Patient-Derived Xenotransplant of CNS Neoplasms in Zebrafish

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Glioblastoma and neuroblastoma are the most common central nervous system malignant tumors in adult and pediatric populations. Both are associated with poor survival. These tumors are highly heterogeneous, having complex interactions among different cells within the tumor and with the tumor microenvironment. One of the main challenges in the neuro-oncology field is achieving optimal conditions to evaluate a tumor's molecular genotype and phenotype. In this respect, the zebrafish biological model is becoming an excellent alternative for studying carcinogenic processes and discovering new treatments. It is possible to maintain glioblastoma and neuroblastoma primary cell cultures and transplant the cells into zebrafish embryos. The zebrafish is a suitable biological model for understanding tumor progression and the effects of different treatments. This model offers new perspectives in providing personalized care and improving outcomes for patients living with central nervous system tumors.

patient-derived xenotransplant zebrafish glioblastoma

1. Introduction

1.1. Neoplasms of the Central Nervous System

Worldwide, 308,102 patients were diagnosed with neoplasms of the central nervous system (CNS) in 2020, causing an estimated 251,329 deaths [1] The World Health Organization (WHO) classifies brain tumors ranging from a genuinely benign tumor (grade I), in which the complete tumor resection can be curative, to a high-grade malignant tumor (grade IV) for which, even with combined treatment, the expected survival is only approximately 22–24 months [2]. Between 2013 and 2017, the annual prevalence of CNS tumors in the United States was 6.4 per 100,000 persons, with an estimated mortality of 4.3 per 100,000 persons [3]. Eighty-five to 90% of the CNS tumors affect the brain [4], the most frequent being anaplastic astrocytomas and glioblastomas (GB), which account for 38% of primary brain tumors, followed by meningiomas and pituitary tumors [4].

Brain tumors are the most common type of solid childhood cancer, second only to leukemia as a cause of pediatric malignancies [5]. The incidence ranges from 0.3–2.9 per 100,000 live births [6][7]. Similar to their adult counterparts, the most common histological type of tumors are gliomas (of which the most common is pilocytic astrocytoma) and embryonal tumors (of which the most common is medulloblastoma) [8]. When considering infants, the most common extracranial solid malignant tumor is neuroblastoma (NB), and it is the most common cancer overall in infants younger than one year, with an incidence rate of 65 per million infants [9][10].

One of the most studied CNS neoplasms is GB [11] since it is the most common primary malignant brain tumor in adults and has one of the highest mortality rates of any neoplasm. The reported GB survival rate is 3.3% at two years and 1.3% at three years [12]. Currently, the standard of care is complete surgical resection combined with radiotherapy and temozolomide [13]. However, recurrences are common [12]. After numerous clinical trials evaluating different treatment strategies, most of them failed to add significant results in improving patient outcomes [14].

1.2. Main Pathways in the Development of Neoplasms of the CNS

Numerous investigations have studied the origin and pathways involved in developing CNS neoplasms [15], with controversies regarding the tumor-initiating cells that undergo mutations to proliferate into actual tumors [16]. The neural progenitor cells, also called neural stem cells (NSC), consists of oligodendrocyte precursor cells and astrocytes. They can proliferate if they receive a specific pathologic insult [15]. Nevertheless, there is no conclusive evidence that the NSCs are necessary or exclusive players in the formation of gliomas [17].

Due to the high heterogeneity of these neoplasms, defining a common pathway is challenging [15]. The Cancer Genome Atlas Project conducted in 2008 showed that 80% of the GB analyzed had altered signaling of the tyrosine kinase receptor, p53, and retinoblastoma protein (RB) [18]. Based on these classifications, Verhaak et al. in 2010 proposed four genomic subtypes, namely mesenchymal, classic, proneural, and neural [19]. One additional subdivision classifies GB in primary (de novo) or secondary (progressing from WHO grades I and II to grade IV) [20].

There is agreement that the critical pathways in the tumorigenesis of these neoplasms are the p53 pathway (p53/MDM2/4/p14ARF), the PTEN/NF1/RTK pathway (EGFR/RAS/NF1/PTEN/PI3K), and the RB pathway (p16INK4a/CDK4/RB) [15]. Mutations in TP53 and Nf1 appear in various grades of astrocytomas and enable them to evade apoptosis [21][22][23]. The PTEN pathway involves receptor tyrosine kinases (EGFR, PDGFR) and their associated pathways, which enable cell growth [24]. PTEN and NF1 modulate cell cycle entry in NSCs [25][26]. Various mutations in these proteins enable high-grade malignant glioma driven by MYC oncoprotein and highly penetrant GB [27][28]. Finally, RB regulates the G1/S checkpoint in the cellular cycle, causing increased mitotic activity [29][30].

1.3. Xenotransplantation in Zebrafish

In cancer research, xenotransplantation is the transfer of human cancer cells into a different species [31]. It is considered a human-in animal disease model with unique advantages and challenges compared to other models [31]. Standard cancer xenotransplant models include mammals, such as mice, rats, rabbits, dogs, and monkeys, due to their genetic and physiologic similarities with humans [32][33][34][35][36]. However, the most common xenotransplantation of human cancer cells employed in preclinical research occurs in immunocompromised mice. Most of the great discoveries in preclinical research in cancer are thanks to mice models due to their multiple benefits, such as their homology to human physiology, but mainly due to the development and maintenance of

specific strains through genetic manipulations and careful breeding under laboratory conditions, such as immunodeficient strains, through many years of research with the model. The model, though very advantageous, also has limitations.

The zebrafish (*Danio rerio*) model has emerged as a meaningful biological model to study cancer due to its genetic, molecular, and histological similarities with humans [32]. It has numerous characteristics that make zebrafish a suitable candidate for xenotransplant, and ingeniously complement and enrich cancer research. Embryos are easy to obtain, breed, and manipulate, with a daily production of hundreds, which facilitates extensive studies and high-throughput screening on in vivo assays [37]. Hundreds of embryos/larvae can be maintained on multi-well plates at a low cost [37][38][39]. Embryos develop rapidly, and in just 48 h, their nervous system is functional, reacting to stimuli through reflexive motility [40]. Zebrafish embryos and larvae are optically transparent, making them useful for dynamic live fluorescent imaging. This feature is particularly advantageous as tumor progression and some cellular processes are readily evident through microscopy techniques [41][42].

In zebrafish, adaptive and innate immune systems are highly similar to mice and humans [43]. Adaptive immunity is functional and morphologically mature between the second to the third week after fertilization, while innate immunity starts to appear on the first day after fertilization [44][45][46][47][48]. Thus, a lack of mature immune response during early larval development facilitates the transplantation of numerous cell types into zebrafish [37][49][50][51]. Although the zebrafish model has several benefits, there are some limitations to this model. Zebrafish larvae thrive in optimal conditions at 28 °C [52], which is below the temperature for proliferation and survival of mammalian cells (37 °C) [53]. Thus, most protocols maintain the larvae at an intermediate temperature of 32–33 °C with no significant consequences. Xenotransplantation can be performed in dozens of adult zebrafish and more than a hundred zebrafish larvae in a single day by a single operator, facilitating high throughput screening in cell transplantation studies [54][55][56][57][58][59][60][61]. Notwithstanding, the technique has many challenges and requires skill and many training hours for a high rate of injection and survival. Despite all the benefits, cancer xenotransplant studies in zebrafish are not numerous.

The first study of transplantation of human cells into zebrafish was published by Lee et al., 2005 [62]. This group injected human melanoma cell lines into the blastula stage of zebrafish embryos [62]. Since then, xenotransplantation using cancer cell lines into zebrafish larvae has been helpful to evaluate multiple diseases (for example, liver cancer [63][64][65], pancreatic cancer [66], colon cancer [49], ovarian carcinomas [67], gliomas [68][69], glioblastoma [70], and breast cancer [71][72], among many other types of cancer).

On the other hand, xenotransplant in zebrafish larvae from patient-derived samples is less common. Four years after the xenotransplantation of the first melanoma cell line in 2009, Marques et al. transplanted small, labeled samples from patients with pancreatic, colon, and stomach adenocarcinomas into the yolk sac of zebrafish [73]. Since then, many other types of primary cells have been transplanted (leukemia [74][75][76], breast cancer [77][78], pancreatic ductal adenocarcinoma [79], melanoma cells [80], gastric cancer cells [81], neuroendocrine tumor cells [82], and colorectal cancer cells [49]. The research in this field continues to grow with xenotransplantation of different types of cancer from both cell lines and patient-derived samples [36].

2. Patient-Derived Xenotransplant of CNS Neoplasms in Zebrafish

2.1. Heterogeneity

Brain Tumor samples from GB are usually very heterogeneous: a mixture of cancer and stem cells- GSCs with complex interactions between them and among different cells within and surrounding the tumor [19][83][84]. This feature represents a unique challenge to investigators and one of the primary considerations of the model. In the case of GB, most cells cannot recapitulate a phenocopy of the original tumor, and only a small subpopulation (GSCs) can form a tumor by themselves [85][86]. This heterogeneity not only can alter results at the laboratory but in the clinical practice, in which GSCs are notorious for their resistance to conventional chemotherapeutic agents and are the source for tumor initiation as well as recurrence [87]. For example, one of the factors that determine the effectiveness of temozolomide at the individual level is the tumor heterogeneity of the patient [88]. For these reasons, one of the main challenges in studying GB is to develop optimal culture conditions that preserve the molecular heterogeneity of the original tumor.

Due to the high heterogeneity, it is unlikely that a single compound will work with the same effectiveness for every patient. Therefore, this scenario makes it necessary to search for new therapeutic agents specific to CNS tumors and pathways in specific patients. A thoroughly described mechanism of action in the pathways could be part of an array of new therapies oriented to personalized medicine. Thus, establishing xenotransplantation of cells derived from patient brain tumors is a reliable and valuable platform with great potential for personalized therapy, similar to T-cell acute lymphoblastic leukemia [74].

2.2. Primary Xenotransplant vs. Cell Lines

Most of the biological characteristics such as proliferation, apoptosis, and drug screening, among others, have been evaluated in vitro using cell lines [70][89][90][91][92][93][94]. Although they have been instrumental in understanding tumor behavior, the mere fact of being a cell line does not represent the actual heterogeneity of a tumor sample from a patient. For example, Allen and co-workers using short tandem repeat analysis and mitochondrial DNA, compared the original U87MGB cell line with the same cell line distributed from the American Type Culture Collection (ATCC) and cell line service (CLS) [95]. They discovered that the original U87MG did not genetically match the U87MG provided by ATCC and CLS; even though the cells are of CNS origin, these cells did not match the reference line of origin [95].

From this point of view, the zebrafish xenotransplant offers the possibility to study main biological aspects such as tumor proliferation, angiogenesis, and metastasis in vivo, while keeping the unique molecular characteristics of the tumor cells. The fundamental importance of evaluating these biological aspects in vivo is that it is possible to evaluate the proliferation pattern (size, cellular projection) of the individual tumors of each patient; as observed for NB patient-derived tumor samples, in which the neuroblastoma cells were mitotically active following implantation. The cells survive and proliferate at rates similar to those inside the patient [96].

Tucker and co-workers emphasized the importance of patient-derived neuroblastoma xenografts to validate tumor progression, molecular targets, and drug resistance [97]. Even though murine systems are highly valuable and effective models, they have limitations such as low engraftment rates, long latency to tumor formation, and the high cost of initiating and maintaining experiments over extended periods [97]. In addition, it is typical to transplant patient-derived tumor cells in murine models after the third cell passage or later, while in zebrafish, it could be carried out directly after cell dissociation [98][99].

Numerous studies have shown that tumor cell xenotransplantation of different origins in zebrafish allows the analysis of different cancerous events such as invasion, metastasis [62][73][100], angiogenesis [50][51][101][102], and cancer therapies [50][51][101][102]. In this way, the xenotransplant of the human cells into zebrafish can address many stages of carcinogenesis and also shows that human cells communicate effectively with recipient fish tissue [103]. Assessing distinct stages of carcinogenesis might be more challenging in murine models since it requires more invasive methods such as biopsies or postmortem analysis, whereas, in zebrafish larvae, distinct features can be appraised under confocal and light-sheet microscopy in living individuals.

2.3. Model Limitations

There are differences between zebrafish and humans that need to be considered [52]. As mentioned previously, the optimal temperature for zebrafish (28 °C) and human cells (37 °C) is different. However, studies using xenotransplantation of tumor cells into the zebrafish model have successfully moved towards clinical studies in personalized medicine [104].

There are other limitations to using zebrafish in xenotransplantation experiments, arising from the phylogenetic distance between teleost fish and mammals. For example, orthopedic implantation is impossible due to the lack of corresponding organs in the model [37][52]. Furthermore, myelinated axonal sheaths do not develop in the zebrafish until four to seven days post-fertilization [105], affecting the invasion of implanted glioma cells [106]. Besides, in zebrafish embryos, the blood-brain barrier (BBB) does not develop up to 3 dpf [107] and is not mature for another seven days [108]. This observation is crucial for testing drugs targeting glioma cells since not all compounds may cross the BBB, and the developmental stage is essential for the experimental observation of pharmacologic effects in the model and clinical settings [52].

3. Conclusions

Based on these findings, the potential of GB isolation and culture cells makes it a valuable method that mirrors the molecular characteristics of the original tumor, with economic, ethical, and experimental advantages compared to xenotransplant in other animal models. Primary xenotransplant of CNS neoplasms in zebrafish remains a growing area of research. The high heterogeneity in the protocols and difficulty in culturing most patient-derived tumors is a challenge to overcome, and the number of studies will surely increase in the upcoming years. Patient-derived CNS tumor cells xenotransplants into zebrafish rise as a valuable platform that can guide clinical treatments in a

personalized way, with the ultimate goal of improving the outcomes for patients living with GB and NB and their complications.

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