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Impact of global DNA methylation on retinal gene expression in various stages of retinoblastoma: A clue for biomarkers

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ABSTRACT

Objectives: Retinoblastoma, the most prevalent intraocular cancer in children, originates from the eye's retina. Multi-omics studies reveal a complex molecular foundation for retinoblastoma, underscoring the crucial interconnected roles of epigenetic and gene expression processes in understanding pathological mechanisms, identifying biomarkers, and exploring potential therapeutic options.

Materials and Methods: The DNA methylation array and gene expression datasets of retinoblastoma were searched in repositories. The relevant datasets were merged based on a common reference platform, and various regulatory patterns were constructed. Notably, canonical patterns containing hyper-DOWN and hypo-UP, localized at critical regions (such as 5′ UTR, 3′ UTR, TSS, shore, and shelves), were selected for mild, moderate, and severe retinoblastomas. Subsequently, common regulators across different disease states were identified, and their protein interactomes were generated and functionally enriched.

Results: We analyzed global DNA methylation in 57 retinoblastoma and 20 normal retinal tissues. Additionally, we collected control, mild, moderate, and severe retinoblastoma gene expression datasets. Among 485,577 probes, 319 exhibited hypermethylation, and 2,846 showed hypomethylation in retinoblastoma. Significant ($P < 0.05$) UP- and DOWN-regulated genes for mild, moderate, and severe conditions were identified and mapped to hyper/hypomethylated probes. Notably, distinct regulation patterns (hyper-DOWN and hypo-UP) at critical regions were chosen for each disease state. Of these, 57 hyper-DOWN and 8 hypo-UP were common across all disease states, contributing to protein interaction networks with 318 extended proteins and 1,345 interactions essential in various neuronal and cancer-related pathways. Alternatively, a few distinct regulators were identified, providing clues for disease biomarkers.

Conclusion: We found a strong association between hyper/hypomethylation and gene expression in retinoblastoma. These findings underscore the downstream regulatory role of DNA methylation, emphasizing stage-specific molecular mechanisms and offering insights into early biomarkers for the disease.

1. Introduction

Retinoblastoma is one of the most prevalent intraocular malignant tumors, accounting for 3% of all juvenile malignancies.[1,2] The incidence of retinoblastoma shows no ethnic, regional, or gender disparities. Diagnosis in newborns is typically possible at 12 and 24 months for bilateral and unilateral retinoblastomas, respectively. Bilateral retinoblastoma is more common, responsible for almost 30%–40% of the overall prevalence. Additionally, 6% of retinoblastomas are familial, while the remaining cases are sporadic.[3,4] Notably, retinoblastoma is highly malignant and results in newborn mortality, primarily owing to cerebral metastasis.[5] Untreated retinoblastoma progresses rapidly, disrupting eyeball structure and causing blindness.[6] Subsequently, it infiltrates the brain via the

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optic nerve,[7] impacting human health and quality of life. This imposes a significant medical and communal burden on individuals and society directly and indirectly. Despite significant advances in therapeutic strategies for retinoblastoma in recent years, patient survival rates remain low.[8] This is predominantly attributed to the difficulty in detecting the disease early. Therefore, attaining a comprehensive multi-omics understanding of the molecular pathogenesis associated with retinoblastoma-related vision impairment and metastasis is crucial, facilitating early diagnosis and prompt treatment.

While the development of retinoblastoma is widely acknowledged to be caused by mutations in the retinoblastoma gene (RB1), emerging evidence suggests that susceptibility to abnormal epigenetic changes contributes to tumor development and progression.[9] The altered RB1 gene in retinal cells promotes various epigenetic modifications, including DNA methylation and histone modification. Numerous oncogenic and tumor suppressor genes undergo alterations owing to these epigenetic modifications, progressing retinoblastoma.[10] Specifically, methylation at CpG islands influences the expression of genes related to retinoblastoma.[11] These findings underscore the critical role that DNA methylation plays in the development of retinoblastoma. Recent attempts to integrate DNA methylation and gene regulation have revealed regulatory patterns involving hyper- and hypomethylation, leading to changes in gene expression. This understanding is pivotal in unraveling the disease mechanism, discovering diagnostic markers, and developing novel treatment strategies.

In this study, we conducted an integrative analysis of a DNA methylation dataset comprising retinoblastoma and normal retinal samples alongside a gene expression dataset aggregated from four cohorts, comparing mild, moderate, severe retinoblastoma, and normal samples. Utilizing these datasets, we delineated two distinct forms of regulatory connections, each exhibiting common and unique characteristics in patterns of hyper and hypomethylation that exert control over gene expression at various stages of retinoblastoma. This implies a significant impact of DNA methylation on gene expression in retinoblastoma. Finally, these identified regulatory patterns were functionally attributed to the core molecular pathogenesis, offering insights that may aid in identifying early biomarkers and developing novel treatment strategies.

2. Materials and Methods

2.1. DNA methylation

We identified DNA methylation datasets using the search term "retinoblastoma methylation" in the Gene Expression Omnibus (GEO) database. Specifically, the GSE57362 dataset, encompassing retinoblastoma DNA methylation data, was selected for this study. This dataset comprises 20 control and 57 retinoblastoma samples, providing a sufficiently large sample size (>10 samples in each group). The analysis encompassed retinoblastoma and normal samples and was conducted using the Illumina HumanMethylation450 BeadChip (GPL13534: HumanMethylation450_15017482).

2.2. Gene expression

Similarly, we manually searched the GEO database for microarray gene expression data related to retinoblastoma in retinal tissue, which presented disease stages categorized as mild, moderate, and severe by the data submitter. Through this search, we selected the GSE110811 dataset, executed on the GPL16686 platform. This dataset encompasses the expression profiles of 28 samples, including three healthy controls, six mild, thirteen moderate, and nine severe retinoblastoma patients. Classifying all diseased samples into mild/moderate or severe categories was based on the Anaplastic Grade designated by the data contributor.[12]

2.3. Data analysis and integration

Utilizing the limma program in R, we identified differentially methylated probes[13] by comparing retinoblastoma samples with their respective controls. Probes with a statistically significant p-value < 0.05 were selected and categorized as hypermethylation (gain) or hypomethylation (loss) based on the differential methylation levels. Similarly, gene expression was assessed using limma and differentially expressed genes with significant p-values were selected across each condition. These genes were labeled UP and DOWN based on their altered expression compared to their respective controls. Regulatory patterns were generated to evaluate the interplay between hyper/hypomethylation of probes and changes in gene expression (UP and DOWN). This involved mapping the gene symbols of probes from the DNA methylation array and gene expression from the microarray gene expression profile using the reference platforms GPL13534 and GPL16686.

2.4. Regulatory patterns and critical region analysis

Our analysis revealed four distinct regulatory patterns: 1) hyper-UP (methylation gain with increased gene expression), 2) hypo-UP (methylation loss with increased gene expression), 3) hyper-DOWN (methylation gain with decreased gene expression), and 4) hypo-DOWN (methylation loss with decreased gene expression). We visualized DNA methylation and gene expression fold changes using scatter plots. Among these patterns, we focused on hypo-UP and hyper-DOWN for further assessment. Utilizing the GPL13534 reference, we obtained the sites of hyper/hypomethylated probes in distinct gene

sections, including 3′ UTR (untranslated region), 5′ UTR, transcription start sites (TSSs), and CPG islands (shelves and shores), from the Hg38 reference genome via the UCSC Browser.

2.5. Protein interaction and enrichment analysis

The identified hypo-UP and hyper-DOWN regulators from mild, moderate, and severe conditions were employed to construct a protein interactome (PPI) network. For this purpose, the HuRI database (www.interactome-atlas.org) was utilized to generate a high-quality protein interactome. Subsequently, the constructed network was analyzed using Cytoscape version 3.8.1. Functional enrichment analysis was conducted to understand the molecular behavior of the hypo-UP and hyper-DOWN regulators in retinoblastoma. The Enrichr application (https://maayanlab.cloud/Enrichr/), which provides molecular pathways based on the KEGG pathway database (https://www.genome.jp/kegg/), was employed for this analysis A significance threshold of $P < 0.05$ was chosen to evaluate the genes associated with pathways.

2.6. Ethical considerations

This study is a computational biology study. However, the study adhered to the tenets of the Declaration of Helsinki, including transparency, rigor, and reproducibility. The study was approved by King Abdulaziz University ethics and research committee (reference No:147-24).

3. Results

First, we examined the DNA methylation array, comprising 485,577 probes associated with genes referenced in GPL13534. Utilizing the limma R module, our analysis revealed that in retinoblastoma, 319 probes exhibited significant methylation gain compared to healthy controls, while 2,846 probes showed significant methylation loss. These probes were linked to 335 and 3,107 genes, respectively (Figure 1 A), based on the GPL13534 reference. A few genes were overrepresented by multiple probes, demonstrating significant hyper/hypomethylation in our analysis. For example, probes cg04814352 and cg15683856 were hypermethylated and linked with the MBNL 2 gene across mild, moderate, and severe retinoblastomas.

3.1. Gene expression

Similarly, we examined the gene expression dataset to identify UP- and DOWN-regulated genes across mild, moderate, and severe retinoblastomas compared to the control. In the mild state, 718 genes were observed to be DOWN-regulated, while 827 were UP-regulated, with a p-value ≤ 0.05. Similarly, 1077 genes were UP-regulated in the moderate condition, and 1437 were DOWN-regulated. In severe retinoblastoma, our analysis revealed 1901 DOWN-regulated genes and 2667 UP-regulated genes (Figure 1 B).

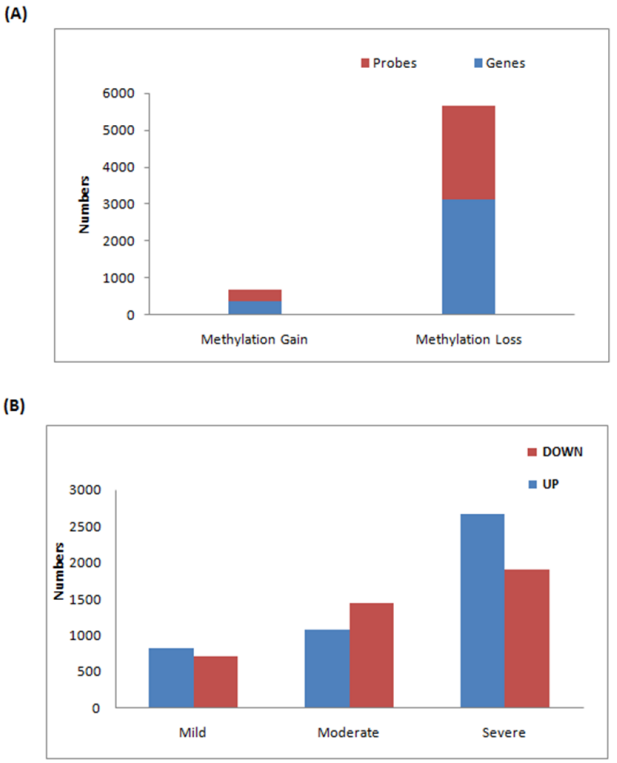

Figure 1: Limma R module analysis. (**A**): Probes that exhibited significant methylation gain and loss in retinoblastoma, with these probes linked to genes based on the reference platform; (**B**): Number of differentially expressed genes (UP and DOWN) in each state of retinoblastoma

3.2. Epigenetic regulation

The DNA methylation results of retinoblastoma (hyper- and hypomethylated probes associated with genes) were combined with differentially expressed genes (DEGs) to identify four distinct regulatory patterns (hyper-DOWN, hyper-UP, hypo-UP, and hypo-DOWN) across mild, moderate, and severe conditions (Figure 2). Among the diverse regulatory patterns, canonical modules such as hyper-DOWN and hypo-UP were selected, comprising 34, 129, and 117 hyper-DOWN patterns in mild, moderate, and severe retinoblastomas, respectively. (Figures 3, 4 and 5) This selection of classic hyper-DOWN patterns elucidates the hypermethylation-induced downregulation of gene expression. Similarly, hypo-UP regulators correspond to the hypomethylation of probes, contributing to the upregulation of their associated genes. Consequently, 161 hypo-UP regulators were identified in mild conditions,

220 in moderate conditions, and 434 in severe conditions. (Figures 3, 4 and 5) Interestingly, some from the hyper-DOWN group exhibited higher methylation fold change, suggesting a more substantial impact on downregulating gene expression—the primary mode of regulation. A similar trend was observed in the hypo-UP group, where a loss of methylation had a more pronounced effect on upregulating connected genes. Our results include many hypomethylated regulators, contributing to increased gene expression (Figure 6).

Figure 2: Scatter plots of the DNA methylation results of retinoblastoma (hyper and hypo-methylated probes associated genes) integrated with differentially expressed genes (DEGs) to form four distinct regulatory patterns (hyper-DOWN, hyper-UP, hypo-UP, and hypo-DOWN)

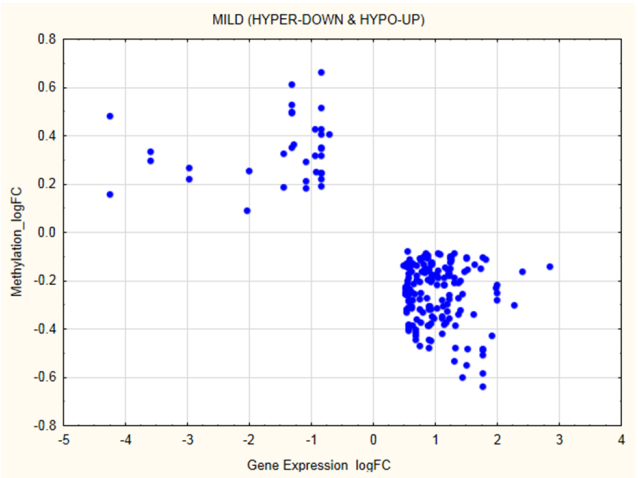

Figure 3: Selected canonical regulatory modules in hyper-DOWN and hypo-UP, presenting fold changes in probes and gene expression specific to mild retinoblastoma

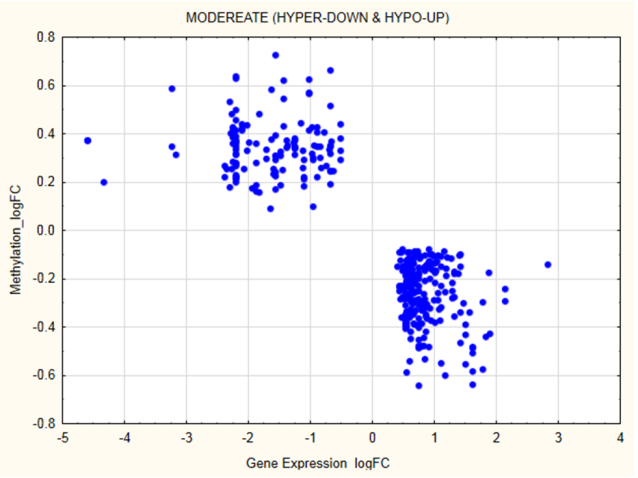

Figure 4: Selected canonical regulatory modules in hyper-DOWN and hypo-UP, displaying fold changes in probes and gene expression specific to moderate retinoblastoma

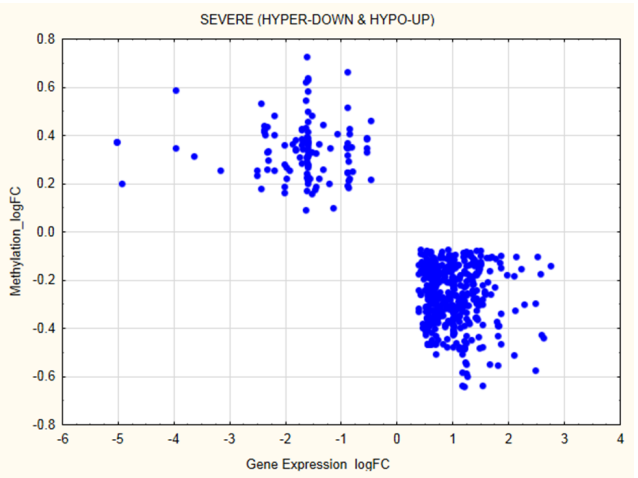

Figure 5: Selected canonical regulatory modules in hyper-DOWN and hypo-UP, displaying fold changes in probes and gene expression specific to moderate retinoblastoma

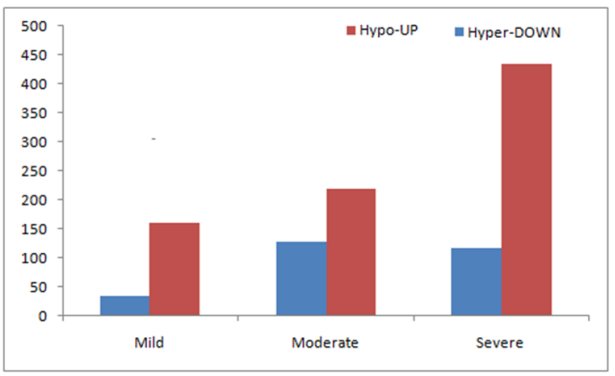

Figure 6: Bar chart illustrating the enriched hypomethylated regulators in mild, moderate, and severe retinoblastomas

3.3. Regulators screening based on critical region assessment

We assessed the proximity between each probe and the critical regions of its associated genes in hypo-UP and hyper-DOWN patterns across all three retinoblastoma conditions, recognizing that probes located closer to the TSS or at the critical regions of the UTR may significantly impact the expression of linked genes. Figures 7 and 8 illustrate the distribution of hyper-DOWN and hypo-UP probes across five key regions (5′ UTR, 3′ UTR, TSS, shore, and shelves). As expected, we observed that probes (hypo or hyper) located at the TSS exerted a more pronounced influence on gene expression. For example, the hypermethylated cg23932332 probe at the TSS led to substantial downregulation of DUSP10 in moderate and severe retinoblastoma (Table 1). Additionally, we investigated probes localized at critical regions such as 3′ UTR, 5′ UTR, shore, and shelves, which impacted 17 genes linked with probes at 5′ UTR, 1 gene contributed to by multiple probes at 3′ UTR, 16 genes at the shore, and 14 genes at the shelves in the hyper-DOWN condition across the disease states. (Figure 7) Similarly, the expression of 294 genes was influenced by probes (2035) at critical regions in the hypo-UP group. This assessment of critical regions provides insight into highly influenced regulatory modules in each disease state. Additionally, we have compiled a list of top genes (Table 1) exhibiting the highest fold change in gene expression in each disease state (mild, moderate, and severe) compared to the control, along with their associated methylation probes and critical regions. These retinoblastoma stage-specific genes offer a promising avenue for diagnosis.

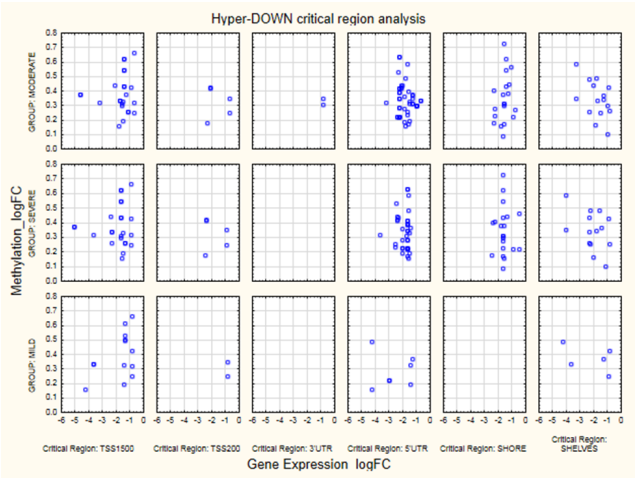

Figure 7: Association of probes localized at critical regions with changes in probe methylation and gene expression in hyper-DOWN across the conditions

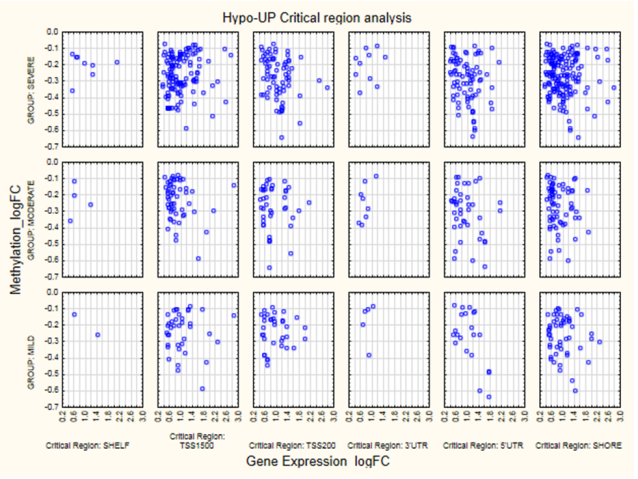

Figure 8: Association of probes localized at critical regions with changes in probe methylation and gene expression in hypo-UP across the conditions

3.4. Protein interaction and enrichment analysis

Within the selected hypo-UP regulation at critical regions, eight UP-regulated genes were consistently observed in all disease states. Similarly, 57 DOWN-regulated genes were consistently identified in hyper-DOWN across all cases of mild, moderate, and severe retinoblastomas. To understand the influence of methylation, the hypo-UP and hyper-DOWN (65; 57 + 8) common genes across disease states were utilized to construct a protein interaction network. The combined 65 genes encoding proteins yielded 1345 interactions with 318 extended proteins from the 65 seed inputs. Furthermore, the functional analysis based on molecular pathways revealed potential involvement in mTOR signaling, axon guidance, ErbB signaling, HIF-1 signaling, gap junction, adherens junction, insulin signaling, and focal adhesion pathways with a significance level of $p < 0.05$.

4. Discussion

Integrated omics data analysis has recently emerged as a promising method for deciphering the molecular pathogenesis of various diseases, aiming to uncover critical genes and molecular pathways. These methods are increasingly utilized for predicting potent diagnostic markers and drug targets.[14,15] In our study, we employed an integrated approach, combining epigenetic methylation data with gene expression profiles of retinoblastoma. This allowed us to identify molecular mechanisms at various disease stages (i.e., mild, moderate, and severe), shedding light on potential early diagnostic markers and developing new treatment strategies. The intricate nature of this disease contributes to its status as a leading cause of mortality in newborns, attributed to the absence of early markers

Table 1: Key genes exhibiting the highest fold difference in gene expression in each disease state, accompanied by the probe ID and critical regions

Disease state	Gene symbol	Gene expression fold change	p-value	Probe ID	Critical region
Hypo-UP					
Mild	COTL1	1.21	0.001	cg03024660	Shore
Moderate	RAI14	0.89	0.01	cg00227578	Shore
Severe	KCNT2	2.52	0.001	cg01768775	Shore
Hyper-DOWN					
Mild	TFPI2	−1.31	0.01	cg19103770, cg13328713, cg19854521, cg09558850	Shore
Moderate	DUSP10	−1.24	0.007	cg23932332, cg13442201	TSS, 5′ UTR
Severe	FBXO32	−2.35	0.01	cg03023681	Shore

and a lack of definitive treatment. Herein, we integrate DNA methylation and gene expression profiles to elucidate the epigenetic consequences that lead to changes in gene expression in mild, moderate, and severe retinoblastomas, classified based on Anaplastic Grade.[12]

The limma R package was utilized to identify hypo- and hypermethylated probes in retinoblastoma. Concurrently, the same package was employed to identify DEGs in each state of mild, moderate, and severe retinoblastomas compared to healthy controls. The hypo- and hypermethylated probes were integrated with DEGs to construct canonical regulators, encompassing hyper-DOWN and hypo-DOWN modules across each condition of retinoblastoma. Based on previous studies on DNA methylation in retinoblastoma, which highlight the vulnerability of critical regions (DNA hyper/hypo methylation) to induce changes in the expression of retinoblastoma-associated genes compared to normal tissue, our analysis identified several significant DNA hyper/hypomethylation probes at critical regions (TSS, UTR, CGP shelves, and shores) impacting expression levels (Figures 2, 3, 4 and 5). These crucial regulators were enriched, participating in 82 molecular pathways common to mild, moderate, and severe retinoblastomas. Many of these pathways likely play a significant role in the initiation and progression of retinoblastoma, as evidenced by our study. For instance, well-established cancer-related pathways such as mTOR signaling, axon guidance, ErbB signaling, HIF-1 signaling, adherens junction, insulin signaling, focal adhesion, and gap junction were observed. In addition to these pathways, the identified crucial regulators were linked with cancer pathways like glioma, pancreatic, bladder, breast, and endometrial cancer. These results underscore the potential molecular linkages between retinoblastoma and the pathogenesis of several cancer types.

Additionally, distinct hyper-DOWN and hypo-UP patterns of DNA methylation and gene expression were identified around the critical regions of linked genes, specific to mild, moderate, and severe retinoblastomas. For example, the tissue factor pathway inhibitor-2 (TFPI2) gene exhibited a hyper-DOWN pattern specific to mild retinoblastoma. TFPI2 is a secretory protein that inhibits several serine proteinases essential for extracellular matrix breakdown and belongs to the Kunitz-type serine proteinase inhibitor family. TFPI2 has been shown to inhibit the formation of new blood vessels.[16] Decreased expression of TFPI2 is associated with the aggressiveness of various cancers and is suggested to play a critical role in tumor formation.[16–19] Similarly, genes such as DUSP10, SLC7A5, SSX2IP, SYNE2, DSCAML1, EXOC8, and MTMR7 were observed in the moderate stage, and three genes (PRPH2, FBXO32, and PANK4) in severe retinoblastoma were downregulated, associated with hypermethylated probes. Likewise, 15 genes (MAN1C1, FXYD5, DAPL1, PDGFC, PRR18, EFNB2, PC, NXPH1, CLCN1, SERPINB6, TRIM5, TXNIP, TRIM71, IRAK3, and COTL1) in mild, 21 genes in moderate, and 130 genes in severe disease states of retinoblastoma were upregulated, related to hypomethylation probes. These genes are specific to retinoblastoma stages, providing insights for diagnosis and treatment. For instance, compared to the control, we have compiled a list of top genes (Table 1) showing the highest fold change in gene expression in each disease state (mild, moderate, and severe).

5. Conclusions

In this study, we underscore the significance of epigenetic mechanisms, specifically hyper- and hypomethylation, in influencing changes in gene expression in retinoblastoma through a series of reproducible computational procedures. Our comprehensive analysis of gene expression data from

mild, moderate, and severe retinoblastomas, combined with genome-wide methylation array data from the cohorts, reveals robust findings that support the canonical association between hypermethylation and gene suppression (hyper-DOWN), as well as hypomethylation's association with gene activation or upregulation (hyper-UP). Our study provides many hyper-DOWN and hyper-UP patterns across various disease states in retinoblastoma. Among these patterns, some are unique, while others are shared across disease stages, providing molecular insights into retinoblastoma that may pave the way for novel diagnostic markers and open avenues for potential early treatment strategies.

6. Ethical Approval and Consent

[The study was approved by King Abdulaziz University ethics and research committee (reference No:147-24)].

7. Informed Consent Statement

This study is a computational biology study.

8. Data Availability Statement

Privacy or ethical restrictions limit the use of data.

9. Source of Funding

None.

10. Conflicts of Interest

The authors declare no conflicts of interest.

11. Author contributions

The author contributed to the conception and design of the study, data acquisition, analysis, and drafting of the manuscript.

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