Identification of core genes of craniopharyngioma angiogenesis based on single-cell nuclear transcriptome sequencing

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Abstract

This study aimed to explore the core genes of craniopharyngioma angiogenesis for targeted vascular therapy based on single-cell nuclear transcriptome sequencing. For single-cell nuclear transcriptome sequencing, we collected six samples from the tumor center and adjacent hypothalamic tumor tissues from three patients with craniopharyngioma, as well as four normal brain tissues based on Gene Expression Omnibus. We screened genes with differential up-regulation between vascular endothelial cells of craniopharyngioma and those of normal brain tissues, performed GO and KEGG analysis, constructed the protein-protein interaction network, and selected key genes verified using immunofluorescence. After data cleaning and quality control, 623 craniopharyngioma endothelial cells and 439 healthy brain endothelial cells were obtained. Compared with normal brain endothelial cells, craniopharyngioma endothelial cells were screened for 394 differentially up-expressed genes (DEGs). GO and KEGG results showed that DEGs probably modulated endothelial cells, adhesen junction, focal adhesion, migration, actin cytoskeleton, and invasion via the PI3K-AKT, Rap1, Ras, Wnt, and Hippo pathways. The core genes screened were CTNNB1, PTK2, ITGB1, STAT3, FYN, HIF1A, VCL, SMAD3, PECAM1, FOS, and CDH5. This study obtained possible anti-angiogenic genes in cranio-pharyngioma. Our results shed novel insights into molecular mechanisms and craniopharyngioma treatment.

Keywords: Craniopharyngioma, Single-cell nuclear transcriptome sequencing, Angiogenesis, Differentially up-expressed genes (DEGs), Protein-protein interaction network (PPI), Enrichment.

1. Introduction

Craniopharyngioma is commonly found in the saddle area and originates from Rathke's capsule or residual pituitary epithelium. It is an intracranial congenital tumor occurring in children, which accounts for approximately 58.3% of childhood saddle area tumors [1]. Based on pathological histomorphology, it is classified as adamantinomatous craniopharyngioma (ACP) or papillary craniopharyngioma (PCP), with ACP accounting for >90% [2, 3]. Although craniopharyngiomas are histologically benign, they have a malignant and aggressive clinical presentation and are prone to attach to important brain architectures, such as the pituitary stalk, optic nerve, hypothalamus, large blood vessels at the skull base, and third ventricle. The main treatment of choice is still surgical resection, with the administration of postoperative adjuvant radiotherapy if necessary. Because it is adjacent to the hypothalamic-pituitary axis, the incidence of postoperative hypothalamic injury and hypothalamic dysfunction is as high as 80%, including a 55% incidence of severe hypothalamic obesity, and the probability of death due to cardiovascular events is 19 times higher than normal [4-6], seriously affecting patient life quality and survival time, making it challenging for neurosurgeons.

Continuous dense and disorderly angiogenesis is an important tumor characteristic closely associated with tumor genesis and supplies continuous oxygen and nutrients to the tumor, but it also carries away a large amount of metabolic waste and carbon dioxide, greatly promoting tumor growth and development. During tumor development, the “angiogenic switch” is almost always activated and kept open, causing the normal resting blood vessels to continuously grow new blood vessels to help maintain tumor spread and growth [7]. Therefore, targeting tumor angiogenesis is a trending topic in anti-tumor research. Previous studies have identified vascular endothelial growth factor (VEGF) as the main effector molecule in tumor angiogenesis, and many studies were conducted to examine the development of VEGF inhibitors. Although drugs, such as bevacizumab, have been successfully developed, their efficacy is not very satisfactory [8-10]. Therefore, further studies should be conducted to explore new anti-angiogenic therapeutic targets.

Craniopharyngioma is usually cystic–solid, with the solid part comprising tumor epithelium, stellate reticulum, palisading epithelium, and whorl-like epithelial cell clus-
Core genes in craniopharyngioma angiogenesis.


2. Materials and methods

2.1. Clinical information
We included three patients with craniopharyngioma from the Department of Neurosurgery, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University. Single-cell nuclear transcriptome sequencing was performed in the central and adjacent hypothalamic tumor tissues of each patient. The ethics committee of The First Affiliated Hospital, Jiangxi Medical College, Nanchang University approved our protocols. All subjects were compensated for the present work provided informed consent for information publication. Single-cell nuclear transcriptome sequencing dataset (GSE159416) of four normal brain tissues was acquired based on the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/).

2.2. Single-cell nuclear transcriptome sequencing
To guarantee RNA quality, the tissue samples underwent flash freezing within liquid nitrogen for 10 min after surgical removal. Nuclei extraction and snRNA-seq were conducted by BerryGenomics (Beijing, China). Subsequently, single-nuclear cDNA library was prepared (Chromium Single Cell 3’ version 3, 10× Genomics) following specific instructions. Thereafter, NovaSeq 6000 for Illumina sequencing was used for library running. Cell Ranger (v6.0.2) was used to process 10× chromium single-cell gene expression profiles. Moreover, the human reference genome (GRCh38) was used to align reads, assign barcodes, and count unique molecular identifiers.

2.3. Single-cell nuclear transcriptome sequencing data cleaning, quality control, and integration
We eliminated cells expressing >7500 or <200 genes and those containing >20% mitochondrial reads out of downstream analysis by using Seurat (v.4.0.3) [12]. After filtering, data were subjected to log normalization and scaling. Cell cycle and mitochondrial read percentage were removed [13]. Single-cell data were integrated using Seurat’s canonical correlation to avoid batch effects across experiments and samples. RunUMAP and UMAP functions were utilized for dimensionality reduction and visualization, respectively. The FindClusters function was also adopted for cell clustering, whereas cell density was defined using the top 30 principal components.

2.4. DEG identification, GO, and KEGG analyses
DEGs in craniopharyngioma endothelial cells versus healthy brain endothelial cells were filtered using the FindMarkers function. Only genes with logfc.threshold = log(2) and min.pct = 0.5 were considered DEGs. GO and KEGG analyses were performed using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/).

2.5. Construction of PPI network
DEGs were imported into the Search Tool for the Retrieval of Interacting Genes (STRING) to obtain gene interaction relationships. Subsequently, protein-protein interaction (PPI) network was constructed using Cytoscape software (Version 3.10.0). CytoNCA was used to predict the core genes.

2.6. Immunofluorescence
Craniohypophyseal specimens were fixed for 24-h using 4% paraformaldehyde. The specimens were paraffin-embedded, and 5-µm-thick sections were made. After conventional dewaxing to water, the sections were placed in sodium citrate antigen repair solution. Subsequently, the slides were incubated in 5% bovine serum albumin (BSA) under room temperature (RT) for 1 h. Following overnight incubation using primary antibodies CD31 (Servicebio, 1:100, Wuhan, China), CD34 (Servicebio, 1:200, Wuhan, China), CTNNB1 (Servicebio, 1:500, Wuhan, China), HIF-1α (Servicebio, 1:1000, Wuhan, China), and STAT3 (Servicebio, 1:300, Wuhan, China) under 4°C, the slides were rinsed thrice and incubated with secondary antibody under RT for 1 h. DAPI was used to stain cell nuclei.

2.7. Statistical analysis
Seurat or R software was used for statistical analysis. Statistical difference was considered with P<0.05.

3. Results

3.1. Neovascularization of craniopharyngiomas
Craniohypophyseal is usually cystic–solid, with the solid part comprising tumor epithelium, stellate reticulum, palisading epithelium, and whorl-like epithelial cell clusters. The typical pathological characteristics associated with craniopharyngioma are cells in whorl-like clusters.

Fig. 1. Immunofluorescence shows (A) CD34 and (B) CD31 expressions in numerous vascular endothelial cells between clusters of whorl-like cells in craniopharyngiomas (200×). (C) Nuclear aggregation of β-catenin protein in craniopharyngioma epithelial tumor cells (200×). (D) HE staining shows that the solid portion of the craniopharyngioma is mainly composed of fenestrated epithelial tumor cells, stellate reticular epithelial tumor cells, and whorl-like cell clusters, as well as a large number of keratinized proteins. Numerous new blood vessels can be seen among whorl-like cell clusters (200×).
showing strong nuclear and cytoplasmic expression of β-catenin. Moreover, numerous new blood vessels can be seen among whorl-like cell clusters (Figure 1).

3.2. Craniopharyngioma single-cell expression profiles and cell fractionation

After multiple quality controls, 59,432 high-quality craniopharyngioma single cells were obtained for downstream analysis, including 30,777 and 28,655 cells from the central tumor tissue and craniopharyngioma tissue adjacent to the hypothalamus, respectively (Table 1). The integrated gene expression profiles were downgraded and clustered with reference to classical marker gene expression into the following nine major cell clusters: astrocytes, B cells, endothelial cells, fibroblasts, tumor epithelial cells, myeloid cells, NK/T cells, neuronal cells along with oligodendrocytes, of which 623 were endothelial cells, accounting for 1.05% (Figure 2 and Table 2).

3.3. Single-cell expression profiles and cell fractionation in normal brain tissue

After multiple quality controls, 43,928 high-quality normal brain tissue single cells were obtained for downstream analysis (Table 1). Subsequently, the integrated gene expression profiles were downsampled and clustered. Based on the classical marker genes, they were mainly categorized into the following six major cell clusters: neuronal cells, astrocytes, microglia, oligodendrocytes, myeloid cells, and endothelial cells. We obtained 439 endothelial cells, representing 0.99% (Figure 2 and Table 3).

3.4. Integration of endothelial cell data and screening of DEGs in craniopharyngioma endothelial cells

We extracted 1062 high-quality endothelial cells, including 623 craniopharyngioma endothelial cells and 439 normal brain tissue endothelial cells. After multiple quality controls to eliminate batch and cell cycle effects, the data were integrated and entered into downstream data analysis (Figure 3). We used the FindMarkers function to

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Table 1. The number of high-quality cells obtained in each sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of high-quality cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td></td>
</tr>
<tr>
<td>Sample 1 (central tumor tissue)</td>
<td>12,320</td>
</tr>
<tr>
<td>Sample 2 (tumor tissue adjacent to the hypothalamus)</td>
<td>13,756</td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
</tr>
<tr>
<td>Sample 1 (central tumor tissue)</td>
<td>9716</td>
</tr>
<tr>
<td>Sample 2 (tumor tissue adjacent to the hypothalamus)</td>
<td>7809</td>
</tr>
<tr>
<td>Patient 3</td>
<td></td>
</tr>
<tr>
<td>Sample 1 (central tumor tissue)</td>
<td>8741</td>
</tr>
<tr>
<td>Sample 2 (tumor tissue adjacent to the hypothalamus)</td>
<td>7090</td>
</tr>
<tr>
<td>Normal brain 1</td>
<td>15,341</td>
</tr>
<tr>
<td>Normal brain 2</td>
<td>11,197</td>
</tr>
<tr>
<td>Normal brain 3</td>
<td>9072</td>
</tr>
<tr>
<td>Normal brain 4</td>
<td>8318</td>
</tr>
</tbody>
</table>

Table 2. Proportion of cells and marker genes in craniopharyngioma.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Count</th>
<th>Ratio(%)</th>
<th>Marker genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid cells</td>
<td>22,384</td>
<td>37.66</td>
<td>CD163, SLC11A1</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>8952</td>
<td>15.06</td>
<td>KRT14, KRT19, CDH1, EGFR</td>
</tr>
<tr>
<td>NK/T cells</td>
<td>8357</td>
<td>14.06</td>
<td>CD96, SKAP1, CD247</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>8323</td>
<td>14.00</td>
<td>GFAP, CLU</td>
</tr>
<tr>
<td>Neurons</td>
<td>5681</td>
<td>9.56</td>
<td>GAD1, SNAP25, MAP2</td>
</tr>
<tr>
<td>B cells</td>
<td>2456</td>
<td>4.13</td>
<td>BANK1, MS4A1</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>2022</td>
<td>3.40</td>
<td>CDK18, MOBP, MOG</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>634</td>
<td>1.07</td>
<td>COL1A1, COL3A1</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>623</td>
<td>1.05</td>
<td>VWF, CD34</td>
</tr>
</tbody>
</table>

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Fig. 2. (A) Based on the classical marker genes, craniopharyngiomas were mainly categorized into nine major cell clusters: astrocytes, B cells, tumor epithelial cells, endothelial cells, fibroblasts, myeloid cells, NK/T cells, neuronal cells, and oligodendrocytes. (B) Based on the classical marker genes, they were mainly categorized into six major cell clusters: neuronal cells, astrocytes, microglia, oligodendrocytes, myeloid cells, and endothelial cells.
screen 394 DEGs in craniopharyngioma endothelial cells.

3.5. GO and KEGG analyses

GO and KEGG analyses on 394 DEGs were performed through the DAVID online website. Based on GO annotation, biological processes were mainly related to angiogenesis, actin cytoskeleton organization, cell differentiation, cell-cell adhesion, and cell migration. Molecular functions were enriched in actin binding, protein binding, cadherin binding, GTPase activator activity, protein serine/threonine/tyrosine kinase activity, and beta-catenin binding. Cellular components were enriched in the cytoplasm, actin cytoskeleton, cell-cell junction, adherens junction, plasma membrane, nucleus, and cytoskeleton. Additionally, KEGG results showed that these DEGs possibly modulated endothelial cells, adherens junction, focal adhesion, migration, actin cytoskeleton, and invasion via the PI3K-AKT, Rap1, Ras, Wnt, and Hippo pathways (Figure 4).

3.6. PPI network establishment

Our study mapped DEGs into the STRING database for PPI analysis. Our established PPI network included 384 nodes and 1547 edges. Besides, the cytoNCA plug-in from Cytoscape was used to search 150 most significant genes. Cytoscape software was used to visualize the PPI network of the top 150 genes (Figure 5). Finally, the critical genes (CTNNB1, PTK2, ITGB1, STAT3, FYN, HIF1A, VCL, SMAD3, PECAM1, FOS, and CDH5) were selected as craniopharyngioma angiogenesis gene for further analysis.

3.7. Immunofluorescence analyses of ACP

Immunofluorescence was used to detect core gene expression in craniopharyngioma endothelial cells. CD31 was used to identify vascular endothelial cells, and CTNNB1, HIF-1α, and STAT3 were highly expressed in craniopharyngioma vascular endothelial cells (Figure 6).

4. Discussion

Since the emergence of single-cell transcriptome sequencing technology, tumor heterogeneity, and gene expression patterns can be investigated at a single-cell level [14, 15]. As single-cell transcriptome sequencing rapidly develops, its application in the study of tumor microenvi-
creatic cancer, and head and neck tumors [16-19]. Certain-
ly, single-cell transcriptome sequencing has greatly im-
proved tumor research into precision medicine and expanded
the dimension of tumor research.

Targeted vascular therapy accounts for a key way to
inhibit solid tumor growth, leading to hypoxic necrosis
within solid tumor tissues by blocking their blood sup-
ply [20]. Additionally, the efficacy of previous anti-tumor
angiogenic therapies targeting VEGF or its receptors has
been unsatisfactory. Therefore, new targeted angiogenic
therapies should be developed. Studies have shown that
tumor vascular endothelial cells have significant hete-
rogeneity and are significantly different from normal vas-
cular endothelial cells in terms of organization, structure,
function, and molecular markers [21-23]. In the future, if
molecules can be developed to target these tumor vascular
endothelial cell-specific molecules, the efficacy of targeted
vascular therapies will greatly improve with reduced side
effects.

Craniopharyngiomas are characterized by malignant and
aggressive growth, often invading the hypothalamus with
finger-like structures. The rate of total surgical resec-
tion is low, and postoperative hypothalamic damage is se-
vere, but effective targeted therapies are limited. This study
focused on craniopharyngioma neovascularization. Many
neovascularized tumor vessels were found between the
characteristic whorl-like structures of craniopharyngiomas.
Targeting and intervention of these neovascularizations are
important in treating craniopharyngiomas. Therefore,
we further compared gene expression differences between
craniopharyngioma vascular endothelial cells and normal
brain endothelial cells and screened genes that are character-
istically up-regulated in craniopharyngioma endothelial
cells. Based on GO and KEGG results, these DEGs might
mainly regulate endothelial cells, adherens junction, focal
adhesion, migration, actin cytoskeleton, and invasion via
PI3K-AKT, Rap1, Ras, Wnt, and Hippo pathways. Finally,
we screened the following endothelial core genes of
craniopharyngioma: CTNNB1, PTK2, ITGB1, STAT3,
FYN, HIF1A, VCL, SMAD3, PECAM, FOS, and CDH5.
Immunofluorescence was used to confirm that some of
these genes were highly expressed in craniopharyngioma
endothelial cells. Thus, these genes can be potential targets
for targeted vascular therapy of craniopharyngioma. This
study mainly provided a reference for craniopharyngioma
angiogenesis from the perspective of bioconfidence analy-
sis, but more research is necessary for verification.

5. Conclusion
In this study, the characteristic genes of craniopharyn-
gioma endothelial cells were screened based on single-cell
transcriptome sequencing analysis, which will provide a
reference for subsequent research on craniopharyngioma
angiogenesis and targeted vascular therapy.

Conflict of Interests
The author has no conflicts with any step of the article
preparation.

Consent for publications
The author read and approved the final manuscript for
publication.

Ethics approval and consent to participate
This study was approved by the ethics committee of The
First Affiliated Hospital, Jiangxi Medical College, Nan-
chang University.

Informed consent
Signed written informed consents were obtained from the
patients and/or guardians.

Availability of data and material
The data that support the findings of this study are available
from the corresponding author upon reasonable request.

Supplemental material
Supplemental material for this article is available online.

Author contribution statement
Jinshi Zhang, Lin Xu, Jiye Ye, Chunming Xu, Bowen Wu,
Jie Wu, Tao Hong were responsible for study conception
and design; experimental implementation; data analysis
and interpretation; reagent, analytic tool, material or data
 provision; and manuscript writing.

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References
1. Kraus R, Schouten-van MA, Finken M, Oostdijk W, van Trot-
of postoperative fluctuations in plasma sodium concentration
after pediatric brain tumor surgery in the sellar region: a national
0886-2
2. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-
Organization Classification of Tumors of the Central Nervous
10.1007/s00401-016-1545-1
3. Muller HL, Merchant TE, Warmuth-Metz M, Martinez-Barbera
5:75. doi: 10.1038/s41572-019-0125-9
4. Daubenbuchel AM, Muller HL (2015) Neuroendocrine Disorders
doi: 10.3390/jcm4030389
Outcome of craniopharyngioma in children: long-term complica-
tions and quality of life. Dev Med Child Neurol 46:220-229. doi:
10.1017/s0012162204000374
bidity in adult craniopharyngioma. Pituitary 16:46-55. doi:
10.1007/s11102-012-0428-2
Therapy: Moving Beyond Vascular Endothelial Growth Factor.
petic agents in cancer: Challenges and future directions. Eur J
Pharmacol 793:76-81. doi: 10.1016/j.ejphar.2016.10.039


