphogenesis" and "GO:0043062-extracellular structure organization", respectively; In the CC group, the 3 most significant enriched terms were "GO:0062023-collagen-containing extracellular matrix", "GO:0031983-vesicle lumen" and "GO:0060205-cytoplasmic vesicle lumen"; and the top 3 enriched items of MF group were "GO:0005178-integrin binding", "GO:0008201-heparin binding" and "GO:0005201-extracellular matrix structural constituent". The researchers reconstructed the interactive network using the clusterProfiler package to show the intrinsic relationships between the GO terms. Cnetplot describes the five richest genetic ontology terms annotated to ATF1 and specific genes were also shown (Fig. 3).

PPI Network

Proteins and their interaction networks constitute the backbone of cell mechanisms, and the connection networks contribute to the full understanding of biological phenomena (Szklarczyk et al. 2019). String database is an online search database for exploring protein-protein interactions (Szklarczyk et al. 2019). The researchers selected genes with fold change greater than 5 in CNE1-DEGs and NP69-DEGs to construct PPI networks, respectively, and the data with a confidence score greater than 0.7 were selected. The researchers identified 60 and 382 nodes from the PPI networks of CNE1-DEGs and NP69-DEGs, respectively. CytoHubba, a Cytoscape plugin, was applied for finding the top 5 hub genes. GNG4, BDKRB2, CXCL11, PTGER3 and GRM7 were considered to be the hub genes of ATF1 regulation in CNE1-DEGs. The hub genes regulated by ATF1 in NP69-DEGs included GNG4, BDKRB1, GNG7, MCHR2 and BDKRB2. A Cytoscape plugin, MCODE, was used to identify sub-networks to understand the modulation of ATF1 at a PPI network level. According to K-score greater than 4, 1 sub-network was recognized in the network of CNE1-DEGs, and there were 6 sub-networks in the network of NP69-DEGs (Fig. 4).

DISCUSSION

Previous studies have identified that multiple genes are regulated by ATF1. However, the related pathways were analyzed separately. It is necessary to consider the synergistic effects of ATF1 on cellular processes. In this research, the mRNA expression pattern of CNE1 and NP69 cells with ATF1 over-expression was identified. The pathways of ATF1 regulation were investigated through GO annotation and KEGG pathway to render the comprehensive perspective of ATF1 regulation.

In the differentially expressed genes between CNE1-ATF1 and CNE1-control cells (CNE1-DEGs), HHLA1, SELENBP1 and FAM24B genes were the most up-regulated, while LOC100 132354, XLOC_004051 and CRYBA4 genes were the most down-regulated. HHLA1 is associated with inhibition of translation of associated fusion transcripts by human endogenous retroviruses (Kowalski and Mager 1998), and according to the Genecards database (https://www.genecards.org/), HHLA1-associated diseases include granulosa cell carcinoma and testicular seminoma. SELENBP1 acts as an enzyme in sulfur metabolism and affects the concentration of bioactive molecules such as H_2O_2 and H_2S. SELENBP1 is a biomarker in some cancers, such as breast cancer, kidney cancer and colorectal cancer (Pol et al. 2018). FAM24B is related to complex structural variation caused by chromosome rearrangement, and also participates in the biological process of esophageal cancer progression (Jin and Miao 2020; Tan et al. 2020). CrybA4 is a human cataract gene that has been implicated in microphthalmia (Billingsley et al. 2006). HHLA1, SELENBP1 and FAM24B genes are involved in the development of various cancers. In CNE1, these three genes were

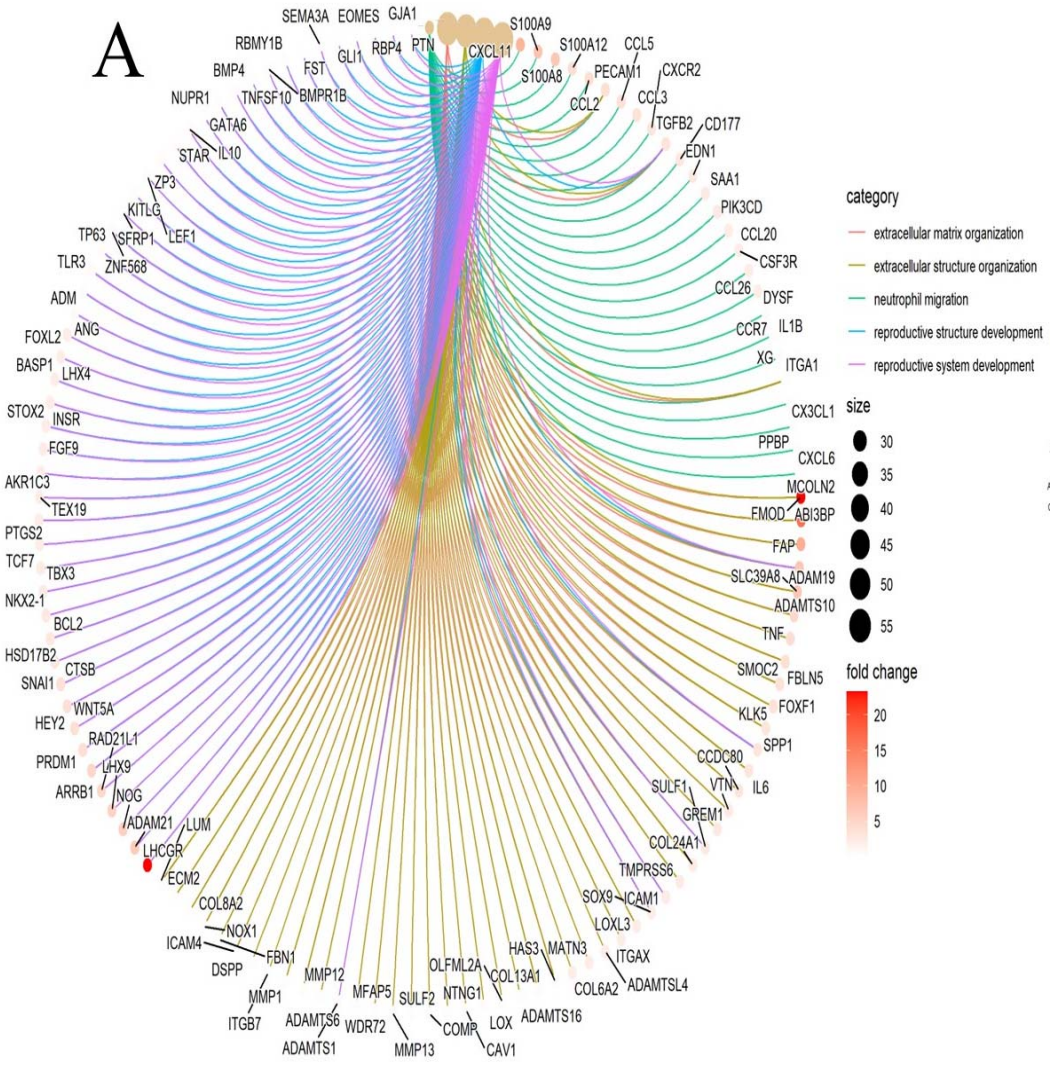

Fig. 3. Cnetplot of GO signal pathway shown the "pathway-gene" network, that depicts the linkages of genes and most important signaling pathways
(A) Cnetplot of GO signal pathway by CNE1-DEGs
Source: Author

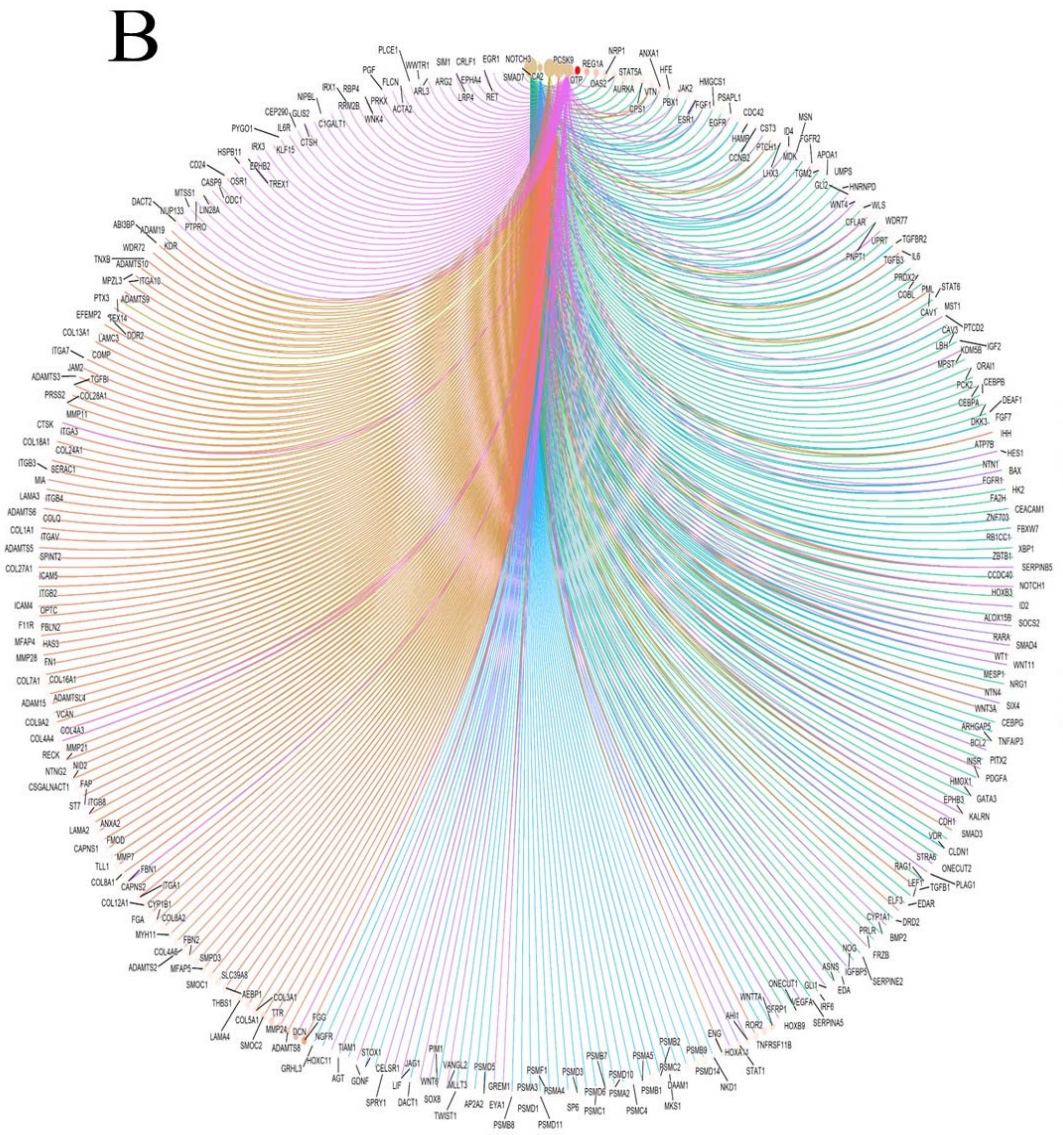

Fig. 3. Cnetplot of GO signal pathway shown the "pathway-gene" network, that depicts the linkages of genes and most important signaling pathways
(B) Cnetplot of GO signal pathway enriched by NP69-DEGs
Source: Author

significantly upregulated by the increasement of ATF1 gene expression. However, HHLA1, SelenBP1 and Fam24B genes were all stably expressed in NP69 with ATF1 overexpression.

In NP69-DEGs, PCSK9, XLOC_013986 and DNMT3L had the largest up-regulation, while XLOC_011183, AFF3 and CA2 had the largest down-regulation. PCSK9 is associated with immune response. Suppression of PCSK9 can enhance cancer immune checkpoint therapy (Liu et al. 2020). DNMT3L is a key modulator in de novo DNA methylation and functions as a positive regulator of DNA methylation (Veland et al. 2019). AFF3 is an important mediator for the resistance of tamoxifen and estrogen-independent growth. It encodes a nucleus protein of retroviral activation potential which confers the resistance of tamoxifen, allowing it to grow independently of estrogen (Shi et al. 2018). CA2 (Carbonic Anhydrase 2) plays an important role in pH regulation, electrolyte balance protection, water transport and other metabolic pathways (Akin et al. 2019). In human, the deficiency of CA2 is related to osteopetrosis, renal tubular acidosis and cerebral calcification (Lai et al. 1998). While in CNE1 cells with ATF1 overexpression, PCSK9, XLOC_013986, AFF3 and CA2 were stably expressed, and DNMT3L, XLOC_011183 were down-regulated. These data

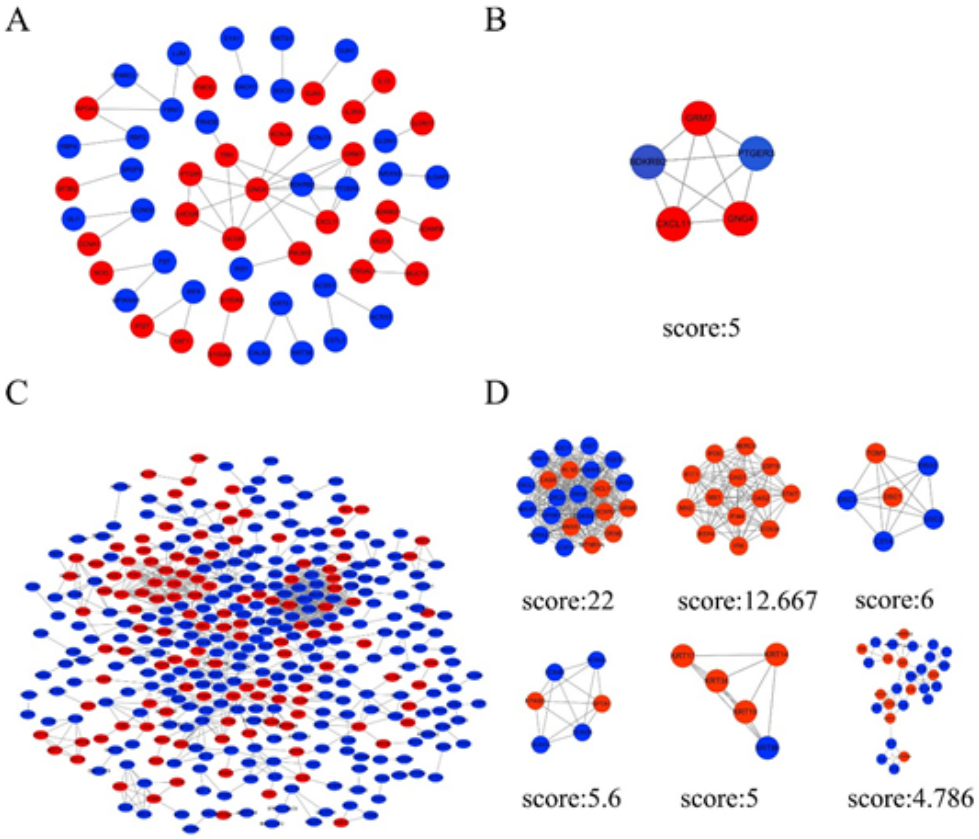

Fig. 4. Protein-protein interaction
(A) PPI networks of CNE1-DEGs
(B) Subnetwork analyzed by MCODE of CNE1-DEGs
(C) PPI networks of NP69-DEGs
(D) Subnetworks analyzed by MCODE of NP69-DEGs
Source: Author

suggested ATF1 regulated different genes between cancer and normal cells.

Enrichment analysis of KEGG signaling pathway of CNE1-DEGs showed that ATF1 affected multiple related signaling pathways in the immune response and tumor. Pathways, including "Cytokine-cytokine receptor interaction", "Viral protein interaction with cytokine and cytokine receptor" and "Malaria" are closely associated with immunity. Viral cytokines and cytokine receptor, as well as a series of cytokine binding or cytokine receptor binding proteins, stand for three crucial molecular strategies that subvert and regulate host cytokine signaling primarily in large DNA viruses. They may promote or suppress cytokine networks and may influence various aspects of immunity (Alejo et al. 2006; Smith et al. 2013; Viejo-Borbolla et al. 2012). Moreover, cytokines are important intercellular regulators and mobilizers involved in cell survival, cell death, differentiation, inflammatory host defense, developmental and repair processes to restore homeostasis, and angiogenesis (Turner et al. 2014). The protozoan Plasmodium is a parasite that infects malaria. The parasite invades red blood cells in a multi-step process mediated by highly specific molecular interactions (Chitnis 2001). Pathways, including "Transcriptional misregulation in cancer" and "PI3K-Akt signaling pathway" are significantly correlated with the occurrence and development of tumor. PI3K-Akt signaling is motivated by various cellular stimulation or toxic injury and modulates essential biological functions including transcription, translation, proliferation and survival (Korkolopoulou et al. 2012). Fusion transcription factors alter the expression of target genes, leading to changes in a variety of cellular properties that contribute to the tumorigenesis process (Suzuki and Matsubara 2011). However, the KEGG pathways enriched by NP69-DEGs are significantly associated with cancer and metabolic diseases. "AGE-RAGE signaling pathway in diabetic complications" is closely related to diabetes, "PPAR signaling pathway" participates in lipid metabolism, and "Basal cell carcinoma" plays an important role in cancer progression. These data indicate that ATF1 is significantly related to various diseases including cancer in both cell lines and that different pathways are found between cancer cells and normal cells.

According to GO enrichment analysis, CNE1-DEGs were mainly enriched in sulfur compound binding, collagen-containing extracellular matrix, glycosaminoglycan binding, integrin binding and heparin binding. NP69-DEGs were mainly enriched in extracellular structure organization, collagen-containing extracellular matrix, gland development, regulation of animal organ morphogenesis and extracellular matrix organization. Several "extracellular" related pathways were enriched in both CNE1-DEGs and NP69-DEGs. These data indicate a constitutive function of ATF1 as a regulator of "extracellular" in both cancer and normal cells. Different pathways regulated by ATF1 were found in normal and cancer cells. Neutrophil migration and receptor ligand activity were modulated by ATF1 in cancer cells only; while phosphoric diester dydrolase activity was modulated by ATF1 in normal cells only. Protein-protein interaction affected by ATF1 was further analyzed. The researchers used Reactome pathway analysis to explain the detailed functions provided by the String database. GNG4 and BDKRB2 were among the top 5 hub genes of both CNE1-DEGs and NP69-DEGs. GNG4 as a subunit of G-protein plays a role as a modulator and transducer of several transmembrane signaling (Downes and Gautam 1999). The function of GNG4 in cancer is still unclear. GNG4 is found to promote tumor progression of colorectal cancer (Liang et al. 2021). Epigenetic modification of GNG4 is indicated mesenchymal glioblastoma and thymic carcinoma (Kishibuchi et al. 2020; Pal et al. 2016). BDKRB2 (Bradykinin receptor B2) is an oncogene of some cancers, such as hepatocellular carcinoma, gastric cancer, colorectal cancer and glioma (Jiang et al. 2011; Wang et al. 2014; Yang et al. 2021; Zhao et al. 2017). The PPI analysis further suggested that CXCL11, PTGER3 and GRM7 were considered to be the hub genes of ATF1 regulation in only CNE1-DEGs. The hub genes regulated by ATF1 in only NP69-DEGs included BDKRB1, GNG7 and MCHR2. The PPI subnetwork of CNE1-DEGs is significantly correlated with Signal Transduction. The subnetworks of NP69-DEGs are significantly related to Signal Transduction, Antiviral mechanism by IFN-stimulated genes, Prolactin receptor signaling, Developmental Biology and ISG15 antiviral mechanism.

CONCLUSION

The researchers identified an expression pattern regulated by ATF1 and the comprehensive signaling pathway was further analyzed using KEGG pathway, GO annotation system and PPI networks analysis. The different role of ATF1 in normal and cancer cells was discussed. These data also provide insights for further investigation on the development of cancer affected by ATF1 and the molecular mechanism of ATF1.

RECOMMENDATIONS

Future studies should evaluate the association of ATF1 with cancer development based on cell experiments and large sample surveys.

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AUTHOR CONTRIBUTIONS

GH designed the study. HC and FW administrated the experiments and analyzed the results. FW, HC, JW, HC and NW analyzed and interpreted the data. FW and HC composed the manuscript. FW, JD and GH revised the manuscript. All authors have read and agreed to publish the current manuscript. Fangfang Wang and Hua Chen have made equal contributions to this work and share first authorship.

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