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Transcriptomic Comparison of Lissencephaly-related Genes: From Correlation to Clinical Relevance

Lizensefali ile İlişkili Genlerin Transkriptomik Karşılaştırması: Korelasyondan Klinik Öneme

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Abstract

Objective: Lissencephaly is a neurodevelopmental disorder resulting from genetic and cellular defects in neuronal migration processes. In this study, correlation-based gene networks were analyzed to understand the functional and cellular relationships of lissencephaly-associated genes.

Methods: Hierarchical clustering and genetic network analyses based on Jaccard indices and Spearman's rank correlation were performed using fetal brain gene expression data from Allen Brain Atlas. These analyses identified functional similarities between gene pairs and suggested potential shared roles in cortical developmental processes.

Results: Hierarchical clustering analysis confirmed known associations, such as those between tubulins and microtubule-associated genes involved in migration and layering processes. Genes that relay extracellular signals to the migrating cells, such as Reelin and very low-density lipoprotein receptor, were observed to form a separate group. Using t-distributed stochastic neighbor embedding analyses of single-cell RNA sequencing data, specific expression patterns of these genes in brain cellular subpopulations were compared. Correlation network graphs showed that genes such as *PAFAH1B1* and *TUBG1* play central roles in cortical development, whereas genes such as *CRADD* and *ARX* have more specific functions.

Conclusion: These findings explain the relationships of lissencephaly-related genes with each other and their functional connections in neuronal migration processes. This study provides new perspectives for understanding the mechanisms of cortical development and points to previously unknown associations.

Keywords: Cortical development, neuronal migration, brain development, transcriptome, gene expression networks

Öz

Amaç: Lizensefali, nöronların göç süreçlerindeki genetik ve hücresel kusurlardan kaynaklanan bir nörogelişimsel bozukluktur. Bu çalışmada, lizensefaliyle ilişkili genlerin fonksiyonel ve hücresel ilişkilerini anlamak amacıyla korelasyon temelli gen ağları analiz edilmiştir.

Yöntem: Allen Beyin Atlası'ndan elde edilen fetal beyin gen ekspresyon verileri kullanılarak, Jaccard indeksleri ve Spearman sıralama korelasyonuna dayanan hiyerarşik kümeleme ve genetik ağ analizleri gerçekleştirilmiştir. Bu analizler, gen çiftleri arasında fonksiyonel benzerlikleri belirlemiş ve kortikal gelişim süreçlerinde olası ortak roller olduğuna işaret etmiştir.

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Öz

Bulgular: Hiyerarşik kümeleme analizi, göç ve tabakalaşma süreçlerinde rol oynayan tübülinler ile mikrotübül ilişkili genler arasındaki bilinen ilişkileri doğrulamıştır. Reelin ve çok düşük yoğunluklu lipoprotein reseptörü gibi dış sinyalleri göç etmekte olan hücrelere ileten genlerin ise ayrı bir grup oluşturduğu gözlenmiştir. Tek hücreli RNA dizileme verilerinin t-dağılımlı stokastik komşu gömme analizleri kullanılarak, bu genlerin beyin hücresel alt popülasyonlarındaki spesifik ekspresyon paternleri karşılaştırılmıştır. Korelasyon ağı grafiklerinde, *PAFAH1B1* ve *TUBG1* gibi genlerin kortikal gelişimde merkezi roller oynadığı, *CRADD* ve *ARX* gibi genlerin ise daha spesifik işlevlere sahip olduğu gösterilmiştir.

Sonuç: Bu bulgular, lizensefali ile ilişkili genlerin birbirleriyle olan etkileşimlerini ve nöron göçü süreçlerindeki fonksiyonel bağlantılarını açıklamaktadır. Ayrıca bu çalışma, kortikal gelişim mekanizmalarını anlamaya yönelik yeni bakış açıları sunmakta ve daha önce bilinmeyen ilişkilere işaret etmektedir.

Anahtar Kelimeler: Kortikal gelişim, nöronal migrasyon, beyin gelişimi, transkriptom, gen ifade ağları

Introduction

Neuronal migration is a fundamental process during brain development, crucial for the proper organization and functionality of the cerebral cortex. Disruptions in this process can lead to various developmental brain diseases, including autism spectrum disorders, schizophrenia, and epilepsy. Studies have shown that aberrant neuronal migration contributes to the pathogenesis of these disorders, characterized by mislocalized neurons and disrupted cortical lamination[1-4]. The mechanisms underlying these migration defects often involve genetic and molecular factors, such as mutations in genes like *PAFAH1B1* and *DCX*, which are essential for cytoskeletal organization and neuronal positioning[5,6]. Furthermore, environmental factors, including in utero exposure to infections or trauma, can also influence neuronal migration, leading to malformations of cortical development[7,8]. Understanding the intricate signalling pathways and cellular interactions that govern neuronal migration is critical for elucidating the etiology of neurodevelopmental disorders and developing potential therapeutic strategies[9,10].

The discovery of genes associated with lissencephaly has significantly advanced our understanding of the molecular pathways involved in neuronal migration and cortical development. Lissencephaly, characterized by an abnormally smooth cerebral cortex due to impaired neuronal migration, has been linked to several critical genes, including *PAFAH1B1*, *DCX*, and *TUBA1A*, which encode proteins essential for cytoskeletal organization and neuronal positioning during embryogenesis[11-13]. For instance, mutations in the *PAFAH1B1* gene, located on chromosome 17p13, have been shown to disrupt microtubule dynamics, leading to severe forms of lissencephaly[14,15]. Similarly, the identification of *DCX* as a key player in X-linked lissencephaly has highlighted the role of microtubule-associated proteins in guiding neuronal migration[16,17]. Recent studies have also uncovered additional genes, such as *CEP85L*, which further elucidate the genetic landscape of lissencephaly and its phenotypic variability[18,19]. These genetic insights have not only improved diagnostic capabilities but have also provided a framework for understanding the complex signalling pathways that regulate neuronal migration, thereby paving the way for potential therapeutic interventions in related neurodevelopmental disorders[19,20].

TUBG1, *PAFAH1B1*, and *DAB1* genes play critical roles in different stages of neuronal migration. *TUBG1* regulates cell polarity by participating in microtubule organization and plays an important role in the initial stages of migration. *PAFAH1B1* supports nuclear movements by interacting with microtubule motor proteins and functions in the intermediate stages of migration. *DAB1* ensures correct placement of neurons by participating in the RELN signalling pathway and is effective in the final stages of migration. It is known that these genes are important in understanding the mechanisms of neuronal migration and in elucidating the genetic basis of neurodevelopmental diseases such as lissencephaly.

The expression weighted cell type enrichment (EWCE) method is a computational approach designed to analyse gene expression data by identifying the specific cell types that contribute to the expression of a given gene list. This method leverages single-cell transcriptomic data to provide insights into the cellular composition underlying various biological conditions and diseases. By applying the EWCE method, researchers can interrogate gene lists derived from diverse sources, such as mouse phenotyping studies, to uncover the cell types associated with specific phenotypes or disorders, including major brain disorders like schizophrenia and Alzheimer's disease[21].

Gene-correlation analyses, which can be performed using various statistical methods, play a crucial role in generating

hypotheses about gene function and interactions. For instance, methods like gene set net correlation analysis allow researchers to assess changes in the correlation structure of gene sets across different conditions, thereby revealing potential regulatory relationships among genes[22]. Additionally, the identification of co-expression networks through correlation-based methods provides a framework for understanding how genes may work together in biological processes, facilitating the generation of hypotheses regarding their functional roles[23,24]. These analyses can lead to the identification of gene modules that are significantly associated with specific traits or diseases, thus guiding further experimental validation and exploration of underlying mechanisms[25,26].

In an effort to develop a consensus on the genetic networks associated with lissencephaly, Di Donato et al.[27] examined the expression profiles and functional pathways of genes using gene-correlation analyses, providing important insights into how genetic networks are organized at the cellular level[27]. In this study, we sought to carry these efforts further by utilizing various computational tools, as well as incorporating single-cell transcriptomic data, to better understand cell types, biological processes, and their relationship with clinical outcomes. Networks created through correlations in gene expressions allowed us to elucidate the associations of genes with specific cellular environments and biological processes. We reveal the hierarchical organization and functional distinctions of genes involved in brain development, and show that microtubule-related genes play a central role in regulating the cytoskeleton, while genes involved in extracellular signaling constitute a separate functional group. Single-cell RNA sequencing analyses show that these genes exhibit differential expression patterns in specific cell subpopulations, indicating their dynamic roles in development. Correlation network analyses confirm that some genes are essential components of cortical development, while others have more specific functions. Thus, we demonstrate that the genetic mechanisms driving neuronal migration and cortical stratification processes should be addressed from a holistic perspective.

Materials and Methods

Databases

In this study, 15 lissencephaly-associated gene sets (*TUBB*, *TUBB3*, *ACTB*, *ACTG1*, *TUBA1A*, *DCX*, *TUBB2B*, *KIF2A*, *VLDLR*, *Reelin (RELN)*, *DYNC1H1*, *ARX*, *KIF5C*, *CRADD*, and *TUBA8*) identified by Di Donato et al.[27] were analysed. Correlation data were obtained from the Allen Brain Atlas and used to evaluate the distribution of gene expressions according to cell types[28,29]. Venn diagrams were created with the PSB Bioinformatics Venn Tool (https://bioinformatics.psb.ugent.be/webtools/Venn/) to visualize the intersections of gene sets. t-distributed stochastic neighbor embedding (t-SNE) analyses were performed using CoDeX Viewer to visualize the high-dimensional distributions of cell types and gene expressions[30]. For single-cell transcriptome analyses, the CellxGene platform was utilized, and gene expression patterns, associated with different cellular environments, were examined[31]. All data were evaluated with an integrated analysis strategy aimed at understanding the roles of genes in biological processes and clinical contexts.

Expression Weighted Cell Type Enrichment Analysis

EWCE analysis is a robust computational method utilized to evaluate the enrichment of gene or gene set expression across distinct cell types. In this approach, gene expression data are first normalized and subsequently compared against a reference cellular atlas. For each gene, the mean expression level across various cell types is calculated and assessed against randomly generated gene lists derived from the reference atlas. This comparison determines whether a given gene is preferentially expressed in specific cell types. The distribution of enrichment scores generated from these random gene lists serves as the null model for statistical evaluation.

The method employs weighted expression profiles of genes within the gene set, coupled with permutation testing, to statistically ascertain the significance of observed enrichments. The results not only identify cell types in which the target genes are significantly enriched but also provide insights into the biological relevance of these expression patterns, highlighting their potential functional roles within specific cellular contexts.

Jaccard Index Calculation

Jaccard indices are a metric used to measure the similarity between two gene sets by expressing the ratio of common genes to total genes. In our analysis, the Jaccard index was calculated for each gene set pair. The Jaccard index between two gene sets is calculated by the following formula:

$$J(A, B)=|A\cap B|/|A\cup B|$$

Where $|A\cap B|$ is the number of genes shared by both sets and $|A\cup B|$ is the total number of genes found in the union

of both sets. For calculation, correlated gene lists were used for each gene set, and intersection and union sizes were carefully evaluated to provide a meaningful gene set match within a biological context. The results numerically represent the similarity level of each gene set pair and allow interpretation of commonalities in genetic function or biological processes. This analysis has been used as an important tool to evaluate the connection of genes through common functional pathways and biological processes[32].

Spearman's Rank Correlation Analysis

Spearman's rank correlation analysis was performed to evaluate the monotonic relationship between the gene expressions associated with lissencephaly. The analysis was used to reveal the interdependence and common patterns of gene expression. First, gene expressions were normalized and made suitable for analysis. Then, correlation coefficients between genes were calculated and a correlation matrix was created using these coefficients. The obtained matrix was represented with a heatmap to visualize the relationships between genes and determine clustering patterns. Hierarchical clustering was performed to group genes with similar correlation patterns[32]. The Python programming language was used during correlation analysis, and the spearmanr function in the scipy library was used preferably for calculations. Strong positive and negative relationships were observed between gene expressions. The observed relationships were used to evaluate the possible commonalities or contrasts of genes in biological processes.

Enrichment Analysis

CellxGene and CoDEx Viewer tools were utilized to support gene expression and functional enrichment analyses. First, CellxGene was used to examine the cell types in which our genes were expressed using single-cell RNA-seq data. This tool was used to visualize the expression levels of selected genes in different cell populations and to explore their potential functions in a cellular context. Then spatial gene expression data were evaluated using CoDEx Viewer to analyse the spatial expression patterns of the genes. This analysis allowed us to understand the localization of cell types within the tissue, cell-cell interactions, and spatial features of gene expression.

Statistical Analysis

We adopted a multi-step approach to statistically evaluate the correlation and enrichment results. The Spearman's rank correlation coefficient was used to calculate the correlations between gene sets. This method allowed us to evaluate the monotonic relationships between the expression levels of genes. For statistical significance assessment, p-values were calculated, and multiple testing correction was performed using the Benjamini-Hochberg method. The corrected p-values (q-values) obtained were evaluated at a threshold of $q<0.05$ to determine significant correlations.

For enrichment analyses, a hypergeometric test was applied for each gene set. To determine significant results, p-values of each test were calculated, and multiple testing correction was performed again using the Benjamini-Hochberg method. After correction, enriched biological pathways, functional categories, and phenotypes were selected using a threshold of $q<0.05$.

Additionally, random permutation tests were applied to increase the reliability of the correlation and enrichment results in a biological context. Background distributions obtained with random permutations were compared to assess the significance of the observed enrichment scores or correlation coefficients. This statistical framework allowed us to interpret the findings of our study in a robust and reliable way.

Results

Jaccard indices obtained from the correlation data of genes associated with lissencephaly enabled an analysis of the functional relationship between lissencephaly genes by evaluating the common gene sets. Network analysis based on Jaccard indices of lissencephaly-associated genes allowed the visualization of functional relationships between genes. *TUBA1A*, *TUBG1*, and *PAFAH1B1* genes stand out as central nodes and form dense connections with other genes (Figure 1a). This suggests that these genes have close functional connections with other genes, especially in processes such as neuronal migration and cortical layering. The central location of genes related to microtubule organization and motor proteins, such as *CDK5* and *KIF5C*, supports their key role in these processes (Figure 1a).

On the other hand, *ARX* and *CRADD* genes are located in a more peripheral position, indicating that these genes have more specific functional relationships with other genes (Figure 1a). *ARX*, in particular, is located further away from the other genes, indicating that it may have a more independent role in cortical development.

This network analysis allows us to understand not only the pairwise relationships between genes, but also their positions and importance within the collective functional network.

To further investigate the relations between lissencephaly genes, we compared the findings of network analysis to those of hierarchical clustering-based analysis. There is a high level of similarity between certain groups of genes. In particular, it was determined that *PAFAH1B1* (w/*ACTB* cor: 0.04; *ACTG1* cor: 0.03; *DCX* cor: 0.03; *DYNC1H1* cor: 0.30; *KIF2A* cor: 0.23; *KIF5C* cor: 0.03; *TUBA1A* cor: 0.04; *TUBA8* cor: 0.03; *TUBB2B* cor: 0.001; *TUBB3* cor: 0.06; *TUBB* cor: 0.05; *TUBG1* cor: 0.04) , *TUBA1A* (w/*ACTB* cor: 0.68; *ACTG1* cor: 0.74; *DCX* cor: 0.61; *DYNC1H1* cor: 0.03; *KIF2A* cor: 0.27; *PAFAH1B1* cor: 0.04; *TUBB2B* cor: 0.50; *TUBB3* cor: 0.71; *TUBB* cor: 0.75), and *DAB1* (w/*ACTB* cor: 0.44; *ACTG1* cor: 0.54; *DCX* cor: 0.66; *DYNC1H1* cor: 0.06; *KIF2A* cor: 0.36; *PAFAH1B1* cor: 0.04; *TUBA1A* cor: 0.50; *TUBB2B* cor: 0.40; *TUBB3* cor: 0.71; *TUBB* cor: 0.60) genes exhibited a strong correlation with other microtubule-related genes involved in cortical neuronal migration processes. These genes were located in the same clusters in the dendrogram, indicating that they are in a common network based on shared gene co-expression (Figure 1b). On the other hand, genes related to the extracellular matrix-associated processes, such as RELN (w/*ACTB* cor: 0.31; *ACTG1* cor: 0.35; *DCX* cor: 0.40; *DYNC1H1* cor: 0.14; *KIF2A* cor: 0.60; *PAFAH1B1* cor: 0.14; *TUBA1A* cor: 0.33; *TUBB2B* cor: 0.18; *TUBB3* cor: 0.35; *TUBB* cor: 0.40; *VLDLR* cor: 0.50; *DAB1* cor: 0.46) and *VLDLR* (w/*ACTB* cor: 0.23; *ACTG1* cor: 0.24; *DCX* cor: 0.33; *DYNC1H1* cor: 0.26; *KIF2A* cor: 0.51; *PAFAH1B1* cor: 0.20; *RELN* cor: 0.50; *TUBA1A* cor: 0.25; *TUBB2B* cor: 0.13; *TUBB3* cor: 0.30; *TUBB* cor: 0.32; *DAB1* cor: 0.37), were grouped separately from other genes, suggesting that these genes regulate cortical layering processes in distinct ways. These results demonstrate the potential of co-expression-based transcriptomic approaches in improving our understanding of the genetic basis of neuronal migration processes and the pathophysiology of lissencephaly.

To further confirm these findings, we compared the gene sets using the Spearman's rank correlation test. The genes were grouped into five groups: Genes including *ACTG1*, *ACTB* and *TUBB* (*ACTG1-ACTB* cor: 0.74; *ACTG1-TUBB3* cor:

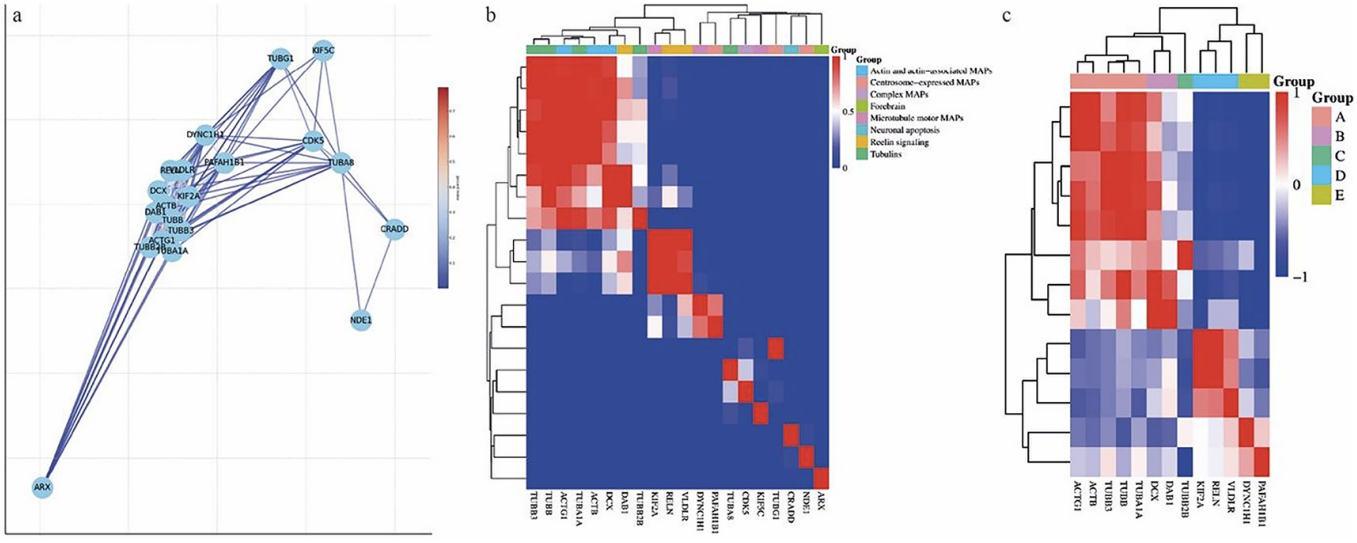

Figure 1. Visualization of lissencephaly-associated genes based on Jaccard index and Spearman's rank correlation analysis. (a) Weighted gene network visualizing the common biological pathways and processes among genes based on Jaccard index scores is represented. Genes are connected according to the proportion of traits they share with each other, and the thickness of the connections represents the magnitude of Jaccard scores. (b) Hierarchical clustering heatmap constructed based on Jaccard index scores shows the grouping of genes based on their biological traits. The color intensity represents the magnitude of Jaccard scores among genes (red: high similarity, blue: low similarity). The upper colored bands reflect the classification of genes according to their biological functions (e.g., actin-associated proteins, microtubule motor proteins, etc.). (c) Hierarchical clustering heatmap based on Spearman rank correlation analysis shows the grouping of genes based on the monotonic relationships between gene expressions. The correlation coefficients are indicated by colors (red: positive correlation, blue: negative correlation). The clustering of genes was determined based on the correlation patterns in their expressions

0.53; *ACTG1-TUBB* cor: 0.73; *ACTG1-TUBA1A* cor: 0.70; *ACTB-TUBB3* cor: 0.53; *ACTB-TUBB* cor: 0.62; *ACTB-TUBA1A* cor: 0.59; *TUBB3-TUBB* cor: 0.83; *TUBB3-TUBA1A* cor: 0.68; *TUBB-TUBA1A* cor: 0.76) in Group A show strong positive correlations (Figure 1c). It is highly likely that these genes are co-regulated, participate in similar functional pathways, or are expressed in similar cellular subtypes or states during neuronal migration. In addition, the *PAFAH1B1* and *DYNC1H1* genes [*PAFAH1B1-ACTG1* cor: -0.21; *PAFAH1B1-ACTB* cor: -0.23; *PAFAH1B1-TUBB3* cor: -0.07; *PAFAH1B1-TUBB* cor: -0.25; *PAFAH1B1-TUBA1A* cor: -0.09; *DYNC1H1-ACTG1* cor: -0.21; *DYNC1H1-ACTB* cor: -0.23; *DYNC1H1-TUBB3* cor: -0.18; *DYNC1H1-TUBB* cor: -0.26; *DYNC1H1-TUBA1A* cor: -0.16] in Group E show negative correlations with most of the genes in Group A, indicating that the expression profiles or activities of these genes may be inversely related (Figure 1c). The fact that genes such as *TUBA1A*, *TUBB2B*, and *DCX* show strong positive correlations with each other indicates that they are critical in microtubule dynamics and neuronal migration. Their clustering indicates their common roles in similar biological processes. The negative correlations of the *RELN*, *VLDLR*, and *KIF2A* genes in Group D with genes in Groups A and B, as evidenced by their correlation scores (*RELN-ACTG1* cor: -0.08; *RELN-ACTB* cor: -0.08; *RELN-TUBB3* cor: -0.06; *RELN-TUBB* cor: -0.02; *RELN-TUBA1A* cor: -0.05; *VLDLR-ACTG1* cor: -0.12; *VLDLR-ACTB* cor: -0.10; *VLDLR-TUBB3* cor: -0.06; *VLDLR-TUBB* cor: -0.03; *VLDLR-TUBA1A* cor: -0.11; *KIF2A-ACTG1* cor: -0.16; *KIF2A-ACTB* cor: -0.14; *KIF2A-TUBB3* cor: -0.11; *KIF2A-TUBB* cor: -0.06; *KIF2A-TUBA1A* cor: -0.11), may reflect that they play a separate role in extracellular matrix interactions and signal transduction, unlike cytoskeletal organization. The negative correlation of the *PAFAH1B1* gene with microtubule polymerization-related genes (Group A and B) may indicate that the pathways regulated by these genes are functionally separated at different stages of neuronal migration (Figure 1c). Spearman's rank correlation measures the monotonic relationship between the expression levels of genes. This relationship may indicate that they follow a similar pattern in the regulation of gene expression.

To further gain insight into the gene-gene associations at a more cellular level, single-cell RNA sequencing data from the human brain cortex were analysed. Microtubule-related genes such as *TUBA1A*, *TUBB*, and *DCX* were found to be highly expressed, especially in neurons (0.79, 0.56, 0.15), neural precursor cells (0.77, 0.53, 0.16), and radial glial cells (0.55, 0.48, 0.08) (Figure 2a). This supports the critical roles of these genes in neuronal migration and cortical development. In addition, cell-membrane and extracellular matrix-related genes such as *RELN* and *VLDLR* were found to be significantly expressed in glial cells (0.07, 0.07) and radial glial cells, with expression levels of 0.07 for both in each cell type (0.07, 0.07) (Figure 2a). This finding confirms that these genes regulate neuronal migration via extracellular signalling.

On the other hand, the expression of genes such as *CRADD* and *KIF5C* was found to be limited to more specific cell types. In particular, the *CRADD* gene showed low but significant expression in cell types such as macrophages (0.09) and glioblasts (0.08), indicating potential interactions of this gene with the immune system and glial differentiation during cortical development (Figure 2a). This analysis sheds light on the possible mechanisms in the pathophysiology of lissencephaly by revealing the cell type-specific expression patterns of the genes.

t-SNE analysis reveals the clustering of cellular groups and the cell type-specific distribution of genes by reducing high-dimensional gene expression data to a two-dimensional plane. The general t-SNE plot of developing human cortex, according to the Codex Viewer [30], shows the cell populations present during cortical development in Figure 2b on the upper left. Among these cell populations, different cell types were identified: radial glial cells, migrating excitatory neurons, maturing excitatory neurons, interneurons, and microglia. In the other panels (c-v), the normalized expression density of each gene is shown in different cellular subgroups. High expression of the *ARX* gene is visible, especially in migrating excitatory neurons and interneurons (Figure 2v). This supports the critical role of the *ARX* gene in neuronal migration and differentiation processes. *VLDLR* showed low to moderate expression in radial glial cells and excitatory neurons, suggesting its role in extracellular matrix signaling (Figure 2m). *KIF2A*, which showed a broad distribution among all cell groups, suggests a universal role in regulating intracellular microtubule dynamics (Figure 2k). The *RELN* gene showed a localized expression profile in radial glial cells and upper layer enriched regions of excitatory neurons, confirming the regulatory function of *RELN* in stratification processes (Figure 2l).

These observations suggest that genes that play critical roles in microtubule dynamics and neuronal migration, such as *TUBB2B*, *ACTB*, *TUBA1A*, *TUBB*, and *DCX*, are widely expressed, especially in migrating excitatory neurons and

radial glial cells (Figure 2d-h). The *DAB1* gene was observed to have high expression levels, especially in migrating neurons (Figure 2i), supporting its critical role in neuronal migration processes in the *RELN* signalling pathway. The expression patterns among all genes, were arranged in accordance with their biological roles associated with cell types. The results reveal the contributions of the analysed genes in different stages of neuronal migration and their cell type-specific functions. These findings provide an important basis for better understanding the functions and cellular effects of lissencephaly-associated genes.

DYNC1H1 and *PAFAH1B1* have a broad expression pattern in most cell types (Figure 2n-o), supporting their role in universal functions such as microtubule dynamics and intracellular transport. *CRADD* and *NDE1* show a more restricted expression profile (Figure 2t-u) suggesting more specific roles in certain cellular subpopulations

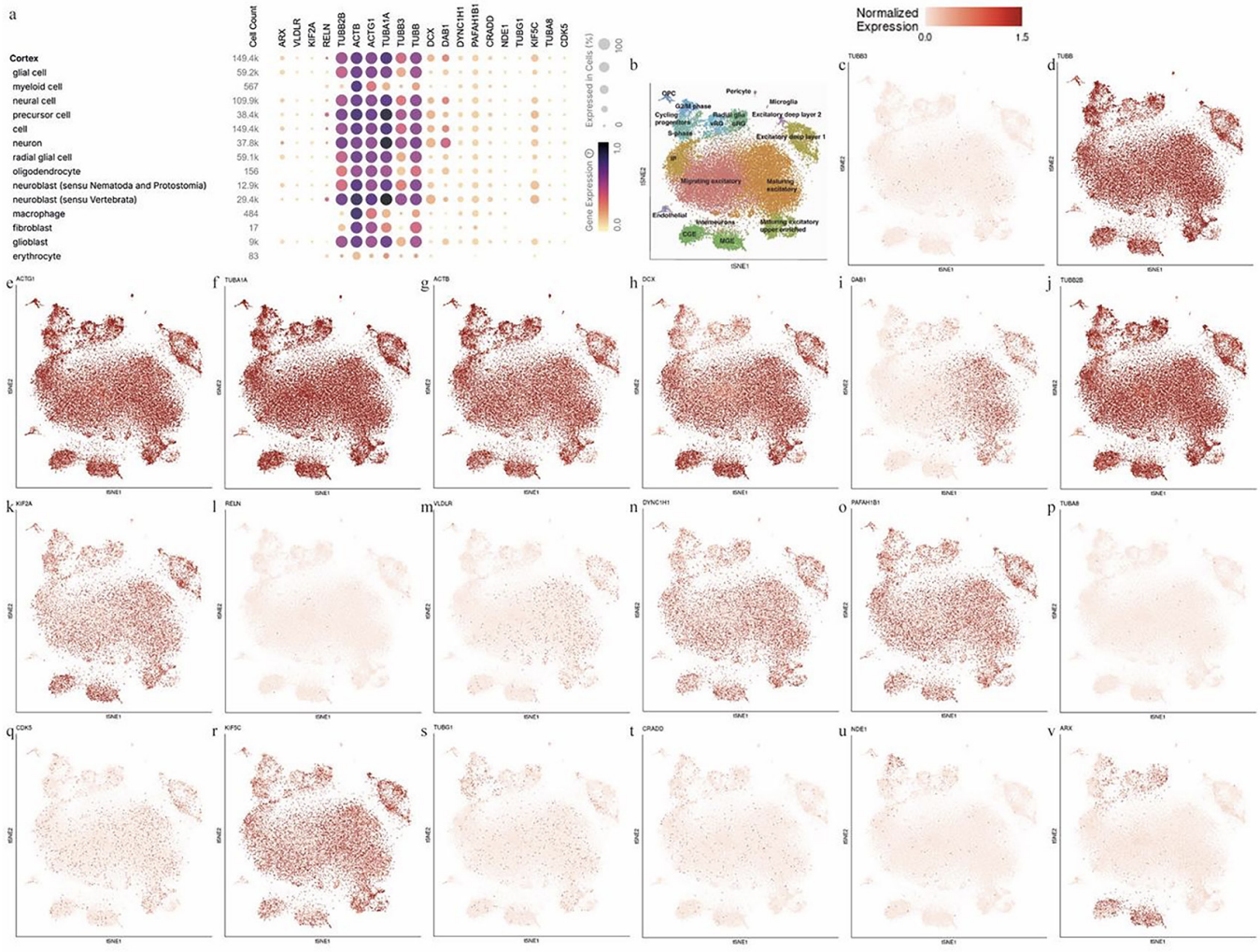

Figure 2. Expression patterns of lissencephaly-associated genes in cortical cell types and cortical cell population. (a) Single-cell RNA sequencing analysis. Each bubble represents the percentage of cells in a given cell type in which the gene is expressed (bubble size) and the intensity of gene expression (color scale). The size of the bubbles reflects the width of the cell population in which the gene is expressed, while the colour scale represents the intensity of gene expression level (0: low, 1: high). The genes studied are shown on the horizontal axis and the cell types on the vertical axis. (b-v) Expression patterns of lissencephaly-associated genes in cortical cell populations by t-SNE analysis. (b) This panel shows cell populations identified during cortical development by t-SNE analysis. Each dot represents a cell, and colors represent different cell types. In the other panels, normalized expression intensities of specific genes [(c) *TUBB3*, (d) *TUBB*, (e) *ACTG*, (f) *TUBA1A*, (g) *ACTB*, (h) *DCX*, (i) *DAB1*, (j) *TUBB2B*, (k) *KIF2A*, (l) *RELN*, (m) *VLDLR*, (n) *DYNC1H1*, (o) *PAFAH1B1*, (p) *TUBA8*, (q) *CDK5*, (r) *KIF5C*, (s) *TUBG1*, (t) *CRADD*, (u) *NDE1* and (v) *ARX*] are visualized on cellular groups. Red tones indicate high levels of gene expression, light tones indicate low levels

(e.g., neuronal precursor cells and interneurons). *TUBG1* and *TUBA8*, involved in microtubule organization, are prominently expressed in populations associated with neuronal migration and cytoskeletal organization (Figure 2p and 2s). *KIF5C*, which is directly associated with neurons, is particularly highly expressed in excitatory neurons (Figure 2r), suggesting an important role in neuronal transport. *CDK5* showed widespread expression across various cellular groups (Figure 2q), suggesting that it may play a regulatory role in cortical developmental processes.

Discussion

A better understanding of the genetic factors leading to lissencephaly and neuronal migration disorders is critical for the elucidation of the mechanisms of the disease and the identification of potential therapeutic targets. In this study, we focused on lissencephaly-related genes and analysed the gene expression correlations of these genes in the developmental brain using the EWCE approach. We determined the common correlations between the expression patterns of 20 genes associated with lissencephaly (*TUBB*, *TUBB3*, *ACTB*, *ACTG1*, *TUBA1A*, *DCX*, *TUBB2B*, *KIF2A*, *VLDLR*, *RELN*, *DYNC1H1*, *ARX*, *KIF5C*, *CRADD*, and *TUBA8*) and performed Jaccard index and Spearman's rank correlation analyses. The data obtained provide important findings on the ways in which these genes may interact at the cellular and functional levels. Our study provides a broader perspective on the mechanisms leading to lissencephaly and contributes to the identification of potential biomarkers that may provide new insights from a clinical perspective.

In lissencephaly and related neurodevelopmental disorders, interactions between *VLDLR*, *KIF2A*, and *RELN* genes are of great importance. *RELN* encodes the Reelin protein, which plays a critical role in the regulation of neuronal migration and cellular organization during brain development. *VLDLR* is one of the main receptors that transmit the Reelin signal[33]. *KIF2A* is a motor protein that regulates microtubule dynamics and is known to be particularly effective in cilium destruction, synaptic function, and neuronal localization processes[34]. The interactions of these genes with each other play important roles in ensuring neuronal positioning and synaptic stability. Mutations in *VLDLR* or *RELN* can lead to impaired cortical layering and serious neurodevelopmental diseases, while mutations in *KIF2A* have been associated with diseases such as epilepsy and autism[35,36].

TUBB2B, which is involved in the organization of microtubules, is distinctly different from other tubulin family members such as *TUBA1A*, *TUBB3* and *TUBB*. *TUBB2B*, which is particularly associated with cortical malformations such as asymmetric polymicrogyria, plays a specific role in neuronal migration and axonal guidance processes[37]. Epigenetic regulations are thought to shape the expression patterns of *TUBB2B* in the brain and cause it to diverge from other tubulin genes. This provides an understanding of the specific functions of *TUBB2B* in neurodevelopmental processes and more clearly demonstrates its relationship with cortical developmental disorders[38].

The *PAFAH1B1* gene, one of the most common causes of lissencephaly, is a key component in the regulation of neuronal migration. Studies show that 87% of classic lissencephaly cases have *PAFAH1B1* mutations. This high prevalence may cause the effects of other lissencephaly-associated genes to become relatively less obvious. While other lissencephaly-associated genes such as *TUBA1A* are associated with additional phenotypes such as cerebellar hypoplasia, *PAFAH1B1* mutations are usually identified by an isolated lissencephaly sequence[39]. This explains the central role of *PAFAH1B1* in cortical development and its differentiation from other genes.

TUBA8 and *TUBG1*, which are involved in the regulation of microtubule dynamics, have functional properties distinct from other tubulin genes. *TUBA8* is more highly expressed in tissues outside the brain, especially in megakaryocytes[40], while *TUBG1* plays an important role in microtubule nucleation and neuronal migration processes[41]. *TUBA8* mutations are associated with phenotypes other than classic lissencephaly, such as polymicrogyria and optic nerve hypoplasia, while *TUBG1* mutations have been observed to cause mild cortical abnormalities. The fact that *TUBG1* does not cause a migration defect as pronounced as *TUBA1A* reveals the different position of this gene in the lissencephaly spectrum.

Our study comprehensively analyzed the positive and negative correlations among genes involved in cortical development and neuronal migration. It was observed that while genes showing positive correlations regulate common biological processes and signaling pathways, negative correlations should not be considered antagonistic interactions regardless of the context. In particular, it was determined that negative correlations sometimes reflect developmental timing differences and expression patterns specific to different cellular subpopulations. For example, genes such as *PAFAH1B1* and *TUBG1* were found to play

central roles in cortical development, whereas genes such as *CRADD* and *ARX* were found to be associated with more specific processes. Hierarchical clustering and correlation network analyses confirmed the expected clustering of tubulin and microtubule-related genes, while genes involved in extracellular signaling such as *RELN* and *VLDLR* formed a different cluster. t-SNE analyses of single-cell RNA sequencing data revealed that these genes exhibit differential expression in specific cellular subpopulations, suggesting that negative correlations may not reflect functional antagonism but rather cell type-specific regulation or differential timing of developmental processes. In this context, the functional significance of negative correlations should be assessed according to the specific roles of the genes in cortical development and the cellular context in which they are located.

Barkovich et al.[42] classified brain malformations into three main groups based on their genetic etiology and developmental stages: atypical proliferation/apoptosis, atypical neuronal migration, and atypical post-migratory development. Clinically, these patients present with a wide spectrum of phenotypes, including neuromotor developmental delay, cognitive impairment, and epilepsy. Although cranial imaging plays a critical role in the diagnosis of the disease, establishing phenotype-genotype correlations is challenging because variable patterns such as agyria-pachygyria, dysgyria, heterotopia, and polymicrogyria can be seen. It is important to carefully evaluate these imaging findings to identify cases that may be candidates for epilepsy surgery.

The spectrum of tubulinopathy can vary widely, including dysgyria, lissencephaly, basal ganglia anomalies, and cerebellar anomalies. Kinesin-related diseases should be considered in the differential diagnosis. The lissencephaly spectrum includes subcortical band heterotopia (SBH), pachygyria, and classic lissencephaly; it is known to be associated with more than 20 genes in this group. Mutations in tubulin genes are not only limited to developmental disorders but have also been linked to degenerative diseases. *TUBB3* and *TUBB2B* mutations can be distinguished by additional clinical findings such as facial dysmorphism, extraocular muscle fibrosis, and peripheral neuropathy[43,44].

From an imaging perspective, three basic patterns of tubulinopathies have been identified: classic lissencephaly, microlissencephaly, dysgyria[44]. Asymmetry due to instability in the tubulin skeleton is common in cases of tubulinopathy and helps distinguish it from other causes. Classical lissencephaly can be classified as posterior dominant types and central dominant types, while microlissencephaly is considered the most severe form and is often seen with corpus callosum agenesis and cerebellar hypoplasia. Pathognomonic findings specific to tubulinopathy include basal ganglia hypoplasia, internal capsule anomalies, and frontal ventricular deformities[45].

In terms of genetic analyses, approximately 150 different variants have been identified in the *TUBA1A*, *TUBB2B*, and *TUBB3* genes. *TUBA1A* mutations are responsible for 4-5% of lissencephaly. Since the changes in the p.Arg402 residue of this gene are located in an area that interact with kinesin and dynein, they can lead to malformations related to motor proteins[45]. *TUBB2B* predominantly affects polymicrogyria, while *TUBB3* mutations have been associated with extraocular muscle fibrosis and peripheral neuropathy. *TUBA8* mutations are less commonly associated with cortical malformations and are thought to play a role in the post-migratory phase and axon maintenance. The fact that *TUBA1A* and *TUBA8* have similar structures but exhibit different electrostatic properties may explain the functional differences in microfilament interactions[45-48].

The lissencephaly spectrum covers a wide range from lissencephaly characterized by thickened cortex and sulcal-gyral anomalies to conditions such as SBH, in which abnormal neuronal clusters form beneath the normal cortex. Involvement within this spectrum can be widespread or regional, anteriorly or posteriorly, dominant. The most common pattern is the presence of pachygyria in the frontal region and agyria in the posterior cortex. Cerebellar hypoplasia is among the most common non-cortical findings in this spectrum. In tubulinopathies, an intermediate form is observed, characterized by dysgyria (such as blurring of the gray-white transition line and the presence of large and small gyria together) is observed[32,43,47].

The genes most commonly associated with lissencephaly are *PAFAH1B1* and *TUBA1A*, although *TUBG1* mutations can also cause posteriorly dominant pachygyria (Table 1). However, TUBG1 mutations do not affect the basal ganglia, cerebellum, and corpus callosum, which would cause a different phenotype compared to *TUBA1A* mutations[49]. *PAFAH1B1* and *NDE1* are proteins that play a role in regulating the cytosolic dynein motor complex and enable the movement of the cell nucleus during neuronal migration. While *PAFAH1B1* mutations are generally associated with more severe phenotypes, *NDE1* mutations may cause milder forms of dysgyria (Table 1)[50,51].

Table 1. Phenotypic effects of genes associated with lissencephaly and other neurodevelopmental disorders

Genes	Diffuse	Partial	P>A gradient	A>P gradient	Temporal> A and P	MicroLIS	Diffuse agyria	Agyria - Pachgyria (P>A)	Pachygyria (P>A)	Agyria - Pachygyria (A>P)	Pachygyria (A>P)	ScBH	PMG	Dysgyria	Diff ScBH thick	Diff ScBH thin	Par ScBH P	Par ScBH A	Pachygyria A/thin CB	Pachygyria A/thin normal CB	CB	CC	BG	Lat vent	Other
TUBA1A	+	+	+			+	+[c]	+[c]	+		+[a]	+	+	+							+	+	+	+	
TUBB3		+	+			+[a]			+					+							+/-	+/-	+/-		
TUBB3		+	+			+[a]			+					+							+/-	+/-	+/-		
ACTG1		+									+[d]	+													
ACTB		+									+[d]	+													
DCX	+	+[a]					+			+[a]	+	+			+	+		+[a]			+/-				
DAB1											+										+	+/-		+/-	
TUBB2B	+[a]	+				+	+[a]		+				+[g]	+							+	+	+	+	
KIF2A		+							+			+[e]										+/-			
RELN		+									+								+		+				
VLDLR		+									+								+		+				
DYNC1H1		+							+	+	+		+[e]	+[a]							+[*]	+/-	+/-		
PAFAH1B1	+	+[a]					+	+	+			+					+	+							
TUBA8		+											+	+[a]								+			**Optic nerve hypoplasia**
CDK5	+[a]	+					+[a]																		
KIF5C									+		+[a]		+[e]	+[h]								+/-			
TUBG1		+	+						+			+[f]										+/-			
CRADD											+								-	+	**N**				
NDE1						+[b]																			
ARX					+																	+	+		**White matter changes**

The symbol "+" indicates a positive association with the phenotype. The symbol "+/-" indicates a limited or variable association. P: Posterior, A: Anterior, ScBH: Subcortical band heterotopia, PMG: Polymicrogyria, CB: Cerebellum, CC: Corpus callosum, BG: Basal ganglia, (a): Rare, (b): Intermediate, (c): R402 variant, (d): With or without band, (e): Frontal, (f): Posterior, (g): Asymmetric, (h): A>P, [*]: Vermis colours indicate similar radiological findings

The interaction between microtubule dynamics and actin filaments plays a critical role in neuronal polarity and migration[52]. *ACTG1* and *ACTB* mutations are usually characterized by anterior dominant lissencephaly and short SBH (Table 1). The doublecortin (*DCX*) gene codes for one of the basic proteins, DCX, that regulate the interaction between microtubules and actin filaments, and studies have shown that *DCX* strongly correlates actin isoforms. *PAFAH1B1* and *DCX* are the major proteins involved in the regulation of microtubule homeostasis, and mutations in these genes are among the most common causes of diffuse agyria[53].

The microtubule-dependent motor proteins kinesin and dynein are the only gene families associated with anterior and posterior dominant lissencephaly. *KIF2A*, *KIF5C* and *DYNC1H1* are involved in many processes from mitotic division to the migration of post-mitotic neurons[54], causing a variety of clinical presentations such as pachygyria, SBH or polymicrogyria (Table 1). *RELN*, *VLDLR*, and *DAB1*, which are involved in the Reelin signalling pathway, are critical for the regulation of cortical layering and cerebellar development[43,52]. Pathogenic variants in these genes can cause anterior dominant mild pachygyria and cerebellar involvement (Table 1).

Study Limitations

This study is based entirely on in silico analyses, and this approach brings with it some limitations. Gene similarity analyses were performed on correlation data, overlap assessments performed with the Jaccard index, cell type level expression abundance analyses performed with the EWCE method, and functional enrichment studies based on Enrichr-KG provide strong predictions based on the literature, however, the biological validity of these findings remains limited without experimental verification. Since the transcriptomic data used may vary depending on parameters such as tissue, developmental stage, and cell type, the biological context of the relationships between genes should be interpreted with caution. In addition, the relevance and scope of the databases used may also affect the accuracy of the results. Therefore, although we believe that the predictions put forward in this study provide an important basis for developing hypotheses, they should be supported by molecular and cellular experiments to definitively reveal the functional roles of the relevant genes.

Conclusion

In conclusion, the lissencephaly spectrum is a group of neurodevelopmental disorders, which are genetically heterogeneous and occur through different mechanisms. Dynamic interactions between tubulin, actin, and motor proteins regulate different stages of cortical development, allowing certain genetic variants to cause specific phenotypes. A better understanding of these genetic interactions may enable new approaches to the diagnosis and treatment of lissencephaly and related disorders.

Future studies may include advanced experimental systems such as patient-derived induced pluripotent stem cells (iPSCs), brain organoids, and animal models to further investigate the biological validity of our findings. iPSC-derived neuronal models may allow us to examine the cellular functions of identified genes and their roles in developmental processes in a patient-specific context. Brain organoids may provide a valuable platform to understand how these genes are organized within the three-dimensional tissue architecture and contribute to cortical layering processes. In addition, *in vivo* validation can be performed using animal models, and the effects of genetic manipulations on neuronal migration and cortical development can be assessed. In addition to elucidating the mechanisms underlying the correlations between genes, these approaches may contribute to a better understanding of neuronal migration disorders and the identification of therapeutic targets.

Ethics

Ethics Committee Approval: As only publicly available data were used, there was no need to obtain ethics approval.

Informed Consent: Not applicable, as no ethics committee approval was needed.

Footnotes

Authorship Contributions

Concept: Y.O., Design: Y.O., Data Collection or Processing: A.K., Y.O., Analysis or Interpretation: A.K., İ.P., Y.O., Literature Search: A.K., İ.P., Y.O., Writing: A.K., İ.P., Y.O.

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