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Original Article

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, adherens 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 craniopharyngioma. 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-

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ters [11]. Our study showed increased neovascularization among whorl-like cell clusters, which is important clinical guidance in investigating the mechanism of craniopharyngioma neovascularization. Recently, as single-cell sequencing develops rapidly, the mechanism of tumor development should be shown at a single-cell level. We screened differentially up-expressed genes (DEGs) in craniopharyngioma vascular endothelial cells versus healthy brain vascular endothelial cells based on single-cell nuclear transcriptome sequencing analysis. We aimed to provide new ideas for subsequent exploration of molecular mechanisms of craniopharyngioma angiogenesis and potential targeted vascular therapy.

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 not 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

Craniopharyngioma specimens were fixated 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

Craniopharyngioma 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

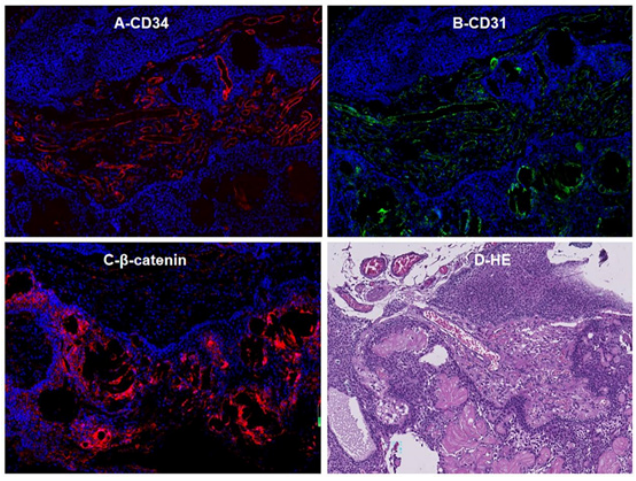

Fig. 1. Immunofluorescence shows (A) CD34 and (B) CD31 expressions in numerous vascular endothelial cells between clusters of whorl-like cells in craniopharyngiomas (200×). (C) Nuclear aggregation of β-catenin protein in craniopharyngioma epithelial tumor cells (200×). (D) HE staining shows that the 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 downscaled 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

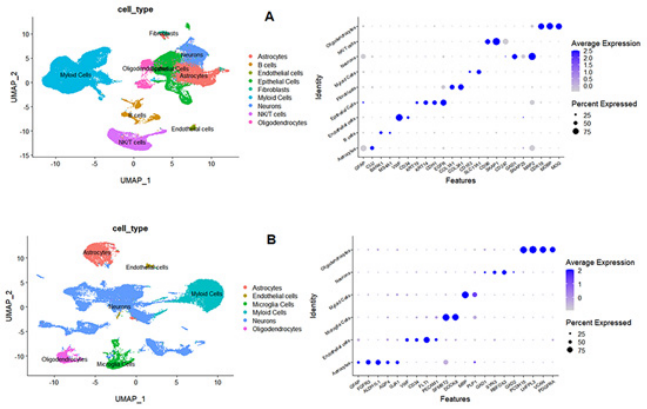

Fig. 2. (A) Based on the classical marker genes, craniopharyngiomas were mainly categorized into nine major cell clusters: astrocytes, B cells, tumor epithelial cells, endothelial cells, fibroblasts, myeloid cells, NK/T cells, neuronal cells, and oligodendrocytes. (B) Based on the classical marker genes,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

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

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

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

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

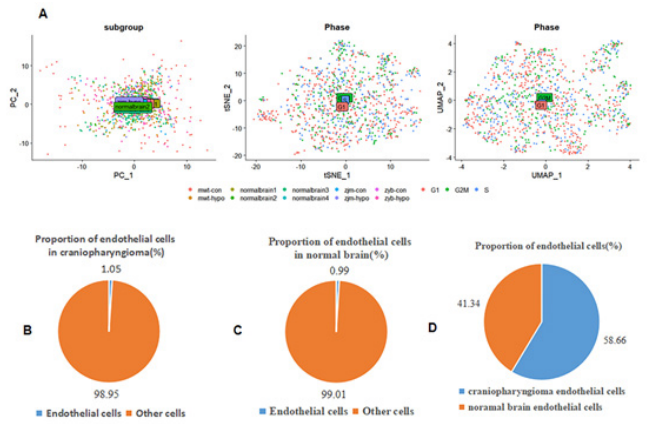

Fig. 3. Elimination of batch effects and cell cycle effects by craniopharyngioma endothelial cells versus normal brain tissue endothelial cells (A). Proportion of endothelial cells in craniopharyngioma (B), normal brain(C), and craniopharyngioma versus normal brain tissue (D).

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-1A, 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 microenvironment has been gradually highlighted. Recently, single-cell transcriptome sequencing has been adopted to resolve the effect of tumor microenvironment on tumor progression, such as lung cancer, nasopharyngeal carcinoma, pan-

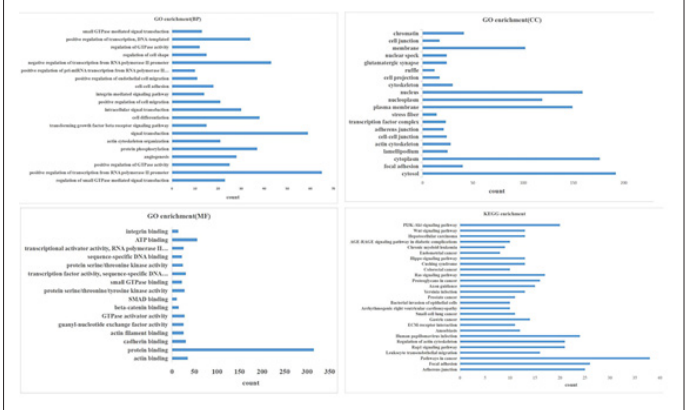

Fig. 4. GO and KEGG enrichment analyses of 394 differentially expressed up-regulated genes in craniopharyngioma endothelial cells.

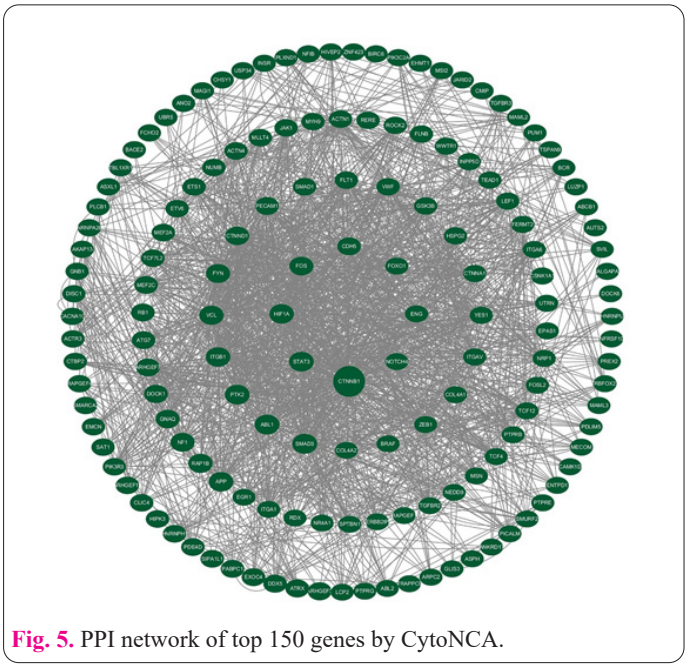

Fig. 5. PPI network of top 150 genes by CytoNCA.

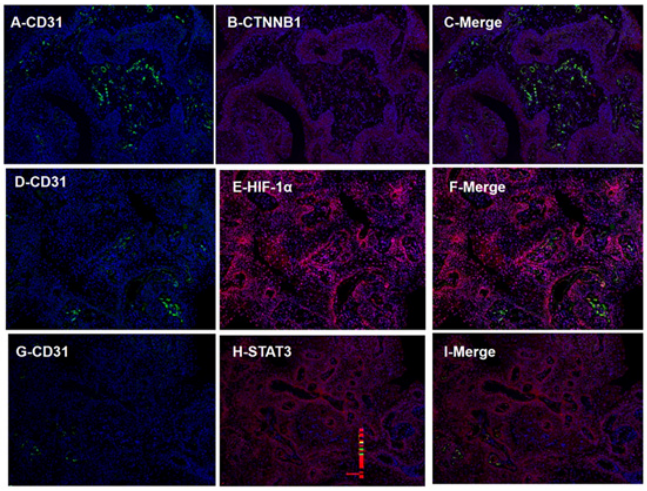

Fig. 6. Immunofluorescence analyses of ACP: (A-C) CD31 and CTNNB1, (D-F) CD31 and HIF-1α, and (G-I) CD31 and STAT3 expression levels were determined in craniopharyngioma endothelial cells with DAPI (200×).

creatic cancer, and head and neck tumors [16-19]. Certainly, single-cell transcriptome sequencing has greatly improved 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 supply [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 heterogeneity and are significantly different from normal vascular 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 resection is low, and postoperative hypothalamic damage is severe, 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 characteristically 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 analysis, but more research is necessary for verification.

5. Conclusion

In this study, the characteristic genes of craniopharyngioma 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, Nanchang 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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