Single-cell analysis reveals link between *FOXD1* and high-risk uveal melanoma

Researchers on behalf of the Rotterdam Ocular Melanoma Study Group[1] connect transcription regulator expression to high-risk uveal melanoma using QIAGEN CLC Genomics Workbench Premium and QIAGEN Ingenuity Pathway Analysis

Introduction

Melanoma is a type of cancer that arises from vmelanocytes, cells that produce melanin and also play a role in the immune system. While this cancer is often associated with the skin, about 5% of all melanomas originate from melanocytes in the eye. The most common type of ocular melanoma occurs in the uvea, the layer of the eye between the white sclera and light-sensing retina (Figure 1). The uvea includes the iris (controls the amount of light that enters the eye), the ciliary body (focuses the eye by adjusting the lens) and the choroid (the connective tissue that provides nutrients to the retina).

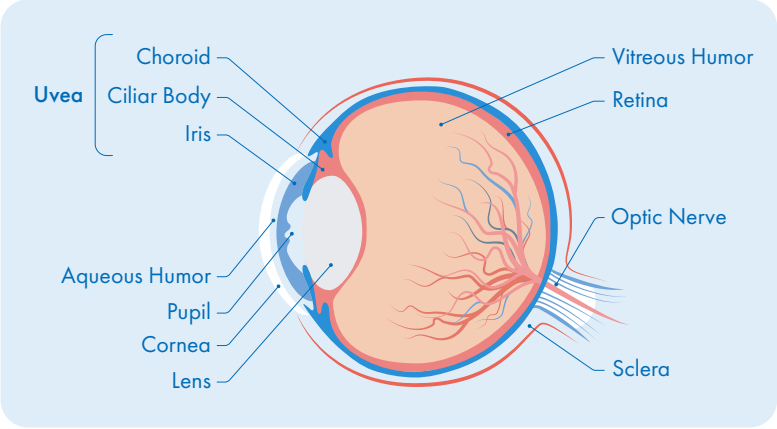

Figure 1. Parts of the uvea.

Since 1973, the 5-year relative survival rate for all types of uveal melanoma (UM) has held steady at 82.8%[2]. About half of patients develop metastatic disease, which is associated with a 1-year survival rate of 50%. There is no standardized treatment for metastatic UM, although the newly developed drug tebentafusp shows significant promise.

Despite this advancement, the mechanisms of metastasis are still largely unknown, making it difficult to prevent progression. Unlike skin melanomas, where driver mutations are usually caused by exposure to ultraviolet (UV) radiation, UM has a completely different mutational profile. Signs of UV-driven mutations are completely absent, with fewer driver mutations and different genes involved than typical melanoma.

One clue could lay in the transcriptome – UMs at high risk of metastasis have a higher expression of transcription factors than low-risk UMs. This expression profile also resembles transcription factor expression in embryonic melanocytes.

Researchers on behalf of the Rotterdam Ocular Melanoma Study (ROMS) Group investigated these developmental transcription regulators as potential mediators of tumor progression and metastasis. Identifying these regulators is easier said than done, though – how can you identify regulators in the developing embryonic ocular melanocytes before the eyes are even formed?

Enter the zebrafish

Although melanocyte cell fate is generally understood, the mechanisms of differentiation are not. This makes it difficult to distinguish uveal melanocytes from their cutaneous and mucosal cousins in human embryos. However, vertebrates like the zebrafish (*Danio rerio*), have unique mechanisms that could help with the identification of ocular melanocytes.

Premelanosome protein (pmel) is present in all human melanocytes, and the homologous protein premelanosome protein a (pmela) is present in all zebrafish melanocytes. But thanks to an evolutionary quirk (see box below), the ocular melanocytes in zebrafish contain a unique protein: premelanosome protein b (pmelb). This makes it easy to locate melanocytes in zebrafish embryos – and as a well-studied species, plenty of data are available.

Using this information, the authors investigated public single-cell RNA-sequencing (scRNA-seq) data from zebrafish embryos to investigate the transcriptome differences in low- and high-risk UM.

An evolutionary quirk

Teleosts are a diverse and fascinating lineage that includes over 26,000 species, from albacore to zebrafish. They represent 96% of all known fish and 50% of all known vertebrates.

Over 310 million years ago, a common ancestor of the teleosts underwent whole-genome duplication, which had significant consequences for the lineage. As a byproduct, the duplicated genes often alter their function. This is why teleosts like zebrafish express different proteins in their ocular melanocytes (pmelb), unlike other vertebrates, whose melanocytes all express the same protein (pmel).

This tweak contributed to the wide variation seen in fish today – and also makes a difference for uveal melanoma research.

A zebrafish (*Danio rerio*)

Methods and results

Finding melanocyte-specific markers

scRNA-seq datasets from zebrafish embryos and human eyes were downloaded from Gene Expression Omnibus (GEO) and processed with CLC Genomics Workbench Premium (CLC) and the Single Cell Analysis Module, using the default settings and genomes.

In CLC, melanocytes were identified in the embryos based on the presence of 5 markers: melanocyte inducing transcription factor a (mitfa), dopachrome tautomerase (dct), tyrosinase (tyr), tyrosinase-related protein 1b (tyrp1b) and pmela. These were separated into 7 clusters with UMAP. Clusters that contained only ocular melanocytes were identified based on pmelb expression, revealing four pmelb+ and three pmelb- clusters. Based on a list of genes expressed in all clusters, 279 genes were unique to pmelb- cells, 637 were unique to pmelb+ and 1817 were found in both (Table 1).

Of the 637 uniquely expressed genes in pmelb+ clusters, 478 were significantly likely to be found in human melanocytes.

Table 1. Unique genes expressed in clusters of melanocytes based on expression or absence of *pmelb*

Gene expression by cluster type	Number of unique genes
pmelb- only (3 clusters)	279
pmelb+ only (4 clusters)	637
Both (7 clusters)	1817

Simplify your single-cell workflow

With QIAGEN CLC, researchers conducted an integrated analysis of large scRNA-seq datasets, combining quality control, visualization, clustering and differential expression in one streamlined workflow. This made it easier to focus subsequent analyses on specific melanocyte populations, supporting biologically relevant discovery.

Identifying human orthologs

The 478 transcription regulators with significant expression in zebrafish were identified and matched to the corresponding human orthologs.

Among these orthologs, QIAGEN Ingenuity Pathway Analysis (IPA) identified 49 that were involved in transcriptional regulation. A Core Analysis was performed to examine how these genes interact with pathways and other functions, which revealed associations with cellular development, embryonic development and cancer.

Further analysis showed that 21 of these regulators were present in both healthy and cancerous human uveal melanocytes.

Guide your decisions with causal knowledge

With QIAGEN IPA, researchers explored how their differentially expressed genes interacted with curated pathways and networks to narrow their focus to a few critical regulators. IPA is built on curated causal knowledge, showing you the complex biological interactions that drive disease.

Exploring promising orthologs

RNA-seq data for 80 UM samples were downloaded from TCGA, and whole-transcriptome sequencing data for 26 UM samples from the ROMS-cohort were obtained. Bulk RNA-seq analysis was conducted, including whole-transcriptome samples and noting secondary driver mutations in eukaryotic translation initiation factor 1A X-Linked (*EIF1AX*), splicing factor 3b subunit 1 (*SF3B1*) or *BRCA1*-associated protein 1 (*BAP1*) where possible. These genes have been shown to correlate with the outcomes of uveal melanoma.

They then assessed how expression of transcription regulators varied based on the presence of mutations in *EIF1AX*, *SF3B1* and *BAP1*. From the list of 49 candidate genes with increased expression, 5 were associated with high risk of metastasis and poor prognosis (Table 2).

Low expression of *ELL2*, *KDM5B* and *REXO4* were associated with poor prognosis. Meanwhile, increased expression of *RBFOX2* and *FOXD1* were correlated with poor prognosis in *BAP1*-mutated UM. *SF3B1*- and *EIF1AX*-mutated UM saw lower expression of *FOXD1* relative to *BAP1*-mutated UM, but in all cases, expression of *FOXD1* was significantly correlated with a poor prognosis (Table 3).

Table 2. Genes associated with poor prognosis in uveal melanoma

Genes	Expression	Prognosis
Elongation factor for RNA polymerase II 2 (*ELL2*) Lysine demethylase 5B (*KDM5B*) REX4 homolog, 3′-5′ exonuclease (*REXO4*)	Low	Poor
RNA binding fox-1 homolog 2 (*RBFOX2*) Forkhead box D1 (*FOXD1*)	High	Poor

Table 3. Median survival rate for uveal melanomas based on the expression of *FOXD1* and the presence or absence of mutations in *BAP1*

Type	Median Survival
FOXD1-positive	26.5 months
FOXD1-negative	129.6 months
FOXD1-positive with BAP1 mutation	26.1 months
FOXD1-negative with BAP1 mutation	41.7 months

Confirming *FOXD1, RBFOX2, ELL2, KDM5B* and *REXO4* as significant biomarkers

To verify that these genes can be used to distinguish high- and low-risk UM, they compared against the genes used in the DecisionDx-UM™ gene expression profiling test. This test includes 12 genes with differential expression in high- and low-risk UM, plus 3 control genes with similar expression in both types. They compared the expression of these genes to expression of their 5 most promising candidates: *FOXD1*, *ELL2*, *RBFOX2*, *KDM5B* and *REXO4*.

In high-risk UM, the upregulation of *FOXD1* and *RBFOX2* resembled the upregulation of DecisionDx-UM genes *CDH1*, *ECM1*, *HTR2B* and *RAB31*. Similarly, the downregulation of *ELL2*, *KDM5B* and *REXO4* resembled the downregulated DecisionDx-UM genes *E1F1B*, *FXR1*, *ID2*, *LMCD1*, *LTA4H*, *MTUS1*, *ROBO1* and *SATB1*.

Finally, they validated these findings on the protein level with immunohistochemistry. They assessed levels of FOXD1 in samples with confirmed mutations in *EIF1AX*, *SF3B1* and *BAP1* and found a significant correlation between FOXD1 expression and lower survival rates.

Discussion

Taking advantage of a quirk of fish biology, researchers from the Rotterdam Ocular Melanoma Study Group analyzed gene expression in zebrafish embryo melanocytes, searching for a protein called pmelb. With CLC, they clustered melanocytes based on the presence of clusters of pmelb, allowing them to analyze ocular melanocytes specifically, rather than melanocytes in general.

They identified transcription regulators and translated these to their human orthologs based on the gene set list derived from this data. With IPA, they narrowed the list to the regulators that were relevant to disease progression. IPA contextualized these candidates within known biological networks, pathways and diseases, revealing that many of the identified regulators were associated with cellular development, embryogenesis and cancer. By applying the right bioinformatics tools, the authors streamlined their biomarker identification process with curated, traceable knowledge.

Integration with bulk RNA-seq datasets from TCGA and the ROMS cohort revealed a significant association between 5 regulators (*ELL2, KDM5B, REXO4, RBFOX2* and *FOXD1*) and high-risk UM. *FOXD1* emerged as the most prominent, showing strong upregulation in high-risk UM and a marked association with poor survival outcomes.

Applying their knowledge of zebrafish to explore uveal melanocyte development in humans, the authors identified multiple new biomarkers associated with metastasis in UM. This discovery points the way towards more personalized care that can focus on prevention instead of reaction.

Combine your single-cell research with curated, causal knowledge to discover new, meaningful biological interactions

Takeways

- Zebrafish embryos reveal developmental transcription factor expression in human uveal melanocytes
- scRNA-seq analysis distinguishes closely related melanocyte populations with confidence
- *FOXD1* emerges as a promising biomarker for high-risk UM

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1. van den Bosch, QCC et al., on behalf of the Rotterdam Ocular Melanoma Study Group. FOXD1 Is a Transcription Factor Important for Uveal Melanocyte Development and Associated with High-Risk Uveal Melanoma. Cancers, 2022;14(15), 3668. https://doi.org/10.3390/cancers14153668
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