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Development of a Heterotopic Zebrafish Model for Real-Time Observation of Retinoblastoma Tumor Growth

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ABSTRACT

Introduction: Retinoblastoma, the most prevalent pediatric eye cancer, arises from mutations in the RB1 gene, leading to the uncontrolled proliferation of retinal cells. This study introduces a heterotopic retinoblastoma model utilizing zebrafish, focusing on injecting the Y79 retinoblastoma cell line into the vitreous cavity for real-time tumor observation.

Methods: By leveraging the transparent embryos and rapid eye development of zebrafish, we tracked the establishment and growth of fluorescently labeled tumors.

Results: Results confirm tumor formation within three days, underscoring the model's relevance for in vivo studies. The zebrafish model capitalizes on the ease of maintenance, transparency for direct visualization, and genetic tractability, offering significant potential for high-throughput screening and therapeutic assessments. **Conclusion**: As the field progresses, this model promises to enhance our understanding of retinoblastoma biology and facilitate the discovery of effective treatments, addressing the critical need for innovative approaches in pediatric oncology.

Keywords: Retinoblastoma, Pediatric ocular tumor, Y79, Orthotopic Transplantation, Zebrafish model.

INTRODUCTION

Retinoblastoma is a malignant tumor that originates in the retina of infants and young children and is recognized as the most common eye cancer in this age group, with an incidence rate of 1 in 15,000 to 1 in 20,000 live births, adding approximately 9,000 new cases each year (1). The most notable symptoms of this cancer include an abnormal white reflex in the pupil, known as leukocoria, and misalignment of the eyes, referred to as strabismus (2). This cancer has a genetic basis and is caused by mutations in both alleles of the RB1 gene located on chromosome 13q14. These mutations impair the function of the retinoblastoma protein (pRB) and prevent it from effectively performing its role in tumor suppression, which involves regulating the transition of cells from the G1 phase to the S phase of the cell cycle. Over 110 unique mutations in the RB1 gene have been identified, producing a non-functional retinoblastoma protein, resulting in uncontrolled cell proliferation and, consequently, the formation of

tumors in the retina. Retinoblastoma occurs in two forms: hereditary, accounting for 40% of cases, and typically affects both eyes (bilateral), and non-hereditary, representing 60% of cases and usually involving one eye (unilateral). In the hereditary variant, the individual inherits a mutated RB1 allele from one parent, found in all cells of the child's body, and a second mutation then develops in a retinal cell, leading to tumor formation. In contrast, in the non-hereditary variant, both mutations of the RB1 gene occur specifically in the retinal cells. Non-hereditary retinoblastoma can occur due to the amplification of MYCN without any mutations in the RB1 gene, which happens in rare cases of these tumors (3).

In 1809, James Wardrop first described retinoblastoma as an independent disease, emphasizing that this tumor predominantly occurs in children and originates from the retina (٤). Cone precursor cells are recognized as the origin of retinoblastoma because, in the absence of the pRb, these cells become susceptible to malignant transformation through specific signaling pathways, including MDM2, RXRγ, TRβ2, and MYCN. These proteins promote cell proliferation and increase resistance to cell death, which contributes to the growth and spread of retinoblastoma tumors. The role of SKP2, which acts as a critical survival signal in pRb-deficient malignancies, further enhances this process. Notably, TRβ2 increases SKP2 activity, undermining the tumor-suppressive effects of TRβ1, thereby promoting the progression of tumors associated with RB1 deficiency (5-8).

Retinoblastoma can initially spread within the eye by seeding into the subretinal space or vitreous (9). The tumor may then invade the choroid and blood vessels, progressing through the optic nerve to the brain and into the subarachnoid space. From there, it can disseminate to the spinal cord and distant organs. Additionally, the tumor may invade surrounding tissue, such as orbital bones and the nasopharynx, and metastasize to other body parts via the bloodstream or, in some cases, through the lymphatic system (10). The staging of retinoblastoma tumors, ranging from stage A (tumors confined to the retina) to stage E (tumors with a high risk of metastatic spread), according to the International Classification System for Intraocular Retinoblastoma, has significantly helped in the management of this cancer (11). Based on the tumor stage, various therapeutic approaches are employed to treat retinoblastoma, including cryotherapy, laser therapy, radiotherapy, chemotherapy, and enucleation. Among these treatments, chemotherapy is the most frequently used option. This therapy can be administered through several methods, including intravenous (IVC), intra-arterial (IAC), intravitreal (IvitC), and intracameral chemotherapy (IcamC). The primary drugs used in this approach include carboplatin, etoposide, vincristine, melphalan, and topotecan, which are employed to combat retinoblastoma (12, 13).

Various animal models of retinoblastoma, such as transgenic mouse models and xenograft models in different species, have been developed to simulate clinical conditions and evaluate drug therapies (14). Additionally, retinoblastoma organoids have developed as innovative tools for investigating new treatments (15-17).

In this study, we developed a heterotopic retinoblastoma model by injecting tumor cells into the vitreous cavity of zebrafish. This model is particularly suitable for studying retinoblastoma due to several unique characteristics:

1. The transparency of zebrafish embryos allows for direct visualization of tumors.
2. The rapid development of the eye facilitates the investigation of early tumor formation stages in young larvae.
3. The structural similarities between the zebrafish retina and the human retina enhance the model's relevance for understanding the disease (18).

Therefore, since retinoblastoma occurs in infancy or childhood, the zebrafish model effectively simulates the early conditions of tumor development in the retina and can help assess potential therapeutic effects.

Furthermore, the ease of maintenance and high reproductive capacity, which enables the production of 100 to 200 eggs weekly, and the ability to maintain small embryos in 96-well

plates, make this model ideal for large-scale studies and the screening of drugs and genetic mutations with minimal drug solution usage (18, 19).

MATERIALS AND METHODS

2.1. Cell culture

The Human Y79 retinoblastoma cell line was obtained from the Royan Institute cell bank. This cell line was originally established by Dr. Reid from a tumor in the eye of a 2.5-year-old white girl (20). Y79 cells, which grow in suspension, were cultured in RPMI-1640 medium (Thermofisher), supplemented with 15% fetal bovine serum (FBS; Gibco), 1% glutamax (Gibco), and 1% penicillin-streptomycin (Gibco), and were maintained in a humidified atmosphere with 5% CO2 at 37°C.

2.2. Labeling tumor cells

Tumor cells were labeled with the PKH67 fluorescent dye (Sigma) to enable tracking and visualization. First, Y79 cells are suspended at approximately 10^7 cells/ml in a PKH67 dye solution and incubated for 5 minutes at 37°C, followed by an additional 15 to 30 minutes at 4°C. After incubation, the cells are washed with phosphate-buffered saline (PBS) or serum-free medium and then resuspended. The labeled cells are now ready for use in vivo experiments.

2.3. Zebrafish Embryo Production

In this research study under reference number IR.ACECR.AEC.1401.005, we used fli1: EGFP transgenic zebrafish, keeping them at 28.5°C under a dark-light cycle of 13 and 11 hours, respectively. To obtain embryos, we crossed adult male and female transgenic zebrafish, collected the fertilized eggs, and incubated them in an E3 medium (a suitable culture medium for zebrafish embryos containing essential salts and nutrients) at 28.5°C. 24 hours after post-fertilization (hpf), we treated the embryos with 0.2 Mm 1-phenyl-2-thio-urea (PTU; Sigma-Aldrich) to stop pigmentation. At 48 hpf, the embryos were dechorionated and anesthetized with Tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) (0.042 mg/ml E3-medium) in preparation for the next step.

2.4. Intravitreal injection of retinoblastoma cells in zebrafish

At 48 hours post-fertilization (hpf), the embryos were dechorionated and anesthetized with 0.042 mg/ml Tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) in preparation for the subsequent steps. To ensure stable injection, zebrafish embryos were placed on a 2% agarose gel. Y79 cells, labeled with PKH67, were accurately counted using a phase-contrast microscope. The cells were then centrifuged at 1,000 rpm for 5 minutes and resuspended in RPMI-1640 medium. A suspension containing 1,000 cells in 0.1-0.2 µl was injected into the zebrafish's right eye's vitreous cavity using Borosilicate Glass Capillary Needles attached to a microinjector under a stereo microscope. Daily evaluations of the injection efficacy were conducted using a fluorescence microscope. The left eye, which remained un-injected, served as the control group, ensuring reliable results for our study.

RESULTS

To simulate the clinical condition of retinoblastoma, we developed an orthotopic retinoblastoma model.

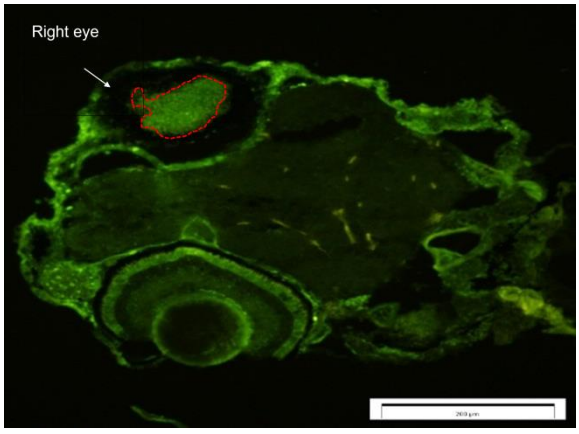

Figure 1. Visualization of tumor cells in zebrafish. Human retinoblastoma cells in the right eye of zebrafish Three days after the intraocular injection of one thousand cells stained with green color. Area of the tumor cells indicated by red dashed line.

In this context, one thousand PKH-labeled Y79 cells were injected into the vitreous cavity of the right eye of a zebrafish, while the left eye served as a control. Tumor formation was monitored daily using fluorescence microscopy. After three days, the tumor cells were clearly visible in the right eye under the fluorescence microscope (Fig.1).The intensity of green fluorescence in this eye confirmed tumor formation. These findings demonstrate the growth and persistence of retinoblastoma cells in the zebrafish model, highlighting the significance of this model for biological and pharmacological studies.

DISCUSSION

Animal models are key tools for cancer research and the development of new treatments. So far, retinoblastoma tumor models have been developed in both transgenic and xenograft forms in different species of animals. These models allow researchers to accurately examine disease mechanisms, tumor growth, and response to treatment. Besides these animal models, retinoblastoma organoids have also been developed as new and efficient tools for evaluating new treatments. In the following, various retinoblastoma models, especially the zebrafish model, will be examined. This model, due to its unique advantages, including the speed of tumor development and the ability to perform rapid screenings, is a valuable tool in the research and treatment of this type of childhood cancer.

LH-β[1] T-Ag models have been instrumental in advancing our understanding of retinoblastoma. By using the LH-β promoter to express the SV40[2] T-antigen oncogene in gonadotrope cells, scientists have created mouse models that develop tumors similar to human retinoblastoma (21). Additionally, other mouse models have been created that express the SV40 T-Ag and T-Ag oncogenes under the control of the IRBP[3] promoter (22, 23). These models have also revealed the role of Müller glia cells in tumorigenesis and the importance of specific genetic alterations in this process (24). However, while valuable, these models have limitations due to the use of viral oncogenes and their inability to fully replicate the complexity of human retinoblastoma.

Unlike humans, mice do not develop retinoblastoma from a single RB1 gene deletion due to compensatory mechanisms involving p107 and p130. To model human retinoblastoma in mice, researchers have combined RB1 deletion with other genetic alterations, such as inactivating p107 or p130. These combined genetic changes lead to uncontrolled cell proliferation and tumor formation (25, 26). Tumor formation occurs at an accelerated rate in mouse models in which pRb and p107 are inactivated, along with deletion of tumor suppressors such as p53 or PTEN or overactivation of the MDM4 gene (27-29). Additionally, increasing MYCN expression, in conjunction with RB1 inactivation, can drive mouse tumorigenesis, although this mechanism differs from human retinoblastoma where MYCN amplification often occurs without RB1 mutations (30).

Organoids, 3D tissue models grown from stem cells, are revolutionizing the study of retinoblastoma, a type of eye cancer (31). Researchers utilized these models to investigate the role of cone cells in tumor growth and identify effective drug combinations, like Topotecan and Melphalan (15). By introducing mutations in the RB1 gene using CRISPR/Cas9, researchers have created organoids that accurately model tumorigenesis, revealing the importance of signaling pathways like PI3-Akt and UPR (16). Additionally, organoids derived from patient-specific iPSCs offer a valuable tool for studying tumor development without relying on patient tissue, enabling the creation of multiple tumor models from a single patient or even from carriers of the RB1 mutation (17). While this method is time-consuming, it holds significant promise for drug discovery and reducing animal testing.

Xenograft models of retinoblastoma involve transplanting human retinoblastoma cells into immunodeficient animals like mice, rabbits, or zebrafish. Heterotopic models, where cells are injected subcutaneously, are useful for assessing treatment effects on tumor growth but lack the

1 Luteinizing hormone β sub-unit
2 Simian virus 40
3 Interphotoreceptor retinoid-binding protein

physiological context of the eye (32-34). Orthotopic models, where cells are injected directly into the eye, more accurately mimic the natural progression of retinoblastoma, including invasion and metastasis (10, 35, 36).

Zebrafish have emerged as a powerful model organism for studying human diseases, including cancer. Their rapid development, high fecundity, and transparent embryos make them ideal for observing cellular processes in real-time (37, 38). The retinal structure in zebrafish starts to develop at 32 hpf[4] and achieves functional maturity by five dpf[5]. This timeline of retinal development, along with the structural and functional similarities between the visual systems of zebrafish and humans, makes them valuable for studying eye diseases like retinoblastoma (18, 19, 39). Additionally, zebrafish are well-suited for drug screening due to their small size and ability to be maintained in large numbers (19).

To date, several studies have been conducted on the development of retinoblastoma tumor models in zebrafish. In a 2013 study, researchers injected human retinoblastoma cells into zebrafish embryos to model the disease. By using fluorescent markers and drugs like carboplatin and melphalan, they were able to observe tumor growth and assess the efficacy of potential treatments. The study demonstrated that the zebrafish model can effectively mimic human retinoblastoma and provide valuable insights into disease progression and therapeutic strategies (40).

In another study by Xiaoyun Chen and colleagues utilized a zebrafish model to investigate the invasion and metastasis of human and mouse retinoblastoma cells. By injecting fluorescently labeled tumor cells into the vitreous of zebrafish embryos, researchers were able to observe the formation of primary tumors, their invasion into surrounding tissues, and their metastasis to distant organs. This model allowed for the evaluation of the effectiveness of drugs like Sunitinib and Vegf-aa morpholino in inhibiting tumor growth and spread (41).

A recent study utilized a zebrafish model to investigate the migration and metastasis of human retinoblastoma cells. By injecting fluorescently labeled tumor cells into zebrafish embryos, researchers were able to track their migration pathways and observe potential metastasis to the brain. While the model proved effective in studying cell migration, the decreasing fluorescence intensity over time limited its utility for long-term drug screening. However, the zebrafish model remains a valuable tool for understanding the early stages of retinoblastoma and identifying potential therapeutic targets (42).

In the present study, we proved an orthotopic zebrafish retinoblastoma model to investigate the feasibility of using zebrafish as a preclinical model for this disease. By leveraging the genetic tractability and transparency of zebrafish embryos, we were able to develop a robust model that recapitulates key features of human retinoblastoma. Our findings provide a foundation for future studies aimed at identifying novel therapeutic targets and developing more effective treatments for retinoblastoma patients.

CONCLUSION

While various animal models, including mouse and rabbit xenografts, have contributed significantly to our understanding of retinoblastoma, zebrafish models have emerged as a particularly powerful tool. Their rapid development, transparent embryos, and genetic tractability offer unique advantages for studying the early stages of tumorigenesis and metastasis.

By directly observing tumor growth and spreading in real-time, researchers can efficiently screen for potential therapeutic targets and evaluate the efficacy of various treatments. While challenges remain, such as the limited time window for drug testing and potential differences in tumor behavior between zebrafish and humans, ongoing research is addressing these limitations. As technology continues to advance, zebrafish models are poised to play an increasingly important role in accelerating the development of effective treatments for retinoblastoma and other pediatric cancers.

4 Hours post fertilization
5 Day post fertilization

DECLARATIONS

Ethics approval and consent to participate: Ethics Approval and Participant Consent: The Institutional Ethical Committee of Royan Institute, Tehran, Iran, approved all animal experiments under reference number IR.ACECR.AEC.1401.005.

AUTHOR CONTRIBUTION

LS contributed to conceptualization, methodology, validation, supervision, and writing (reviewing and editing); SF contributed to investigation and original draft preparation; RM contributed to investigation and original draft preparation.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

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