

Comprehensive Analysis of Angiogenesis subtype of Squamous Cell Carcinoma

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Research

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Abstract

Background: Squamous cell carcinoma (SCC) is a disease with distinct management complexities as it displays a remarkably heterogeneous molecular subtype. However, the landscape of angiogenesis for SCC is not fully investigated.

Method and materials: The integrated analysis for the angiogenesis-related subtype for SCC and was performed using the ConsensusClusterPlus package based on angiogenesis-related genes and TCGA data respectively. We analyzed the alternation of the genes and miRNAs as well as pathways associated with angiogenesis. Next, we evaluated the prognostic value of the SCC subtype, the mode of regulation, the correlation with genomic characteristics, immune microenvironment, and clinical features of the angiogenesis subtypes.

Results: Totally, a total of 1368 SCC samples were included in this study. Two angiogenesis subtypes were then identified based on the one hundred and sixty-three angiogenesis-related genes with subtype1 of 951 SCC patients and subtype2 of 417 SCC. GSEA revealed that angiogenesis and epithelial-mesenchymal transition, inflammatory response, hypoxia was enriched in the subtype1 and suggested that the subtype 1 were angiogenesis-subtype of SCC. Eight of the 15 immune checkpoints (ADORA2A, BTLA, CD276, CYBB, HAVCR2, SIGLEC7, SIGLEC9, and VTCN1) were significantly up-regulated while C10orf54 were significantly down-regulated in the angiogenesis subtype. The survival analysis revealed that the patients in the angiogenesis subtype have a poorer survival outcome than that in the non-angiogenesis subtype ($P=0.017$ for disease free interval and $P=0.00013$ for overall survival).

Conclusion: Our analysis suggests that the importance of the angiogenesis pathway in SCC and may represent an underappreciated hallmark of SCC progression.

Introduction

Squamous cell carcinoma (SCC) represents the most common human solid tumor and is a major cause of cancer mortality [1]. The occurrence of these tumors and the disturbance of the genome, gene mutation, and/or squamous cell differentiation are closely related to the key changes in molecular expression at different stages. Fundamental changes in mesenchymal cells in the development of these tumors also play an important role, recent evidence suggests that they might even be a major determinant of promoting escape immune surveillance and chemotherapy drug resistance [2]. In recent years, more and more genomic research has promoted the development of clinical medicine. PAM50, a classification of breast cancer widely used in gene expression profiling, can divide different clinical outcomes into five subtypes [3]. On the other hand, changes in certain cancer-related genes EGFR and TP53 pathway are present in different cancers and subtypes [4]. These facts suggest that cancer treatment can benefit from pan-cancer analysis. Early diagnosis and treatment of SCC are of great importance because small early tumors have a good prognosis after treatment, and surgical resection is

considered to be the best treatment among many treatments [5]. Therefore, the discovery of new molecular mechanisms for clinical diagnosis and treatment from pan-cancer research is essential.

Angiogenesis is a change in the balance between pro-angiogenesis and anti-angiogenesis factors [6]. Increased angiogenesis is associated with tumor progression, metastasis, and outcome [7]. Numerous studies have shown that solid tumors are "angiogenesis-dependent" [8]. For example, in oral squamous cell carcinoma (OSCC), keratinocytes, and inflammatory cells directly produce a variety of molecules that can induce angiogenesis. Besides, in many different tumors including head and neck squamous cell carcinoma (HNSCC), the increased expression of VEGF protein may contribute to the induction of angiogenesis in tumors that secrete high levels of this cell [9, 10]. The relationship between angiogenesis and tumor is a recognized factor. However, the angiogenesis and the effect of SCC of the related research is still limited. If we are to fight the deadly disease from all aspects, to understand the potential of anti-angiogenesis therapy is very important.

In this study, we aimed to define here the molecular characterization of SCC by exploring the development of a categorization system that is based on the gene expression profile of angiogenesis genes.

Materials And Methods

Data download and preprocessing

TCGA pan-cancer data were downloaded from the UCSC Genome Browser (<https://genome.ucsc.edu>), including batch effects normalized gene expression transcription data, clinical data, single-sample gene set enrichment analysis (ssGSEA) score data, drug-target data, homologous recombination deficiency (HRD) score and genome-wide DNA damage data, immune signature scores data and RNA based stemness scores data. All data processing is described on the official website. The pan-cancer study combined with clinical data included a total of 1368 SCC samples, including 252 cervical squamous cell carcinomas (CESCs), 95 esophagi squamous cell carcinomas (ESCA), 520 head & neck squamous cell carcinomas (HNSCs), 501 lung squamous cell carcinomas (LUSCs).

Angiogenesis Subtypes

First of all, we obtained 507 angiogenesis genes from the AmiGO2 website (<http://amigo.geneontology.org/amigo>). Combined with gene expression data, 474 angiogenesis genes were finally obtained for analysis. Univariable Cox analysis was used to filter the angiogenesis genes which had the prognostic value for SCC patients ($P < 0.05$). Based on the prognostic angiogenesis-related genes, ConsensusClusterPlus R-package was used to identify subtypes in SCC tumor samples using 1000 iterations, 80% sample resampling from 2 to 7 clusters (k2 to k7) using kmdist with average linkage algorithm and correlation as the similarity metric.

Gene set enrichment analysis and pathway

To study the changes in gene sets, Gene Set Enrichment Analysis (GSEA) was performed on all [11]. We analyzed the correlations between angiogenesis subtypes with cancer hallmark pathways and the Encyclopedia of Genes and Genomes (KEGG) pathway in each tumor sample. GSEA can highlight genes associated with the subtypes through pathway analysis, even if this link is weak. After differential Expressed Genes (DEGs) analysis, the Gene Ontology (GO) analysis and KEGG pathway enrichment analysis were also performed.

Genomic correlations with angiogenesis subtypes

Aneuploidy and LOH Scores and ABSOLUTE purity/ploidy file were obtained from research by Thorsson, V et al. [12]. All purity, ploidy, LOH, and CNV invocation used to create the DNA damage scores utilized in this study as well as those summarized below were derived by the TCGA Aneuploidy AWG using ABSOLUTE[13], respectively. Moreover, HRD and HRD-loss of heterozygosity (HRD-LOH) score from the UCSC genome browser. The copy number burden fraction change and the number of segments represent the base fraction deviating from the baseline multiplicity and the total number of segments in each sample's copy number profile, respectively. Each fragment was designated as amplification, deletion, or neutral based on its number of copies relative to the circular ploidy of the sample. All data have calculated the correlations of angiogenesis subtypes (subtype1 and subtype2) by t-test. In addition, we calculated oncoplot, mutation panorama and OncogenicPathways based on TCGAmutation and maftools R packages.

Differentially expressed genes and regulation associated with angiogenesis

For the analysis of differentially expressed genes and miRNA, the Mann-Whitney U test was performed to derive the differential genes between subtype1 and subtype2 (FDR <0.05, absolute logFC > 1). Abnormal vascular networks due to tumor cells can secrete a large number of pro-angiogenesis factors, which is characterized by vascular disease, immaturity, and permeability [14]. To clarify the regulation of angiogenesis subtypes, we performed a computational analysis to identify two "master regulators": transcription factors (TF) and miRNA. For TF, we first downloaded 318 TFs from the Cistrome Bowser (<http://cistrome.org/>). Correlation test of 163 angiogenesis genes obtained from batch survival analysis with TF (person correlation: $R^2 = 0.4$, $P < 0.05$). For miRNA, use t-test to calculate the differential miRNA, (FDR=0.05, log FC > 1). And the resulting five miRNAs to the site of the target gene prediction TargetScan (http://www.targetscan.org/vert_72/). Each of the predicted miRNA target genes is the result of the correlation test which intersected angiogenesis genes. By TransmiR v2.0 database (<http://www.cuilab.cn/transmir>) of predicted miRNA each TF, and then intersected with the TF of the

correlation test. Finally, the TF-miRNA-target regulatory network was constructed using Cytoscape (<https://cytoscape.org/>) software.

The microenvironment in the angiogenesis subtypes

Tumor infiltrating leukocytes (TILs) have been shown to be associated with tumor prognosis and treatment response and are a crucial component of the tumor microenvironment [15]. CIBERSORT [16], a common computational method for quantification of cellular components by gene expression profiling (GEP) from bulk tissues. Therefore, we upload the gene expression data to the CIBERSORT website, which can be obtained free of charge (<https://cibersort.stanford.edu/>). we used a leukocyte gene signature matrix, termed LM22 and 1000 permutations. LM22 contains 547 genes, can be distinguished 22 kinds of human hematopoietic cell subtypes, including 7 types of T cells, naive and memory B cells, plasma cells, NK cells, and myeloid subpopulations[15]. We used the ssGSEA [17] which ranks the gene expression values of a given sample and then uses the empirical cumulative distribution function to calculate the enrichment score (ES) [18] method to calculate the enrichment score of angiogenesis genes in each sample. In the ssGSEA analysis, we used only genes associated with angiogenesis for our calculations. R packages have used GSVA, limma, and GSEABase. Application of the Estimating Stromal Cells and Immune Cells in Malignant Tumor Tissues Using Expression Data (ESTIMATE)[19] Algorithm to Calculate Stromal Cells, Immune Cells, and Estimated Scores.

The clinical implication of angiogenesis subtypes

The survival outcome of these patients with different subtypes was calculated by Log-rank test. To investigate whether there is a significant correlation between clinicopathological characteristics and SCC subtypes. We further studied the relationship between angiogenesis subtypes and gender, clinical stage (I~IV), tumor status, and histological grade (1~3). According to the grouping established by angiogenesis classification (K=2), subtype1 has 957 samples and subtype2 has 417 samples and analyzes the prognostic difference between the two groups of samples.

Statistical analysis

Pearson was performed to evaluate the immune signatures level for each sample. The enrichment levels of 68 immune signatures and angiogenesis were quantified by the heatmap R package. We compared the differences in drug target types between subtype1 and subtype2 and visualized the differences using the "pheatmap" R software package. A two-sided P value less than 0.05 were set as a statistical significance threshold. All the Analysis were performed based on R version 3.4.2 (2020-04, <https://www.r-project.org/>). The Student's t-test was used to compare subtype1 and subtype2 with differential expression genes.

Results

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Discussion

In this study, SCC cohorts were merged into a metadata set of 1368 patients, and one hundred and sixty-three angiogenesis genes were clustered based on batch survival analysis. A comprehensive analysis of SCC angiogenesis-related subtypes was performed using the ConsensusClusterPlus software package based on angiogenesis-related genes.

Angiogenesis is a complex process that plays a vital role in organ and tissue regeneration, growth and development, and many pathological conditions [20]. On the one hand, angiogenesis contributes to the development of malignant subtype traits. It is believed that the transition to the angiogenesis subtype is caused by the change in the balance of positive and negative regulators of angiogenesis [21]. On the other hand, tumors require a blood supply to grow and may be generated by the expression of pro-angiogenesis growth factors. SCC treatment is frustrating and prolongation of survival is slow. SCC is one of the main human common cancers. At present, the molecular mechanisms underlying squamous cell carcinoma and angiogenesis need to be further elucidated. Although in recent years it has made many SCC classification based on gene expression, but not yet a consensus on angiogenesis molecular taxonomy established. To determine the SCC subtypes associated with the angiogenesis process and good prognosis. This study established the SCC classification based on 474 angiogenesis genes screened from the AmiGO2 website. Two angiogenesis subtypes and non-angiogenesis subtype of subtypes of SCC were identified. The prognostic value of subtypes, regulation methods, immune infiltration, immune characteristics, and genome mutations were studied.

Results showed that subtype1 displayed distinct angiogenesis characteristics and subtype2 had a better prognosis. Angiogenesis was characterized by subtype1 than subtype2. EMT has been closely linked to 'stemness' in development and cancer [22]. Zhang et al study showed that endothelial cells secrete factor may be attracted to the epithelial tumor cells by blood vessels, allowing it to pass through the EMT connective tissue transfer, and to enhance its potential by imparting tumorigenic tumor cells involved in metastasis and proliferation of stem [23]. Inflammatory responses increase the risk of developing many types of cancer. The Hallmark of cancer-related inflammation includes inflammatory cells and tissue expression of inflammatory mediators (e.g., chemokines, cytokines, and prostaglandins), and tissue remodeling, and angiogenesis, chronic inflammatory diseases, and similar reactions and tissue repair [24]. Furthermore, hypoxia is a key factor in the tumor angiogenesis process. Studies have shown that tissue is upregulated by HIF-1a promoter steady-state reaction, to adapt to hypoxia, which may further facilitate tumor growth and tumor angiogenesis [25]. In summary, subtype1 is linked to the characteristics of angiogenesis closely and promotes the occurrence and development of cancer.

Also, cancer cells have various mechanisms to evade the local immune attack, including upregulation of immune checkpoint proteins [26]. Immune checkpoint proteins are activated by ligand-receptor, resulting in a dynamic balance between stimulatory and non-stimulatory, and inhibitory signals that regulate the immune response [27]. This phase of equilibrium is ensured primarily by the PD-1/PD-L pathway, which inhibits the activation and proliferation of T-cell as well as cytokine production. And the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) pathway, which induces cell cycle arrest and apoptosis in Tregs and T cells activation. However, once tumor antigens expressed by tumor cells can bypass the immune system, the balance is altered [26, 28] and the final result showed the progression of cancerous tumors in clinical practice [29]. We examined 15 immune checkpoint genes and showed that eight of the 15 immune checkpoint genes (ADORA2A, BTLA, CD276, CYBB, HAVCR2, SIGLEC7, SIGLEC9, and VTCN1) were significantly up-regulated, whereas C10orf54 was significantly down-regulated in subtype1. Therefore, angiogenesis subtype is closely associated with tumorigenesis and development. To be sure, the appropriate subtype classification may be key to the selection of appropriate adjuvant therapy.

Conclusions

In general, this study explored the angiogenesis landscape of SCC. Our analysis suggests that the importance of the angiogenesis pathway in SCC and may represent an underappreciated hallmark of SCC progression.

Abbreviations

SCC: Squamous cell carcinoma; OSCC: Oral squamous cell carcinoma; HNSC: Head and neck squamous cell carcinoma; ssGSEA: Single-sample gene set enrichment analysis; CESC: Cervical squamous cell carcinoma; ESCA: Esophagi squamous cell carcinoma; LUSC: Lung squamous cell carcinoma; HRD: Homologous recombination deficiency; GSEA: Gene set enrichment analysis; KEGG: Encyclopedia of genes and genomes; HRD-LOH: HRD-loss of heterozygosity; TF: Transcription factors; FDR: False

discovery rate; TCGA, The Cancer Genome Atlas; ESTIMATE: Estimation of STromal and Immune cells in Malignant Tumor tissues using Expression data; ECM: Extracellular matrix; E2F: E2 factor; EGF: Endothelial cells secrete factor; VEGF: Vascular endothelial growth factor; EMT: Epithelial-mesenchymal transition; MCY: Microcystin synthetase; TGF-beta: Transforming growth factor beta; CTLA-4: T-lymphocyte-associated antigen 4; Tumor infiltrating leukocytes (TILs); gene expression profiling (GEP)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Data and materials of this work are available from the corresponding author on reasonable request.

Competing interests

Not applicable

Funding

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Authors' contributions

KD, JW and ZT performed data analyses and helped prepare the manuscript. YS and JQ provided study materials. FQ, ZX, and LY conceived the research, determined the appropriate analyses to be performed, and wrote the manuscript. SL designed this study. All the authors read and approved the final manuscript.

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Not applicable

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

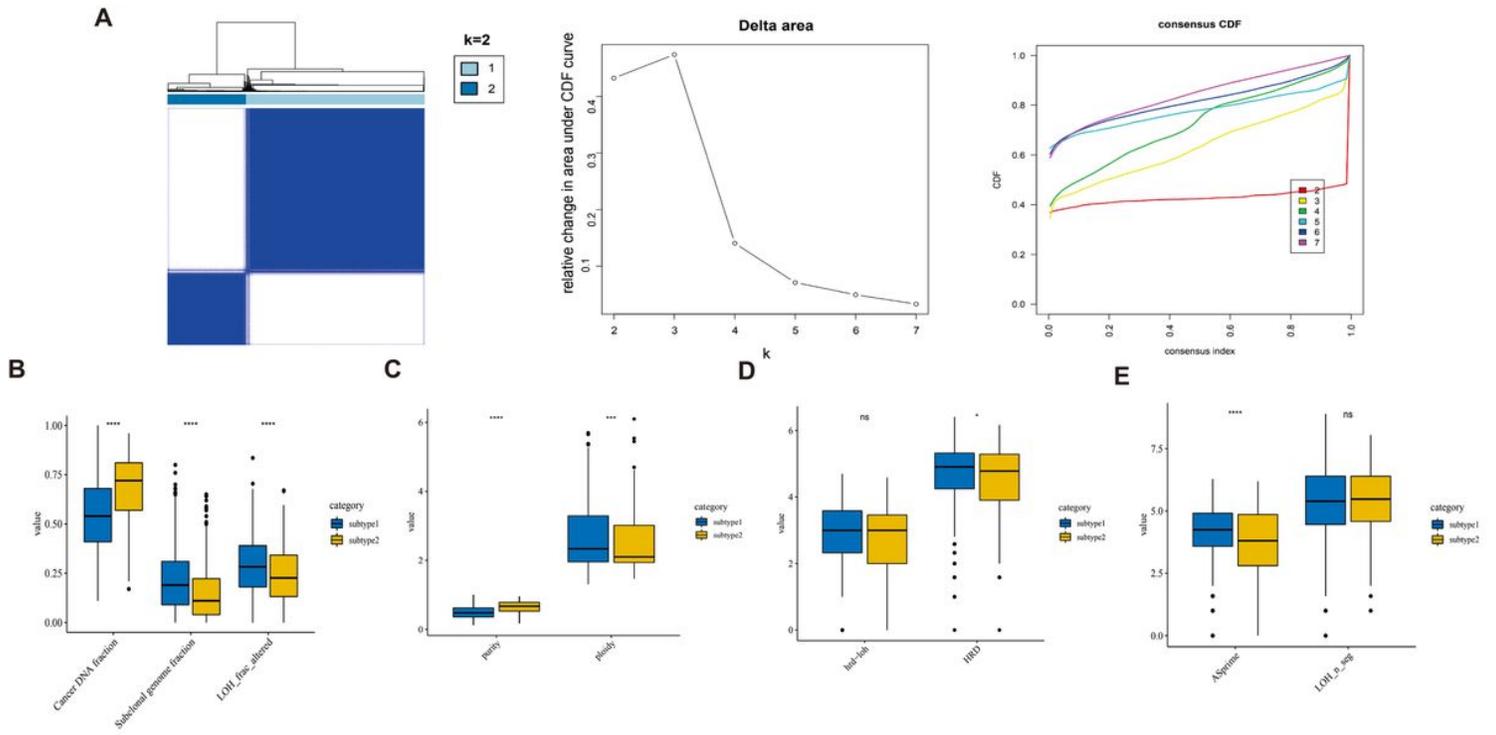


Figure 1

Clusters and genomic alteration of different subtypes in SCC. (A) Consensus Cluster for SCC patients based on prognostic angiogenesis genes. (B) DNA damage scores of different subtypes of SCC. (C) Tumor purity and ploidy of different subtypes of SCC. (D) Homologous recombination deficiency (HRD) and HRD-loss of heterozygosity (HRD-LOH) scores. (E) Prime loss of heterozygosity and fractions in different subtypes of SCC.

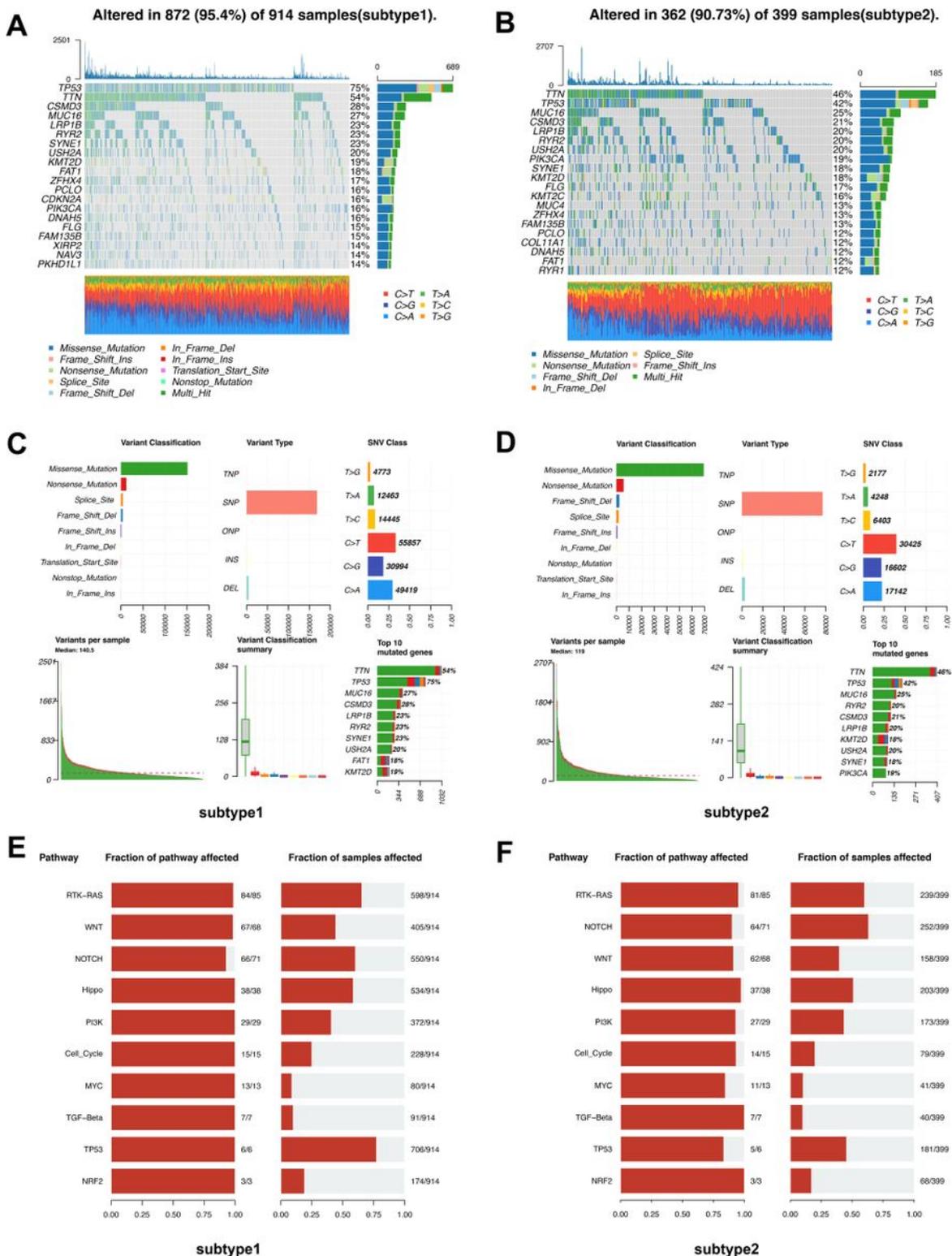


Figure 2

Mutations between two subtypes (A) The ratio of the top 20 genes in the number of mutations in subtype1. (B) The ratio of the top 20 genes in the number of mutations in subtype2. (C) The landscape of mutation panorama for the number of mutations and mutation types in subtype1. (D) The landscape of mutation panorama for the number of mutations and mutation types in subtype2. (E) The number of

mutated genes contained in the number of mutated samples per pathway in subtype1. (F) The number of mutated genes contained in the number of mutated samples per pathways in subtype2.

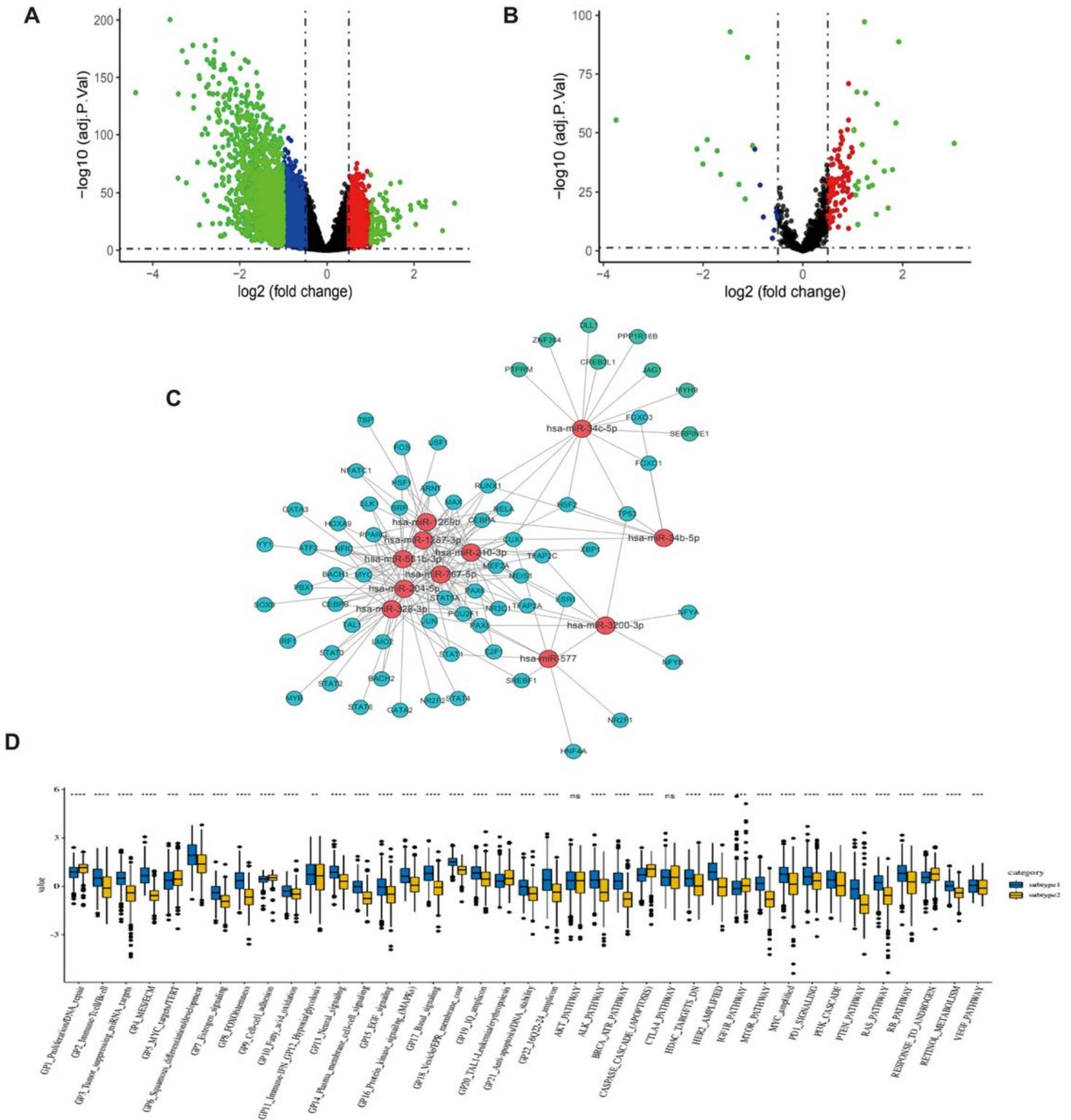


Figure 3

The regulation of genes and networks of angiogenesis subtype. (A) Differently expressed genes between angiogenesis subtype and non-angiogenesis subtype; (B) Differently expressed miRNAs between

angiogenesis subtype and non-angiogenesis subtype; (C) The regulation network of genes-miRNAs-TFs that were altered by angiogenesis.

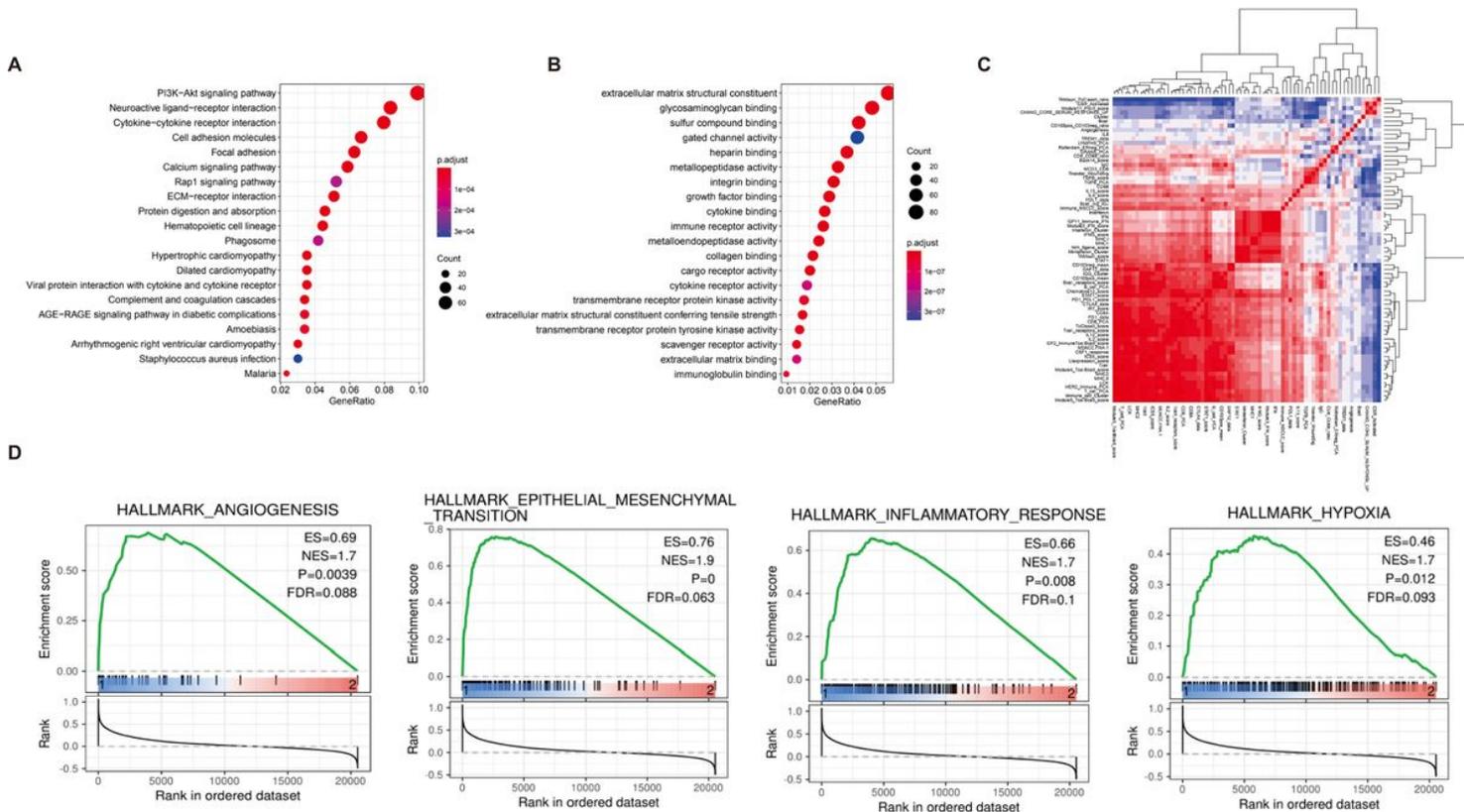


Figure 4

Alteration of pathways for the different subtypes of SCC. (A) KEGG pathway analysis associated with DEGs. (B) GO pathway analysis associated with DEGs. (C) The correlation of different pathways and angiogenesis subtype. (D) Four hallmark pathways of cancer enriched in angiogenesis subtype.

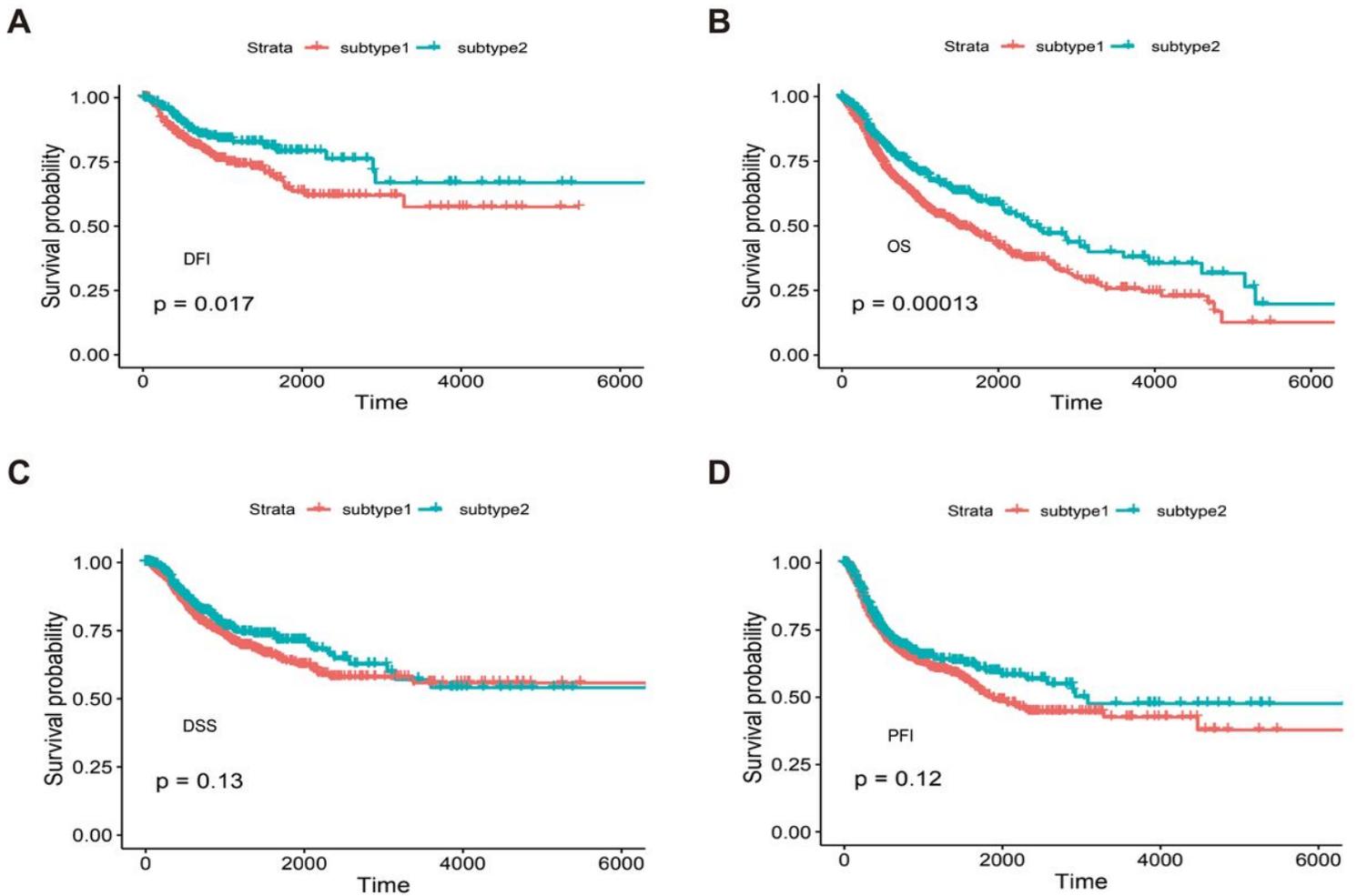


Figure 6

Prognostic of angiogenesis subtype for SCC patients. (A) Disease-free interval (DFI). (B) Overall survival (OS). (C) Disease-specific survival (DSS). (D) Progression-free interval (PFS).

Supplementary Files

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- [Table1.xlsx](#)