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IDENTIFICATION OF POTENTIAL REGULATORY GENES ASSOCIATED WITH CHONDROSARCOMA USING INTEGRATED BIOINFORMATIC ANALYSIS

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ABSTRACT
Identifying the novel critical regulatory genes in the molecular processes driving chondrosarcoma (CS) growth is essential for establishing targeted therapeutic approaches. Objective: This study aims to investigate the core regulatory genes implicated in the molecular mechanisms of CS progression. Method: We conducted a dataset search from the Gene Expression Omnibus (GEO) database using "chondrosarcoma" as the keyword. DAVID database was utilised to obtain the Gene Ontology (GO) and pathways enrichment of DEGs. Interaction between the proteins network was constructed using the STRING database and visualised by Cytoscape (3.10.0) software. Subsequently, the essential genes were identified as the intersected genes from cytoHubb and MCODE plugin. Furthermore, we analysed these genes based on their expression and survival using the UALCAN database. Additionally, the cBioPortal database and Tumor Immune Estimation Resource (TIMER) were utilised to obtain the genetic alteration and immune cell infiltration associated with the hub genes. Moreover, the NetworkAnalyst database was deployed to construct the interactions between microRNAs (miRNAs) and the hub genes. Results: 114 common DEGs were found between two datasets (GSE30844 and GSE48418). These genes are predominantly associated with Focal Adhesion. Seven hub genes were identified which include CCND1, CDK6, CAV1, MLC1, SQSTM1, GAPDH, and FOXO1. The validation analysis revealed a diagnostic value amidst the hub genes, particularly CDK6 and FOXO1 genes associated with unfavorable outcome in sarcoma patients. The miRNAs analysis demonstrated that miR-15a-5p has a potential binding with CDK6 and FOXO1. Conclusions: This study revealed seven core genes and indicated a putative regulatory molecule associated with CS progression. Taken together, this study's findings suggest that the CDK6, FOXO1, and miR-15a-5p have a potential role in regulating CS progression.

Keywords: bioinformatic analysis; chondrosarcoma; differentially expressed genes

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INTRODUCTION

Chondrosacoma (CS) is an unusual tumor that arises from cartilage with strong local invasion capabilities and can metastasize to other distant organs. CS is the most frequent primary osseous malignancy after Osteosarcoma, representing about 30% of all bone malignancies. The estimated incidence of CS range around 1:200.000 and shows more dominant predilection in the male populations, with the peak age in the seventh decade (Jami et al., 2021; Amer et al., 2020). Clinical symptoms of CS include deep pain and a gradually enlarging mass that arise in any bone derived from enchondral ossification, with the most

frequent location being the pelvis, ribs, proximal femur, and proximal humerus (Suyasa et al., 2019; Zulfariska et al., 2020; Apley, 2019). Chondrosarcoma categorized into primary and secondary. Primary CS refers to the development of chondrosarcoma without any history of malignancy, whereas secondary CS arises from pre-existing conditions such as enchondroma or osteosarcoma. While the progression of CS is often gradual and metastasis occurs seldom, the resistance to chemotherapy and radiotherapy frequently observed in this particular type of tumor can be attributed to the presence of extracellular matrix constituents, a low rate of cell division, and inadequate vascularization. A lack of a better understanding of therapeutic targets adds to the limitations in the clinical management of this tumor (Urdinez et al., 2020; Dai et al., 2011; Quan et al., 2021; F. Li et al., 2020).

Several studies reported that mRNAs govern a fundamental part in malignancy. Many studies also documented multiple microRNAs dysregulated in CS development and progression (Urdinez et al., 2020; Chen et al., 2020). There are two primary categories of chondrosarcoma (CS) based on genetic factors. The mutation of isocitrate dehydrogenase genes (IDH1 and IDH2) is attributed in the central CS. The other category is secondary peripheral CS, characterised by changes in the exostosin glycosyltransferase 1 and 2 genes (EXT1 and EXT2). Mutations in the IDH1/IDH2 or EXT1/EXT2 genes have been observed to be generally implicated in the development of common central or peripheral chondrosarcomas, respectively. Furthermore, the inactivation of the Rb and p53 pathways is commonly present in most tumors and is likely to play a significant role in chondrosarcoma development. Additional sporadic findings suggest that certain genes and pathways involved in normal cartilage development are disrupted in the development of chondrosarcomas. Research that aims to elucidate the molecular events that uphold the pathogenesis of this rare bone malignancy and identify new molecularly targeted therapies, especially for chemotherapy-refractory CS, is highly desirable (Chow, 2018; Scotlandi et al., 2020; Zhu et al., 2020).

Cancer is a multifaceted affliction that emerges from the intricate interaction of diverse tiers of information. The correlation between the formation of a tumour and the manifestation of genomic and transcriptomic alterations, as well as interactions with the tumour microenvironment, are pivotal factors contributing to the complexity of tumour treatment (Jiménez-Santos et al., 2022). High-throughput platforms for gene expression analysis, like microarrays, are gaining attention and offer potential clinical applications in medical oncology, including diagnosis, prognosis prediction, and identification of therapeutic targets. Recently, researchers have utilised microarray technology combined with bioinformatics to analyse the expression changes of mRNA in the occurrence and development of CS (Wu et al., 2020). Hence, in order to develop an optimal knowledge about molecular process in CS tumorigenesis, this study aim to identify the novel regulatory genes through utilisation of integrated bioinformatic analysis.

METHOD

Gene Expression Data Extraction

Two different gene profiles (GSE30844 and GSE48418) were gained from the Gene Expression Omnibus (GEO) database. The dataset GSE30844 used Illumina HumanWG-6 v3.0 expression beadchip (GPL6884) which contained 14 chondrosarcoma tissue samples and 6 normal cartilage samples, while the dataset from GSE48418 used Illumina HumanHT-12 V4.0 expression beadchip (GPL10558) which contained 3 chondrosarcoma cell lines samples and 3 normal chondrocyte samples (Pansuriya et al., 2011; Galoian et al., 2014).

Differentially Expressed Genes Identification

The GEO2R tool was utilised in order to obtain the matrix files for GSE30844 and GSE48418 from their corresponding GSE databases. Additionally, genes that possessed an adjusted p-value less than 0.05 and a [log2FC] greater than +0.5 or less than -0.5 were deemed to be significant DEGs. These DEGs were then depicted in a heat map, which was generated through the utilisation of the online bioinformatic resources provided by SRplot. To visualise the upregulated, downregulated, and unique DEGs that were identified from the two datasets, the Venn Diagram was employed.

The analysis of Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway

The DEGs functional analyses were obtained through utilising the Database for Annotation, Visualization and Integrated Discovery (DAVID) database. This analysis was conducted based on the three main component annotation including biological process (BP), molecular function (MF), and cellular component (CC) of the DEGs (Huang et al., 2009). Online bioinformatic tools, including The Kyoto Encyclopaedia of Genes and Genomes (KEGG) program, were utilised to conduct a metabolic pathway enrichment analysis (Kanehisa & Goto, 2000). The significance of pathway enrichment was determined by considering a cutoff score of the adjusted p-value being less than 0.05.

Protein-protein Interaction (PPI) and Hub Gene Identification

The online software application STRING was employed to extract interconnected genes with the aim of generating a protein-protein interaction (PPI) network (Szklarczyk et al. 2015). The PPI analysis was conducted using Cytoscape (V3.10.0). To identify the significant genes in the subnetwork, a Cytoscape plugin called Molecular Complex Detection (MCODE) was utilized, characterized by the K-score (2), degree cutoff score (2), node cutoff score (0.2), and 100 maximum depths parameters (Bader & Hogue, 2003). Furthermore, the Cytohubba plugin integrated the PPI-MCODE modules and was employed to determine the most intersected essential genes and modules (Chin et al., 2014). The network was enhanced by incorporating several topological attributes, including Maximal Clique Centrality (MCC), Density of Maximum Neighbourhood Component (DMNC), Maximum Neighbourhood Component (MNC), Edge Percolated Component (EPC), Closeness Centrality, and Degree, in order to facilitate the identification of hub genes and modules.

Validation of Hub Gene

The analysis of the expression of hub genes that were identified was conducted by employing UALCAN, a publicly available database that profiles the gene expression of cancer and normal samples and enables interactive analyses (Chandrashekar et al., 2022). A p-value less than 0.05 was deemed to possess statistical significance in the event of a discrepancy or outcome. In order to corroborate the prognostic importance of these hub genes for patients with sarcoma, the Kaplan-Meier plotter was employed.

Genomic remodeling analysis

The cBioPortal database was employed to investigate genetic remodeling in the hub genes. The types of genomic remodeling and the frequency of remodeling in sarcoma were then addressed (Barretina et al., 2010; Gao et al., 2013; Nacev et al., 2022).

Immune Infiltration in Sarcoma Associated with Hub Genes

The investigation of genetic modules and somatic copy number alterations (SCNA) pertaining to hub genes was executed using the Tumour Immune Estimation Resource (TIMER) (T. Li et al. 2017). By employing the gene module, we were capable to identify indispensable hub genes and examining their association with immune infiltration in sarcoma. The "SCNA" module was utilised to contrast the levels of tumor infiltration in tumors with diverse SCNAs of the hub genes. TIMER classifies SCNAs into seven groups employing GISTIC v2.0, which are deep deletion [-2], arm-level deletion [-1], diploid/normal [0], arm-level gain [1], and high amplification [2]. Box plots were generated to portray each immune subset at every copy number status in selected cancers. A two-sided Wilcoxon rank-sum test was executed to compare the infiltration level for each SCNA category with the normal control.

Establishment of miRNA and hub gene networks

Further relation of miRNAs and the hub genes was analysed using the NetworkAnalyst database (Xia et al., 2014). The NetworkAnalyst constituted an internet-based repository consisting of the miRTarBase, TarBase, and miRcode databases. The miRTarBase database, in particular, was frequently employed for the purpose of investigating the associations between miRNAs and essential hub genes. Subsequently, the Cytoscape platform was employed to execute the visualisation of regulatory networks involving miRNAs and hub genes. To further validate the accuracy of predicted miRNA, interaction between miRNA and core hub genes were analysed with ENCORI online database.

RESULTS

Identification of Significant Differentially Expressed Gene from Datasets

The GEO2R online tool from the GEO database revealed 506 DEGs (279 upregulated and 227 downregulated) from GSE30844, 6374 DEGs (3035 upregulated and 3339 downregulated) from GSE48418, which were differentially expressed between CS group and normal cartilage group. The Venn diagram demonstrated that a total of 114 DEGs were found to be shared between the two datasets, encompassing 48 upregulated genes and 66 downregulated genes (Figure 1A). Furthermore, the heatmap visually depicted the presence of these 114 overlapping DEGs in both GSE30844 and GSE48418. (Figure 1B).

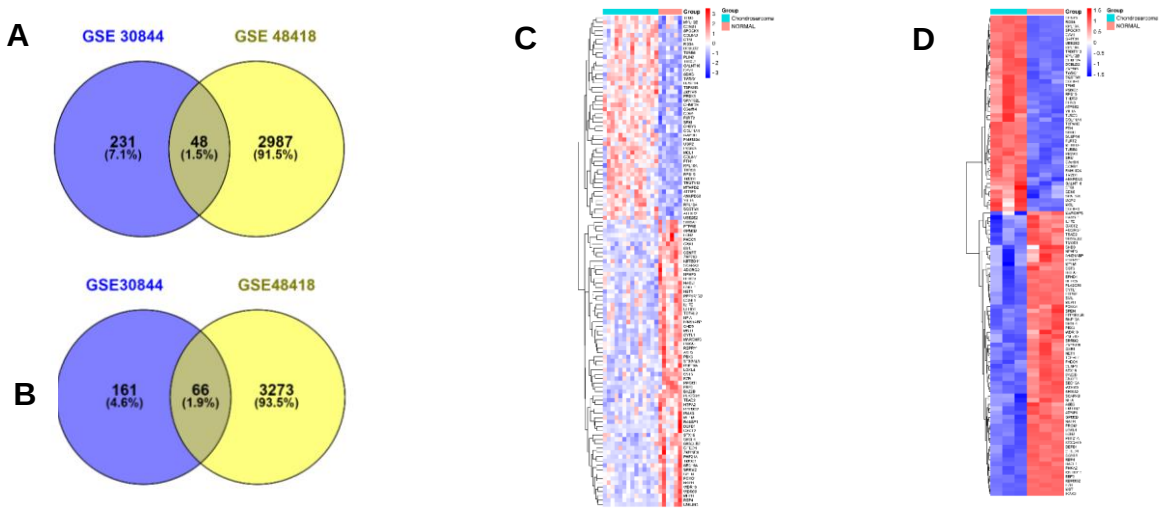


Figure 1. (A) There were 48 differentially expressed genes (DEGs) that were up-regulated with a log fold change (logFC) greater than 0.5. (B) On the other hand, 66 DEGs were down-regulated with a logFC less than 0.5. (C, D) The integrated microarray analysis of the GSE30844 and GSE48418 datasets resulted in a heat map of the DEGs. In this heat map, each column represents a sample, and each row represents a gene. The color spectrum, ranging from blue to red, indicates the extent of downregulation or upregulation.

The GO and KEGG pathway analysis of DEGS

To explore the functions of the identified 114 intersecting DEGs, we utilised the DAVID database to conduct GO and signaling pathway enrichment analyses on the DEGs. The analyses revealed that in terms of biological process, the 114 intersecting DEGs were mainly enriched in the negative regulation of protein ubiquitination (p=0.003), ureter development (p=0.03), ISG15-protein conjugation (p=0.03), cellular response to hyperoxia (p=0.03), negative regulation of transcription from RNA polymerase II promoter (0.03), response to estrogen (p=0.03), positive regulation of mammary gland epithelial cell proliferation (p=0.04), and embryonic heart tube morphogenesis (p=0.04) (Figure 2A). For cellular component, it was uncovered that most DEGs were enriched in the extracellular exosome (p<0.001), cytosol (p=0.002), extracellular space (p=0.002), focal adhesion (p=0.006), actin filament (p=0.01), GAIT complex (p=0.02), cytoplasm (p=0.02), golgi apparatus (p=0.03), cilium (p=0.03), and perinuclear region of cytoplasm (p=0.03) (Figure 2B). In terms of molecular function, it was revealed that the DEGs were mainly enriched in disordered domain-specific binding (p=0.001), protein binding, bridging (p=0.003), ISG15 transferase activity (p=0.02), extracellular matrix structural constituent conferring tensile strength (p=0.02), and cadherin binding (p=0.03) (Figure 2C). Regarding to signaling pathway enrichment analysis provided by KEGG pathway, significantly enriched pathways of DEGs were enriched in focal adhesion (p=0.04) (Figure 2D).

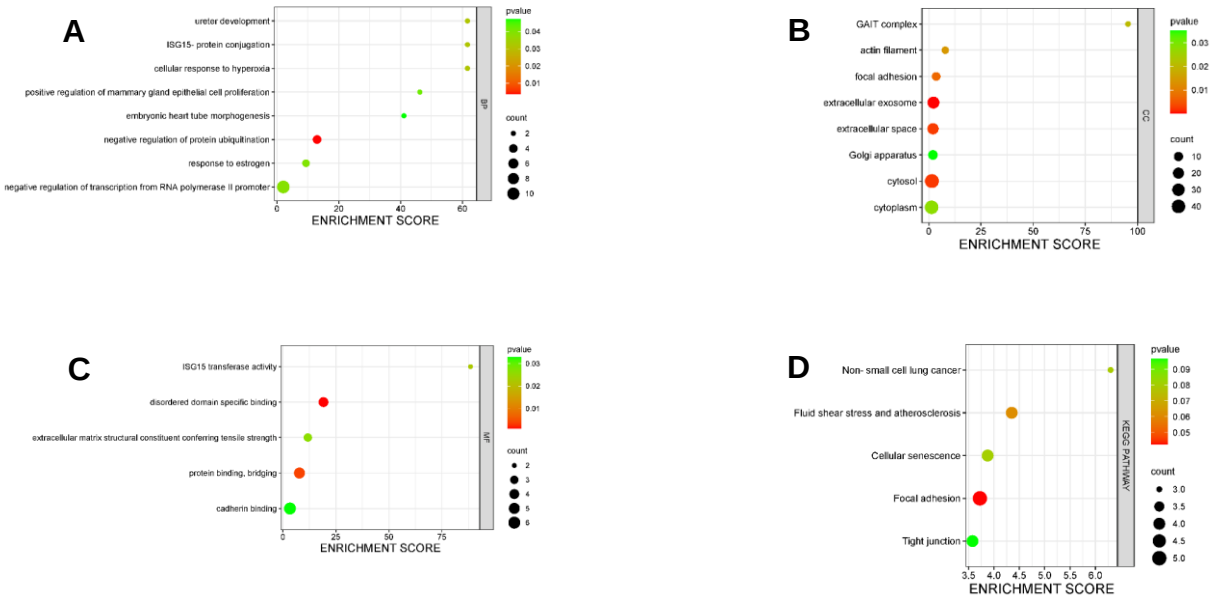


Figure 2. (A) Gene Ontology comprising of Biological Process (BP) mainly enriched in negative regulation of protein ubiquitination (p=0.003). (B) Cellular Component (CC) mainly enriched in extracellular exosome (p<0.001), cytosol (p=0.002), and extracellular space (p=0.002). (C) Molecular Function (MF) mainly enriched in protein binding bridging (p=0.003) and disordered domain specific binding (p=0.001) and (D) KEGG pathway analysis of the overlapping DEGs in chondrosarcoma mainly enriched in focal adhesion (p=0.04).

Protein Interactions and Hub Genes Identification

The STRING database was utilised to construct the PPI network amidst the 114 DEGs. An appropriate PPI network was successfully constructed, encompassing 114 nodes and 82 edges. Subsequently, this PPI network was visualised through the utilisation of Cytoscape software (Figure 3A). Module analyses were subsequently carried out employing the MCODE plugin of Cytoscape based on the entire network and revealed the most crucial PPI network modules, consisting of 7 nodes and 18 edges. In order to further identify the hub

genes, the cytoHubba plugin of Cytoscape was applied to rank the top 10 nodes in the aforementioned PPI network based on six topological analysis methods, namely MCC, DMNC, MNC, degree, closeness, and EPC. A total of seven overlapping hub genes were determined for further analysis, namely CCND1 (Cyclin D1), CDK6 (Cyclin Dependent Kinase 6), CAV1 (Caveolin 1), MCL1 (Myeloid Cell Leukemia Sequence 1), SQSTM1 (Sequestosome 1), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), and FOXO1 (Forkhead box O1) (Figure 3B). Having conducted the aforementioned procedure, we utilised the DAVID database to subsequently produce notable enrichment pathways, biological processes, cellular components, and molecular functions that are linked to the seven hub genes, respectively. With respect to KEGG enrichment pathway analysis, the core hub genes were mainly expressed in the cellular senescence, microRNAs in cancer, human papillomavirus infection, PI3K-KT signaling pathway, viral myocarditis, pathways in cancer, melanoma, and non-small cell lung cancer (Table 1, Figure 3C).

Table 1.
Hub Gene Attributed Signaling Pathway

Category	Term	Count	p Value	Genes	Fold Enrichment	FDR
Biological Process	negative regulation of transcription from RNA polymerase II promoter	5	9.21E-05	CDK6, CCND1, CAV1, SQSTM1, FOXO1	14.03556969	0.023299338
Biological Process	cellular response to hyperoxia	2	0.002	CAV1, FOXO1	924.4762	0.23397
Biological Process	cellular response to DNA damage stimulus	3	0.003	CCND1, FOXO1, MCL1	28.49413	0.23397
Biological Process	positive regulation of apoptotic process	3	0.004	SQSTM1, FOXO1, MCL1	25.4443	0.23397
Biological Process	negative regulation of epithelial cell differentiation	2	0.005	CCND1, CAV1	326.2857	0.23397
Biological Process	negative regulation of anoikis	2	0.005	CAV1, MCL1	291.9398	0.23397
Biological Process	temperature homeostasis	2	0.006	SQSTM1, FOXO1	264.1361	0.23397
Biological Process	protein phosphorylation	3	0.01	CDK6, CCND1, SQSTM1	15.52292	0.286184
Biological Process	lactation	2	0.01	CCND1, CAV1	135.2892	0.286184
Biological Process	apoptotic process	3	0.01	SQSTM1, FOXO1, MCL1	13.50696	0.286184
Biological Process	energy homeostasis	2	0.01	SQSTM1, FOXO1	115.5595	0.286184
Biological Process	response to calcium ion	2	0.02	CCND1, CAV1	102.7196	0.286184
Biological Process	cell differentiation	3	0.02	CAV1, SQSTM1, MCL1	12.05839	0.286184
Biological Process	negative regulation of protein ubiquitination	2	0.02	CAV1, SQSTM1	97.31328	0.286184
Biological Process	response to estrogen	2	0.02	CCND1, CAV1	94.01453	0.286184
Biological Process	response to ischemia	2	0.02	CAV1, SQSTM1	94.01453	0.286184
Biological Process	G1/S transition of mitotic cell cycle	2	0.02	CDK6, CCND1	84.04329	0.301035

Category	Term	Count	p Value	Genes	Fold Enrichment	FDR
Biological Process	cellular response to starvation	2	0.02	CAV1, FOXO1	73.9581	0.322707
Biological Process	fat cell differentiation	2	0.02	CCND1, FOXO1	69.33571	0.325894
Biological Process	positive regulation of autophagy	2	0.026	SQSTM1, FOXO1	63.75698	0.336386
Biological Process	protein localization	2	0.038	CAV1, SQSTM1	44.37486	0.458053
Biological Process	autophagy	2	0.046	SQSTM1, FOXO1	35.78618	0.54008
Cellular Component	cytosol	6	0.0068	CDK6, CCND1, GAPDH, SQSTM1, FOXO1, MCL1	3.160686	0.224461
Cellular Component	cytoplasm	6	0.0073	CDK6, CCND1, GAPDH, SQSTM1, FOXO1, MCL1	3.108443	0.224461
Cellular Component	cyclin-dependent protein kinase holoenzyme complex	2	0.012	CDK6, CCND1	137.0365	0.237716
Cellular Component	nucleoplasm	5	0.015	CDK6, CCND1, SQSTM1, FOXO1, MCL1	3.654535	0.237716
Cellular Component	lipid particle	2	0.03	CAV1, GAPDH	55.07076	0.374924
Molecular Function	macromolecular complex binding	3	0.005	CCND1, CAV1, SQSTM1	22.7431	0.182596
Molecular Function	enzyme binding	3	0.0057	CCND1, CAV1, SQSTM1	21.36654	0.182596
Molecular Function	protein kinase binding	3	0.01	CCND1, CAV1, SQSTM1	15.55419	0.225277
Molecular Function	protein binding, bridging	2	0.035	CAV1, SQSTM1	47.90139	0.564207

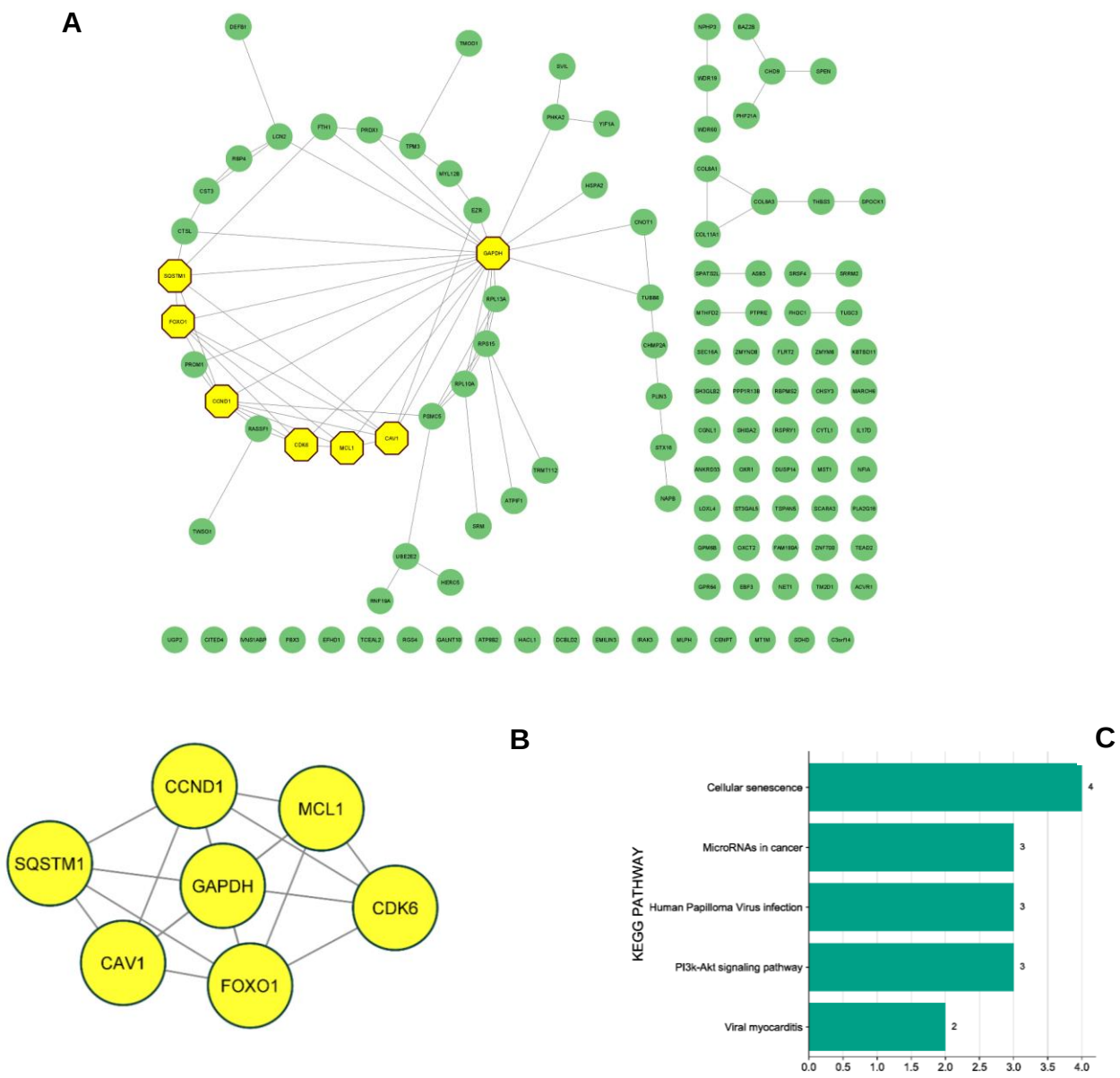


Figure 3. (A) A total of 114 differentially expressed genes (DEGs) were observed within the intricate protein-protein interaction (PPI) network of DEGs. In this network, the nodes symbolize proteins or genes, while the edges symbolize the interaction between the proteins, resembling a hexagonal configuration. This network was further analysed using the MCODE algorithm. (B) Hub genes overlapping between Cytohubba and MCODE plugin in Cytoscape. Module analysis using MCODE: degree cutoff =10, node score cutoff = 0.2, k-core = 2, max depth = 100. (C) Significant enrichment KEGG pathway associated with hub Genes.

Validation of Seven Selected Hub Genes

The UALCAN tool database was deployed to corroborate the expression of these seven genes. The database demonstrated that the expression of CCND1, CDK6, CAV1, MCL1, SQSTM1, GAPDH, and FOXO1 presented significant dissimilarities between sarcoma samples and normal samples, only CCND1 presented a statistical significance ($p<0.0001$) (Figure 4). Survival analysis of the aforementioned genes further demonstrated that among the seven hub genes, CDK6 ($p=0.016$) and FOXO1 ($p=0.042$) exhibited a significant correlation with unfavorable patient survival. (Figure 5).

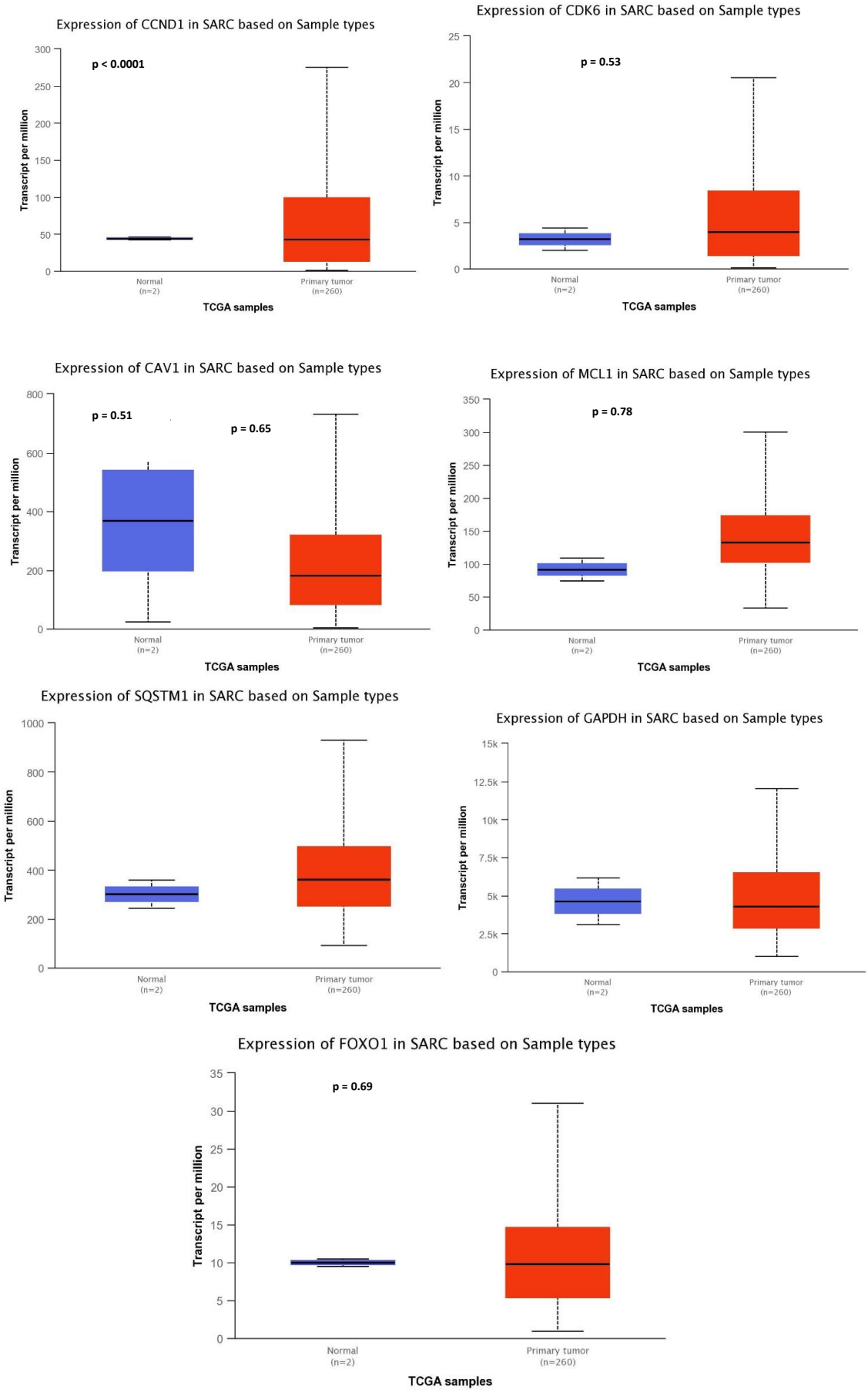


Figure 4. Hub genes validation by using UALCAN database revealed the expression level of hub genes between sarcoma and normal tissue. CCND1 has a significant expression level ($p<0.0001$) in sarcoma tissue compared to normal tissue sample.

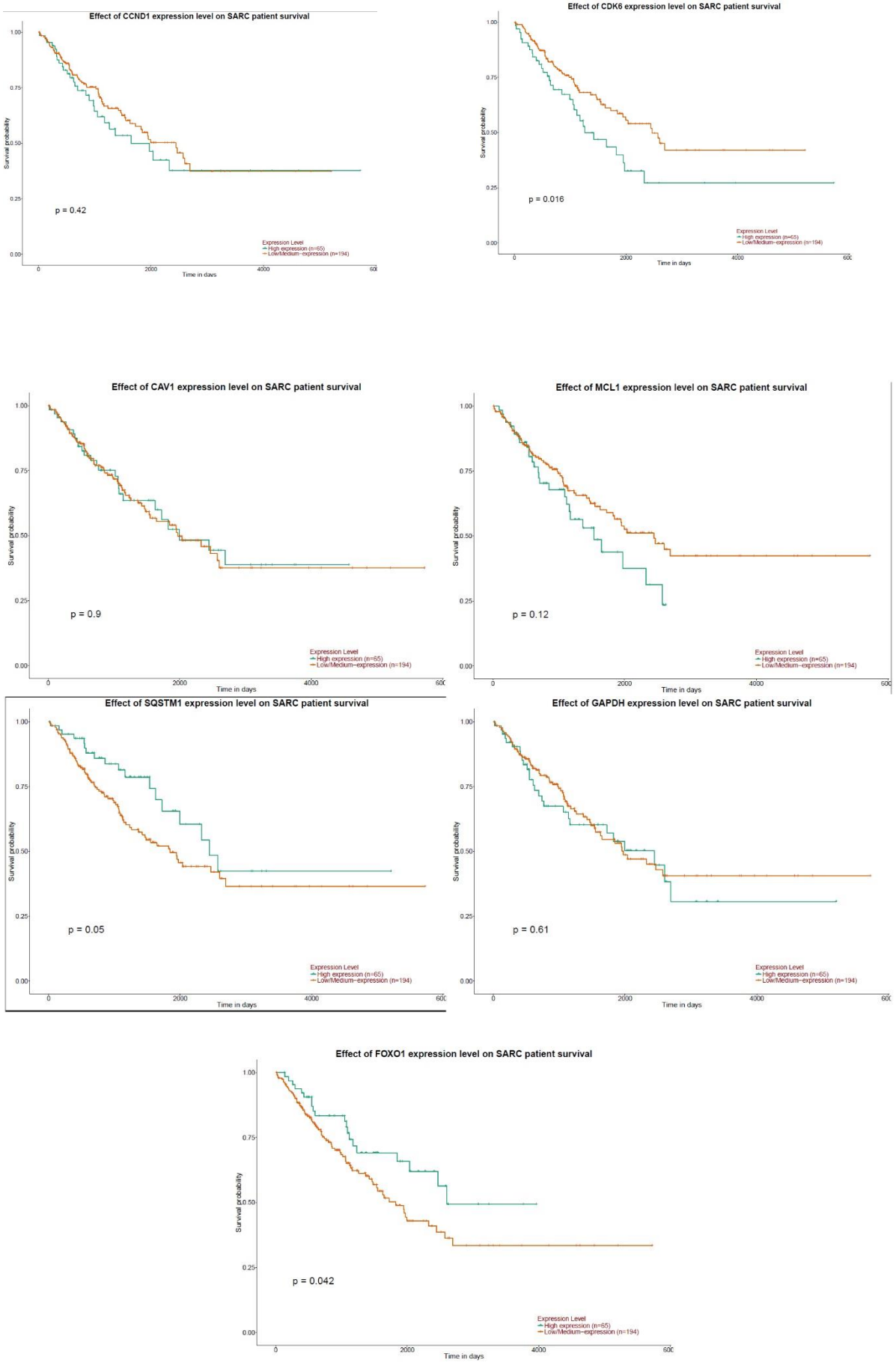


Figure 5. The prognostic value of the hub genes and their correlation with poor survival in sarcoma patients were assessed, revealing that CDK6 (p = 0.016) and FOXO1 (p = 0.042) were significantly associated with unfavorable prognosis in sarcoma patients.

Genomic Alteration Analysis of Hub Genes

The occurrence of genomic mutations has a strong correlation with tumorigenesis. As such, this research delved into the genomic mutations of the top seven hub genes in sarcoma patients. The findings revealed the presence of genetic alterations in CCND1, CAV1, CDK6, MCL1, SQSTM1, FOXO1, and GAPDH genes, which constituted approximately 1.5%, 2.6%, 0.8%, 3%, 2.8%, 2.4% and 2.1% of the mutations respectively. The genetic mutations observed encompassed various types such as missense, splice, truncating mutations, structural variants with uncertain implications, amplification, and deep deletion (Figure 6A). Moreover, there were notable dissimilarities in the genetic mutation types and frequencies of the seven hub genes in sarcoma (Figure 6B). This implies that these genetic mutations in central genes may have a crucial role in the development of tumors.

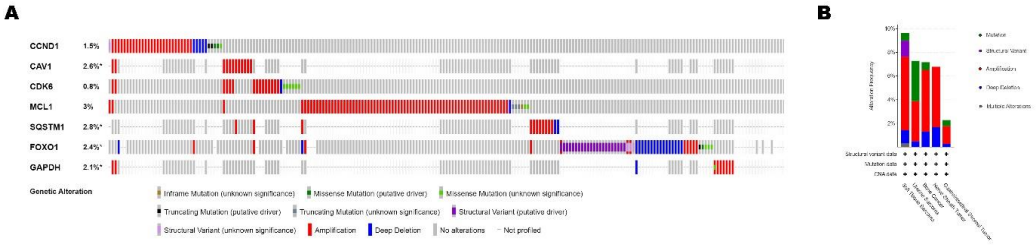
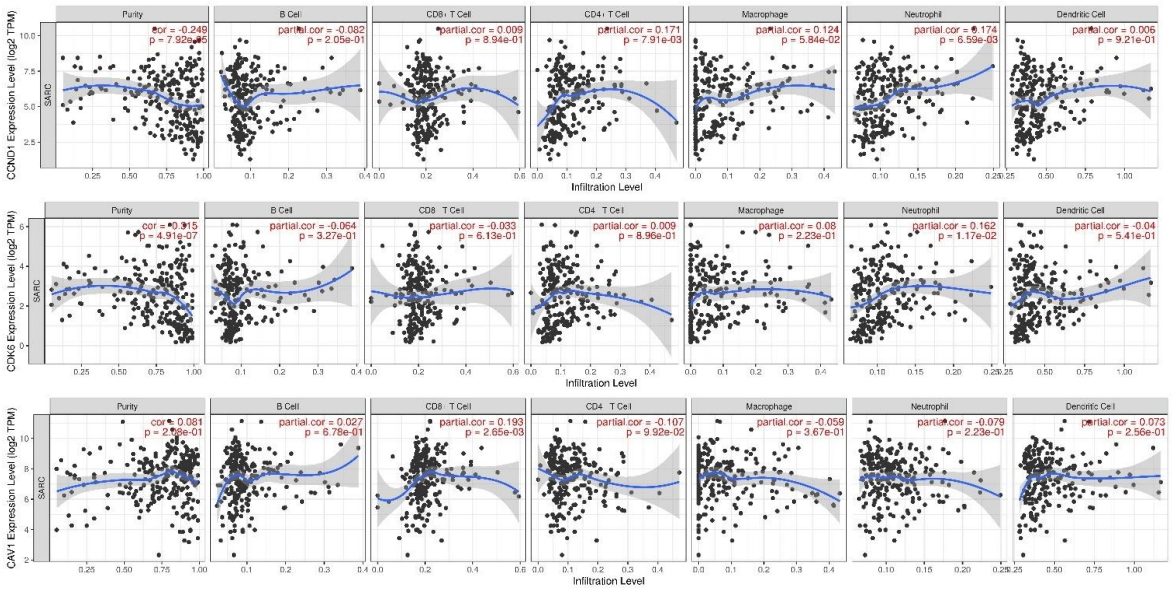


Figure 6. (A) The Genetic modifications linked to hub genes in the TCGA-Sarcoma dataset are visually summarized. This summary provides insights into the genetic alterations of seven hub genes in 2610 patients diagnosed with sarcoma. (B) The overall frequency of alterations in the seven hub genes among patients with sarcoma is presented.

Relationship Between Immune Cell Infiltration and Hub Genes

The infiltration of immune cells into tumors is a robust predictor of both cancer therapy and prognosis. We sought to explore the correlation between the expression of seven hub genes and the infiltration of various immune cells in sarcoma. Our findings revealed that all hub genes expression manifested a significant correlation with neutrophil cells, with the exception of CAV1. Moreover, CCND1, CDK6, MCL1, SQSTM1, and FOXO1 exhibited a significant positive correlation with neutrophil cells (r=0.174, r=0.162, r=0.416, r=0.14, r=0.144, respectively), whereas GAPDH alone demonstrated a negative correlation with neutrophil cells infiltration (Figure 7).



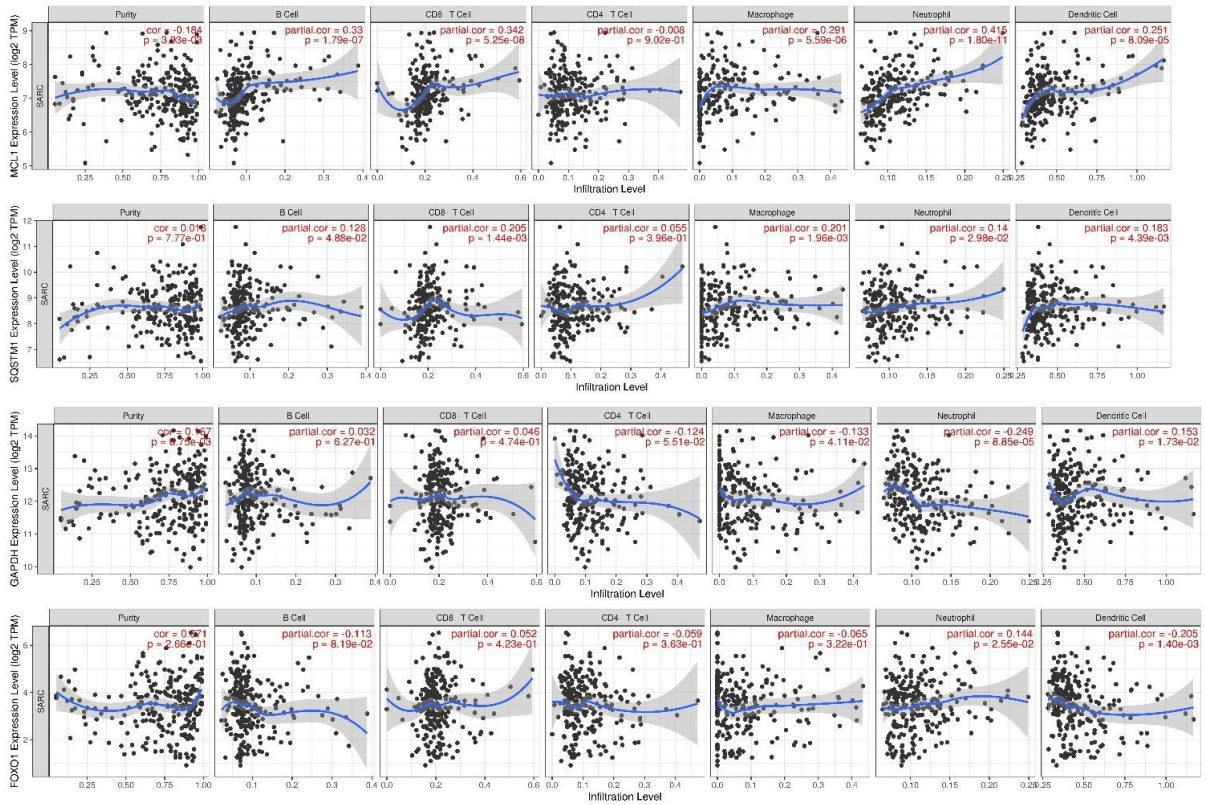


Figure 7. The present study aimed to investigate the relationship between the expression levels of hub genes and the populations of immune cells,

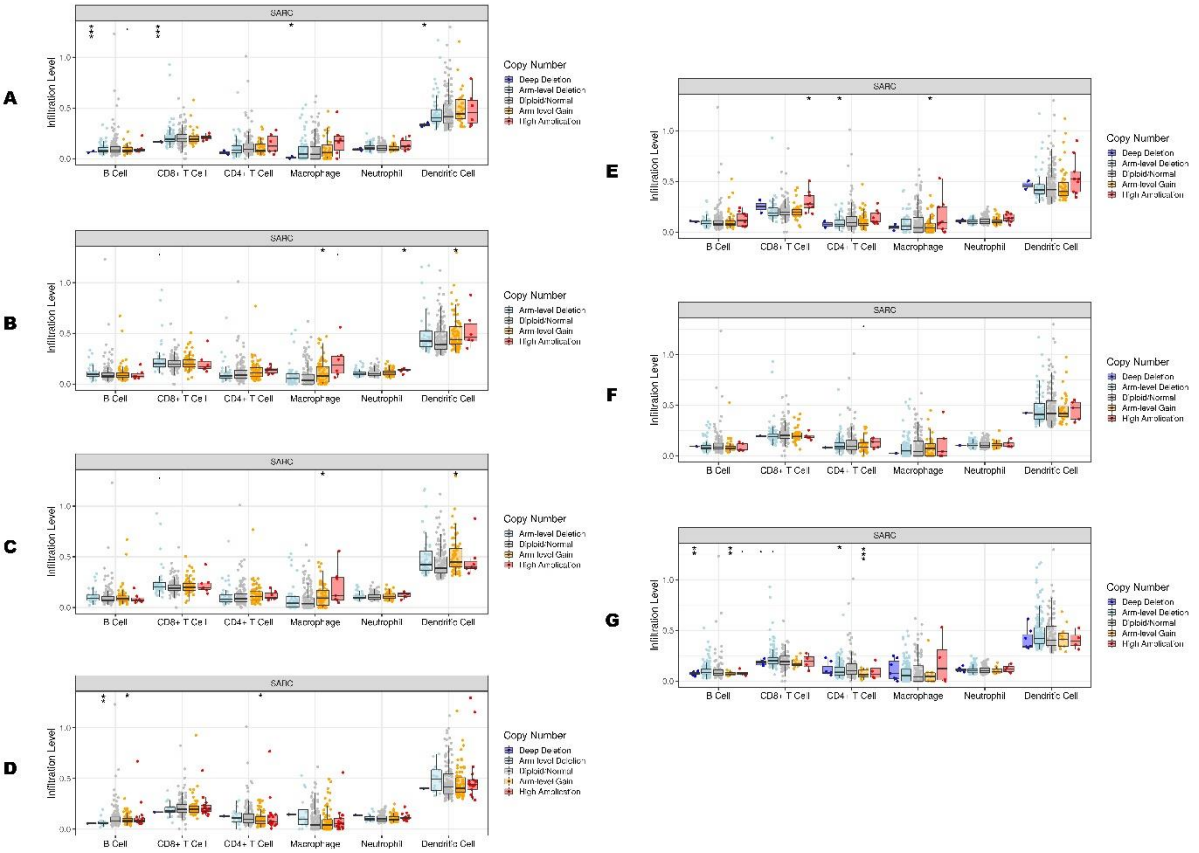
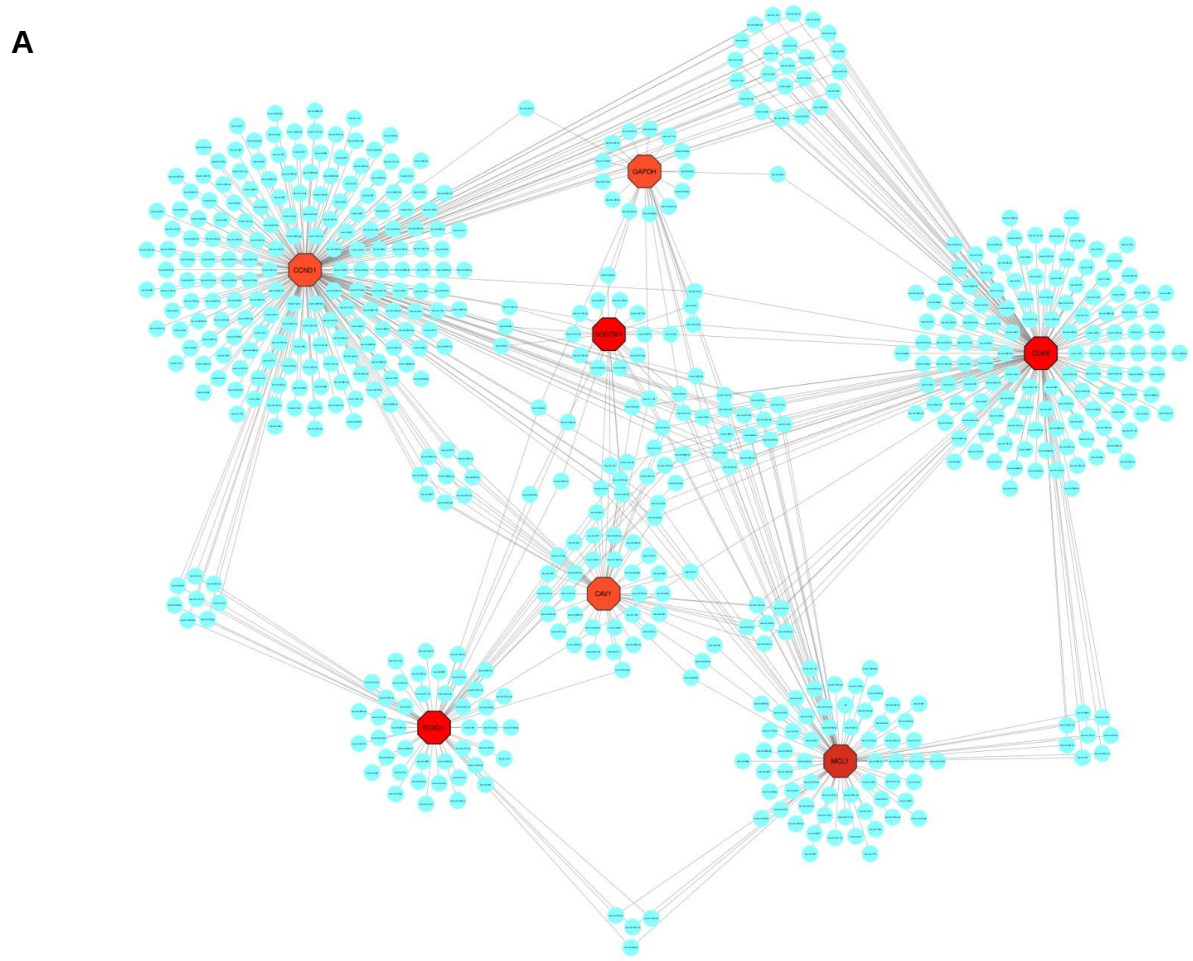


Figure 8. The SCNAs of the seven hub genes and immune cell infiltration in sarcoma were examined using GISTIC 2.0 in TIMER. (A) *CCND1*, (B) *CDK6*, (C) *CAV1*, (D) *MCL1*, (E) *SQSTM1*, (F) *GAPDH*, (G) *FOXO1*. The SCNAs of these genes were classified into five types in the genomic datasets. The infiltration of six immune cell types in sarcoma was analysed. The immune cell types included B cells, CD8+ T cells, CD4+ T cells, macrophage cells, neutrophil cells, and dendritic cells. The statistical significance was indicated by *P<0.05, **P<0.01, and ***P<0.001. SCNA

The widely acknowledged concept of the importance of immune surveillance in determining the prognosis of different types of cancers has prompted further investigation into the connection between the genomic measurements of the seven central genes and the level of immune infiltration in sarcoma. In order to accomplish this, the somatic copy number alterations (SCNAs) of these seven hub genes were established using GISTIC 2.0 in TIMER. Consequently, it was observed that there was a significant variation in immune cell enrichment in sarcoma depending on the gene SCNAs (Figure 8).Moreover, it was found that sarcoma with the SCNA of CDK6 exhibited a significant increase in macrophage, neutrophil, and dendritic cell infiltration (Figure 8B). Conversely, the SCNA of FOXO1 showed a significantly lower infiltration in B cell and CD4+ T cell (Figure 8G).

The Analysis of miRNA Targeting the Hub Genes

The miRNAs that target the hub genes were acquired from the NetworkAnalyst web-based database and were graphically represented using Cytoscape during this stage (Figure 9A). By using the cytoHubba plugin with multiple topological analysis (betweenness, and stress) we evaluated the essential miRNA. Topological analysis revealed that miR-15a-5p has a potential binding with CDK6 and FOXO1 gene (Figure 9B,C). To validate the accuracy of predicted miRNA, we subsequently analysed the correlation between mir-15a-5p expression with CDK6 and FOXO1 gene using miRNA-target co expression platform in ENCORI. The results of this validation denoted that mir-15a-5p has a significant positive correlation with



CDK6, in the other hand it negatively correlate with FOXO1 expression (Figure 9D,E).

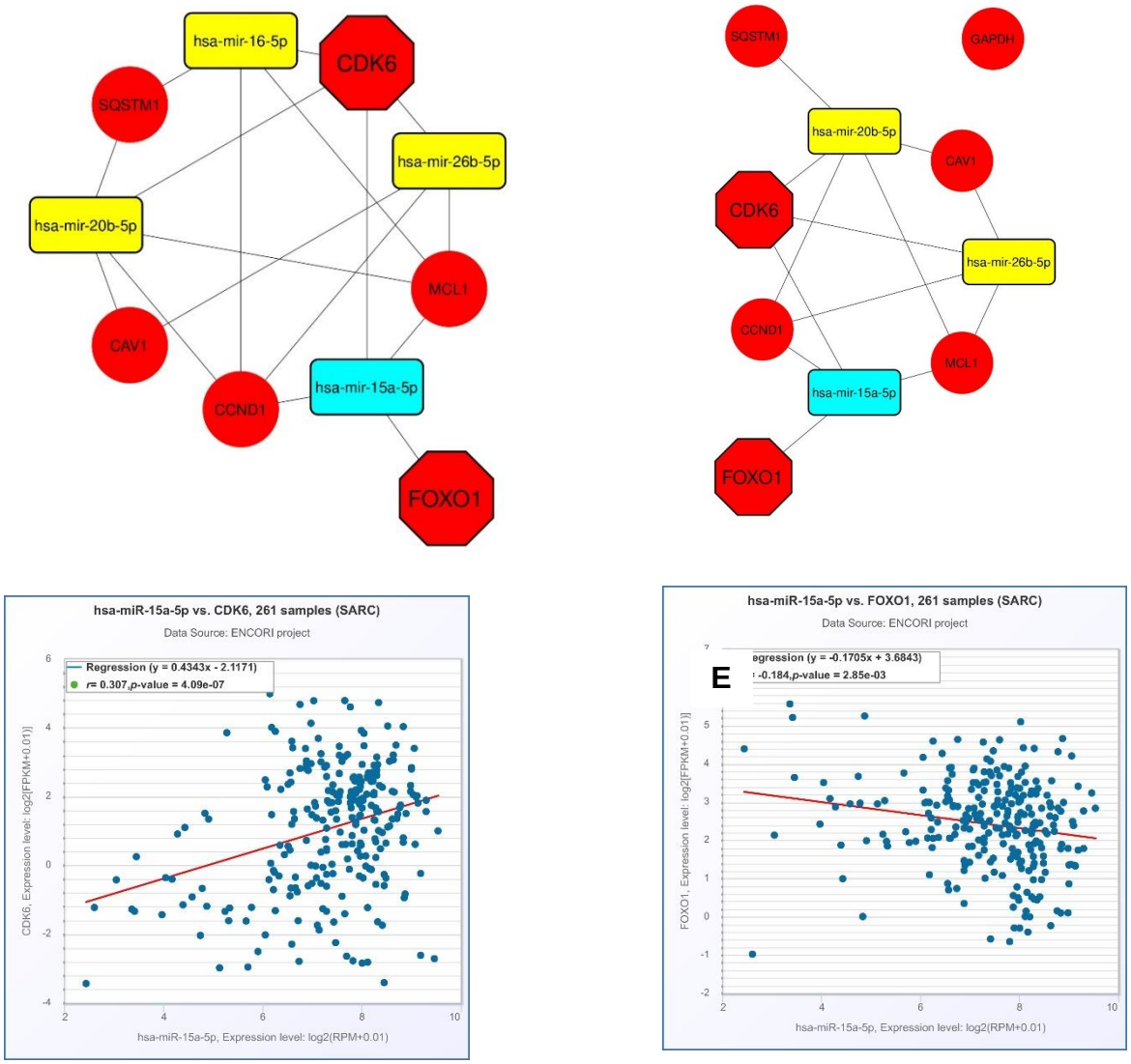


Figure 9. (A) The network of miRNA and the hub genes. Red hexagons show the hub gene, while the blue circle represent miRNA targeting the hub genes and (B,C) Red hexagons show the core hub genes (CDK6 and FOXO1) which associated with poor survival in sarcoma patients and blue rectangles show miRNA that has potential binding with them according to Cytohubba plugins (betweenness and stress). (D) Correlation between miRNA, miR-15a-5p expression and CDK6 show a significant positive correlation. (D) Correlation between miRNA, miR-15a-5p expression and FOXO1 show a significant negative correlation

DISCUSSION

Chondrosarcoma current treatment options are limited to surgery because the tumor tends to be resistant to chemotherapy (CT) and radiotherapy (RT). Previous study showed that surgery with RT or surgery with CT did not prolong the patients survival time, therefore new therapeutic approaches are urgently needed (Hua & Hu, 2020; Polychronidou et al., 2017). Recently, gene expression analysis has yielded significant advantages for oncological research due to advancements in sequencing technologies. In the present study, high-throughput datasets of CS (GSE30844 and GSE48418) were procured from the GEO database to facilitate subsequent comprehensive bioinformatics analyses.

The progression of CS-related DEGs were screened out and explored, and the related biological processes and signaling pathways that make a better understanding of their functions were also investigated. Terms like negative regulation of protein ubiquitination,

ISG15-protein conjugation, and autophagy were significantly enriched in biological processes. Ubiquitination is a molecular process occurring after protein translation, in which ubiquitin molecules are added to specific proteins, resulting in a remodeling effect. Ubiquitination is executed by a group of enzymes known as ubiquitin ligases, which transport ubiquitin molecules from an activating enzyme to a lysine residue located on the target protein. This process plays a critical part in regulating the cell cycle by targeting proteins for degradation, altering protein function, and regulating protein localization. Dysregulation of the ubiquitination modification led to uncontrolled cell cycle progression and eventually resulted in tumorigenesis (Dang et al., 2021).

ISG-15 encrypts an IFN-inducible, ubiquitin-like protein. ISG15 expression exposes heterogeneity in tumors, in addition to a small number of cancers, ISG15 expression is obviously upregulated in a substantial number of tumors, such as bladder cancer, breast cancer, and ovarian cancer (Nguyen et al., 2023). ISG15, similar to ubiquitin, has the ability to facilitate a modification resembling ubiquitin by covalently modifying other proteins, a process referred as ISGylation. The presence of ISG15 demonstrates a dual role in tumor promotion and suppression across various tumor systems, indicating the intricate nature of ISG15 regulation in tumorigenesis and development. In summary, ISGylation of proteins can enhance the progression of cancer by influencing signaling pathways such as Ki-Ras, EGFR recycling, and AKT in breast cancer, as well as being associated with cell proliferation in prostate cancer and pancreatic ductal adenocarcinoma. Furthermore, the effect of protein ISGylation on lung cancer has two roles; it can promote tumorigenesis and development by affecting the YAP-proteasome pathway but also inhibit tumor growth by affecting the ubiquitination and degradation of c-Myc (Yuan et al., 2023). Meanwhile, cervical cancer cell viability was inhibited by both interferon-alpha and ISG15-siRNA, suggesting that the IFN-alpha/ISG15/p53 axis may be a potential target for cervical cancer treatment (Zhou et al., 2017).

Autophagy, as a cellular phenomenon, upholds the equilibrium of normal cell functioning through the degradation and recycling of cellular constituents. Autophagy is also an important mechanism to prevent cancer development in both cell autonomous and non-cell autonomous methods (Mulcahy Levy & Thorburn, 2020). However, the role of autophagy in cancer is still controversial and depends on the stage of the tumor, biology, and the ambient microenvironment (Debnath et al., 2023). Autophagy may act as a tumor suppressor in the early stages of cancer development by removing damaged organelles and proteins that can cause DNA damage and mutations (Patergnani et al., 2021). In contrast, autophagy may act as a mechanism of cell survival for established tumors by providing nutrients and energy to cancer cells under stress conditions, such as hypoxia and nutrient deprivation (Mulcahy Levy & Thorburn, 2020; Patergnani et al., 2021). Numerous studies have demonstrated that suppressing autophagy can promote cancer cell death and sensitize cancer cells to chemotherapy and radiation therapy. For example, the promotion of chemotherapeutic agent-induced apoptosis during nutrient deprivation is facilitated by the suppression of autophagy in hepatocarcinoma cells. The growth of non-small cell lung cancer cells is inhibited by a small molecule that targets the autophagy kinase ULK1, thereby impacting both autophagy and apoptosis pathways. Suppression of autophagy by FIP200 deletion impairs DNA damage repair and increases cell death upon treatments with anticancer agents (Mulcahy Levy & Thorburn, 2020). The association between autophagy and cell proliferation in CS has recently been documented. Notably, autophagic inhibitors, such as 3-MA, hydroxychloroquine, and bafilomycin A1, have demonstrated the ability to interrupt drug-induced autophagy and reduce cellular proliferation in various human chondrosarcoma cell lines, including SW1353,

HCS-2/8, and Hs819T. In addition, the siRNAs directly against ATG3 were able to block antitumor drug-induced autophagosome formation in human chondrosarcoma cells (Reumann et al., 2016; Min et al., 2017).

The presence of abnormal cyclin expression and activity is often observed in malignancy, and these modifications are believed to contribute to the growth and advancement of neoplasms. The involvement of cyclins in the initiation of tumor formation can fluctuate depending on the particular cancer subtype and the phase of the ailment. However, some general roles of cyclin in tumorigenesis include promoting cell cycle progression, inhibiting apoptosis, promoting genomic instability, and DNA repair regulation (Casimiro et al., 2012). CDK6 belongs to the family of cyclin-dependent kinase proteins, which are essential for the regulation of the cell cycle. CDK6 plays a crucial role in facilitating the transition of cells from the G1 phase to the S phase, during which DNA replication takes place. The involvement of CDK6 in chondrosarcoma pathogenesis is believed to be of significant importance, as it promotes the growth and survival of tumor cells. Specifically, a previous study demonstrated that CDK6 facilitates cell cycle progression by phosphorylating and deactivating the retinoblastoma protein (RB), a tumor suppressor protein responsible for regulating the G1-S checkpoint. CDK6 has also been shown to promote angiogenesis, or the formation of new blood vessels, which is essential for tumor growth and metastasis (Fassl et al., 2022; Sherr et al., 2016).

The dysregulation of the CDKN2A-CCND-CDK4/6-Rb pathway is a prevalent phenomenon observed in various sarcomas. In the case of chondrosarcoma specifically, the tumor suppressors cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase inhibitor 2B (CDKN2B) play a significant role in the regulation of the cell cycle. These tumor suppressors encode p16 and p15Ink4b, respectively. They function by inhibiting cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and subsequently activating the retinoblastoma (Rb) family of proteins. The activation of these proteins leads to the prevention of progression from the G1 to S-phase of the cell cycle. Notably, deletions of CDKN2A and CDKN2B are frequently observed in chondrosarcomas and various other cancer subtypes. Palbociclib is an example of CDK4/6 inhibitor that has been approved for breast cancer patients. Meanwhile, a recent ongoing study still investigating the use of CDK4/6 inhibitor in chondrosarcoma patients (Tlemsani et al., 2023; Hsu et al., 2022).

FOXO (Forkhead box O1) exerts its influence on an array of cellular processes, encompassing developmental pathways, metabolic functions, stem cell preservation, and longevity. Several studies demonstrated that FOXO possessed the capacity to govern genes that are indispensable for the proliferation of cells, cessation of cell activity, ageing of cells, formation of blood vessels, movement of cells, and the spread of cancer to other parts of the body. It is widely acknowledged that FOXO plays a role in suppressing tumor development. The expression of FOXOs varies within different tissues and FOXO1, FOXO3, FOXO4, and FOXO6 are widely expressed in mammals (Farhan et al., 2017; Jiramongkol & Lam, 2020). FOXO1 is considered to be a key tumor suppressor due to its involvement in a broad range of cancer-related functions, including cellular differentiation, apoptosis, cell cycle arrest, and DNA damage (Gheghiani et al., 2020). Several tissue culture experiments have shown that FOXO1 is down-regulated in a wide variety of cancers such as breast, cervix, renal, and prostate cancer (Han et al., 2019). In gastric cancer, phosphorylated FOXO1 is correlated with higher overall survival and lower tumor angiogenesis, suggesting that active FOXO1 supports tumor suppression (Hornsveld et al., 2018). There is no clear evidence of FOXO1 involvement in chondrosarcoma development but a preliminary study showed FOXO1

involvement in another primary osseous malignancy. The upregulation of FOXO1 expression during the process of osteoblast differentiation and its decrease in osteosarcoma indicate a potential tumor suppressor role of this gene in the context of osteosarcoma. Meanwhile, suppression of FOXO1 is responsible for a growth regulatory repressive transcriptional sub-signature of EWS-FLI1 on ewing sarcoma (Guan et al., 2015). Our bioinformatic analysis has uncovered that the expression of FOXO1 is diminished in the CS group in comparison to the normal cartilage group. This discovery aligns with a prior investigation that showcased the tumor-suppressing capabilities of FOXO1. All these processes involving CDK6 and FOXO1 are appropriate with several cancer hallmarks including sustained proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, and avoiding immune destruction (Hanahan, 2022).

In our investigation, it was observed that miR-15a-5p exhibits the highest level of interaction with CDK6 and FOXO1. MiRNAs are known to regulate protein translation inhibition or targeted mRNA cleavage (Iorio & Croce, 2009). According to numerous studies, the search for biomarkers is focused on miRNA, which are involved in various cellular pathways, such as proliferation and differentiation by affecting the expression of their target genes (Van Rooij & Olson, 2012; Iorio & Croce, 2012; Bartel, 2009). Several studies have observed how miR-15a-5p was involved in cancer progression, including colon and lung cancer. The expression level of miR-15a-5p was significantly decreased in clinical colon tumor samples and cell lines suggesting that miR-15a-5p may act as a tumor suppressor (Z. Li et al., 2021). Meanwhile another study in lung cancer unveiling the role of miR-15a-5p in inhibiting metastasis by suppressing lipid metabolism via suppression of ACSS2 mediated acetyl-CoA activity and histone acetylation (Ni et al., 2020). However, there exists an absence of definitive evidence regarding the association between miR-15a-5p and the progression of CS. Therefore, additional experimental study is imperative in order to elucidate this matter.

CONCLUSION

In conclusion, through the utilisation of bioinformatics analysis, we have successfully detected seven resilient hub genes associated with the development of CS. These encompass CCND1, CDK6, CAV1, MCL1, SQSTM1, GAPDH, and FOXO1. This study demonstrated that CDK6 and FOXO1 genes exhibit a significant association with the unfavorable survival outcomes of patients diagnosed with sarcoma. These particular genes are closely connected with miR-15a-5p. Further experimental study is needed to reveal the potential role of CDK6, FOXO1, and miR-15a-5p in regulating CS progression.

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