AN INVESTIGATION OF THE HIPPO SIGNALING PATHWAY IN ATYPICAL TERATOID RHABDOID TUMOR

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ABSTRACT

Atypical teratoid rhabdoid tumor (ATRT) is a highly malignant pediatric central nervous system tumor. The prognosis is often poor, with a 2-year survival rate estimated at 15%. This dismal prognosis highlights the need to develop new treatment modalities for this devastating pediatric tumor. Recently, a tumor suppressing signaling pathway known as Hippo has emerged as a possible cancer treatment target. The Hippo signaling pathway is involved in organ growth and maintenance, and is dysregulated in many diverse cancers. We used quantitative real-time PCR to evaluate the mRNA expression profile of Hippo pathway genes. We then used determined the protein expression of various Hippo components using Western blots. The results of this study suggest that Hippo plays a definite role in atypical teratoid rhabdoid tumor.

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INTRODUCTION

Atypical Teratoid Rhabdoid Tumor

Atypical teratoid rhabdoid tumor (ATRT) is a rare, highly malignant embryonic central nervous system tumor that often strikes children less than two years of age [1–3]. ATRTs represent 1-2% of all pediatric brain tumors, but 10-20% of all brain tumors in children less than three years of age [4]. ATRTs are histologically heterogeneous, marked by rhabdoid (rod-shaped) cells with mesenchymal and epithelial elements [1,3]. In some cases, the classic rhabdoid elements can be missing entirely [5]. The heterogeneity within ATRT and the histological similarities to other central nervous system neoplasms often cause ATRTs to be misdiagnosed as medulloblastoma, primitive neuroectodermal tumor (PNET), choroid plexus carcinomas, and high-grade gliomas [2,6]. Thus, misdiagnosis of ATRTs has led to an underestimation in its prevalence and poorer treatment outcomes due to the necessity of aggressive treatment for this disease. ATRTs can be found in any part of the central nervous system, but are most commonly found in the posterior fossa [2]. Less commonly, ATRT can be found in the pineal gland, the pons, or the dorsal midbrain [3]. The median age at diagnosis is 20 months, with a slight male predominance. ATRTs have a dismal prognosis, significantly worse than other embryonal brain tumors [2]. A recent report from the Surveillance, Epidemiology, and End Results Database from 1973-2008 estimated an overall survival time of just 10 months from diagnosis. It has been estimated that nearly 20% of ATRTs present with metastatic disease, which greatly lessens survival time [4].

Atypical teratoid rhabdoid tumor is therapeutically challenging. Diagnosis of the disease is made based upon imaging techniques such as X-ray computed tomography and magnetic resonance imaging, clinical symptomatology, and biopsy (Figure 1). The frequency of diagnosis in patients less than three years of age complicates therapeutic options, as radiation therapy is often avoided in these patients due to the high risk of severe long-term side effects [4,7]. Children treated with high doses of cranial radiation often have great declines in intelligence quotient scores, lower quality of life measures, delayed neurodevelopment, and show significant neurocognitive dysfunction [8,9]. Other effects of cranial radiotherapy include decreased cerebral white matter, decreased rate of white matter development, hearing loss, endocrine

impairment, and secondary malignancies [8,10]. However, the exact pathophysiology of radiation-induced central nervous system damage is unknown [8]. There has been a recent push to use craniospinal irradiation therapy with a focal tumor bed boost in ATRT because of poor prognosis with surgical intervention and chemotherapy alone. Due to the rarity of ATRT, there is no consensus on effective chemotherapy regiments or protocols [4]. Currently, the role of high-dose chemotherapy followed by bone-marrow transplantation, or myeloablative chemotherapy, is being evaluated. The rationale of this treatment modality is to increase the dosage of the drugs and to lessen the protective effects of the blood-brain barrier. The effectiveness of myeloablative chemotherapy in ATRT, however, remains unknown [4,7]. The uncertainty in the optimal treatment for this disease highlights the need to develop new therapeutic options for the treatment of atypical teratoid rhabdoid tumor.

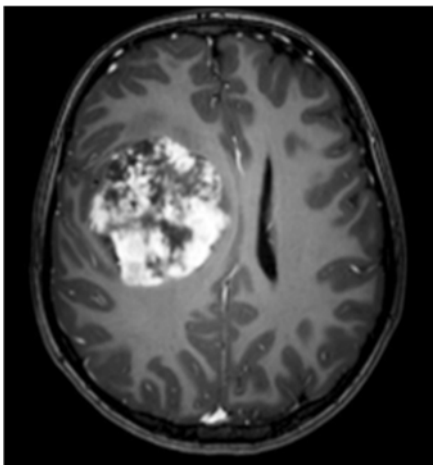

Figure 1. A Gadolinium-enhanced magnetic resonance image showing a large heterogeneously enhancing neoplasm in the right posterior portion of the frontol lobe. Histology confirmed the tumor to be an atypical teratoid rhabdoid tumor (reproduced from Yousaf et al, 2013)[11].

The SWI/SNF Complex

A defining feature in ATRT is a biallelic deletion of SMARCB1, a gene which encodes the evolutionarily conserved SWI/SNF chromatin remodeling complex on chromosome 22q11.2 [2,12]. While it is clear SMARCB1 deletion drives oncogenesis in ATRT, the exact mechanism in which it does so is unknown (ADD Lee, R. S. & Roberts, C. W. Rhabdoid tumors: an initial clue to the role of chromatin remodeling in cancer. Brain Pathol. 23, 200–205 (2013). DNA and its protein scaffold make up a complex tertiary structure known as chromatin. The central unit of chromatin is known as a nucleosome, which is made up of 146 base pairs of DNA wrapped around an octamer of histone proteins. Linker DNA connects adjacent nucleosomes, and these structures are tightly coiled to form high-order chromatin structures. The nucleosome provides both an organizational structure for DNA and modifier for gene expression by creating a physical barrier that can allow or block transcriptional access to DNA. Chromatin remodeling complexes regulate local chromatin structure and play a crucial role in regulating gene expression. These chromatin remodeling complexes can be grouped into two broad classes by their mechanisms of action: histone modifiers and ATP hydrolyzers. The SWI/SNF chromatin remodeling complex uses ATP hydrolysis to shift nucleosomes along a DNA strand, regulating transcriptional access to DNA [5,13]. The SWI/SNF complex is evolutionarily conserved and consists of a minimum of nine different protein subunits [13]. This shows its importance across various organisms.

Recently, the complex has been implicated in carcinogenesis [12,13]. Genetic knockdown mice have shown that loss of the BRG1 subunit cause the formation of tumors resembling breast adenocarcinomas, and mutations in this gene have been seen in prostate, breast, lung, and pancreatic cancer cell lines [5,13]. In addition, nearly 30% of non-small-cell lung cancers do not express BRG1 and BRM, both members of the SWI/SNF chromatin remodeling complex. It has been shown that four of the subunits – BRG1, SMARCB1, BAF155, and BAF170 – are required to remodel nucleosomes *in vitro*. The function of the other subunits is unknown, but it is believed they have a role in complex specificity by facilitating protein-protein interactions [13]. The SMARCB1 protein is also highly conserved, with identical amino acid sequences in mice and humans. However, there are no homologues of the gene and the amino acid sequence gives

little insight into how the protein functions. While the precise pathogenesis of ATRT is uncertain, SMARCB1 is thought to play an important role in the disease [2,3,6]. Loss of SMARCB1 has been shown to cause the dysregulation of several key cell cycle regulatory proteins in the G_1/S phase transition of the cell cycle [3,5].

The Hippo Signaling Pathway

In order for a solid tumor to grow, it must hijack the machinery involved in organ development and maintenance[14]. It is now clear that tumor cells are driven not only by genetic and epigenetic factors, but also by their microenvironment[15,16]. Thus, tumor growth is related to a much more fundamental question: how do organs "know" their final size? The recently discovered Hippo signaling pathway has emerged as a key regulator of organ size by controlling aspects of cell proliferation and apoptosis[17–19]. The Hippo pathway is composed of a highly conserved group of kinases that regulate transcription factors yes-associated protein (YAP) and PDZ-binding motif (TAZ) (Figure 2). The Hippo pathway is active when a group of four serine/threonine kinases, LATS1, LATS2, MST1, MST2, are phosphorylated. MST1/2 activity is regulated by the scaffolding protein Salvador (SAV). Similarly, LST1/2 is regulated by a co-molecule known as MOB1. Together, these kinases are responsible for regulating YAP and TAZ activity, co-activators of the tumor-suppressing pathway. When phosphorylated, YAP and TAZ are sequestered and degraded on the cytoplasm. In their unphosphorylated state, they translocate to the nucleus and bind with the TEA domain-containing sequence-specific transcription factors 1-4 (TEAD 1-4). These YAP/TAZ/TEAD complexes drive cellular proliferation [14,20,21]. TAZ has been shown to be the master regulator of glioblastoma, and excessive Hippo activity in the developing brain has been shown to increase neural progenitors[22,23]. When YAP and TAZ are unphosphorylated, they are active and translocate into the nucleus to serve as coactivators for genes that promote cell proliferation and inhibit apoptosis[14,18,19]. Thus, overactivation of the Hippo pathway leads to tumorigenesis[24,25]. Upregulation of Hippo pathway factors have been seen in several cancers, including medulloblastoma, glioblastoma, meningioma, B-cell acute lymphoblastic leukemia, ovarian, breast, and gastric cancer[17,23,25–29].

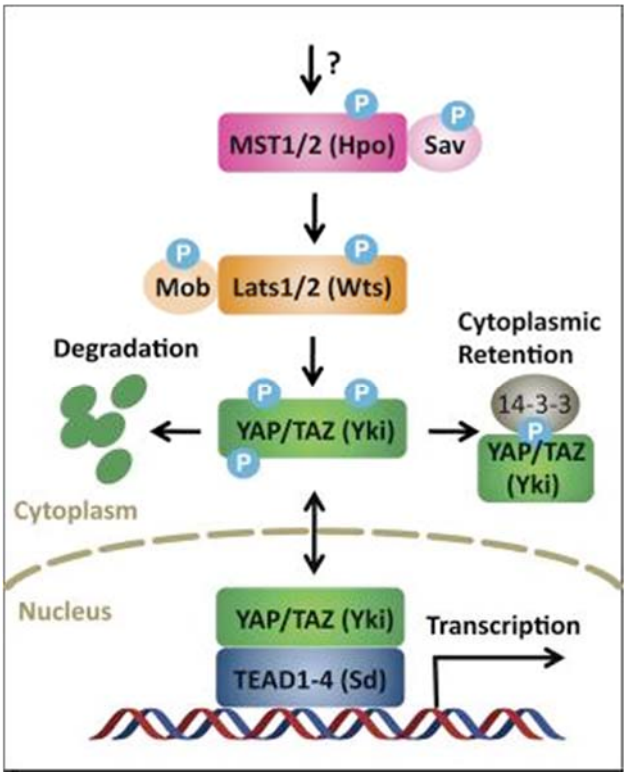

Figure 2. The Canonical Hippo Signaling Pathway. Reproduced from Yu and Guan, 2013.

Study Aims

Previous work from the Bhardwaj lab has shown aberrations in other signaling pathways in ATRT. Some ATRT possess overexpression of the Wnt5b ligand, a part of the Wnt signaling pathway. This pathway has been previously implicated in the carcinogenesis of numerous malignancies, including medulloblastoma, leukemia, breast, hepatocellular carcinoma, lung, and pancreatic cancer[30–34]. In addition, the Bhardwaj lab has found dysregulation in the Sonic hedgehog signaling pathway, another driver of oncogenesis. Other sources indicate that aberrant Sonic hedgehog (Shh) signaling is implicated in various tumors, including medulloblastoma, basal cell carcinoma, pancreatic, prostate, lung, and gastrointestinal cancers [35–39]. There is growing evidence that Wnt and Shh interact with the Hippo pathway, leading to cancer cell invasion and self-renewal[14,22].

Poor prognoses highlights the desperate need to develop new treatment strategies for ATRT and other malignant pediatric neoplasms. This study will investigate the Hippo pathway as a potential therapeutic target for ATRT and determine its relationship to other drivers of carcinogenesis, including Wnt and Shh. No previous group has investigated the role of Hippo in ATRT. We hypothesize that ATRT will possess overactive Hippo pathway activity.

MATERIALS AND METHODS

Cell Lines

Three ATRT cell lines were used in this study (CHLA-04-ATRT, 20-month old male; CHLA-05-ATRT, 2-year old male; CHLA-06-ATRT, 4 month-old female). The guardians of each patient signed informed consent for their scientific use. Tissues were prepared as described (Xu et al) and were initially cultured as neurospheres in modified neuro-basal medium consisting of 1:1 Dulbecco's modified Eagle's medium: F12, HEPES (15 mM), sodium pyruvate (110 mg/L), sodium bicarbonate (1.2 g/L), 1xB27 (Invitrogen), epidermal growth factor (20 ng/mL; Invitrogen), and bovine fibroblast growth factor (20 ng/mL; Cell Sciences). Gentamycin 25 mg/ml was used during the first 2 weeks of growth. Passaging was performed at a ratio of 1:2–3 with 25% conditioned medium (Erdreich) Loss of SMARCB1 was confirmed by immunohistochemistry, Western blotting, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and G-band karyotyping.

Human Tissue

Brain regions (frontal cortex, temporal cortex, white matter) from 2 age-matched patients' non-tumor brain tissues were used as a control. The non-tumor brain tissues were acquired from the NICHD Brain and Tissue Bank for Developmental Disorders (The University of Maryland, Baltimore, MD).

Quantitative Reverse Transcriptase PCR

Total RNA was isolated from all tissues using RNeasy Minikit (Qiagen) and cDNA was synthesized using the Superscript III cDNA Kit (Life Technologies) according to manufacturer protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad) and the CFX96 Real Time System (Bio-Rad). The mRNA expression levels were compared to non-tumor brain using the comparative Ct method, normalized to GAPDH.

Western Blot Analysis

Protein expression levels were analyzed by Western blot according to manufacturer protocol. 30 μg of total protein were separating by SDS-PAGE using 4-12% Bis-Tris gels (Life Technologies) and transferred by nitrocellulose (Invitrogen). The membrane was incubated with YAP1 (1:500; Cell Signaling), phospho-YAP1 (1:500; Cell Signaling), MST1 (1:1000, Cell Signaling), MST2 (1:1000, Cell Signaling), LATS1 (1:1000, Cell Signaling), LATS2 (1:1000, Cell Signaling), MOB1 (1:1000, Cell Signaling), phospho-MOB1 (1:1000, Cell Signaling), or Salvador (1:1000, Cell Signaling). All membranes were incubated with the GAPDH as loading control (1:1000, Cell Signaling). HRP-linked anti-rabbit IgG was used as secondary antibody (1:2000, Cell Signaling) and detected using SuperSignal West Dura Extended Duration Substrate (ThermoScientific).

RESULTS

Hippo pathway components are overexpressed in pediatric atypical teratoid rhabdoid tumor

The role of the Hippo signaling pathway, thought to be responsible for organ growth and maintenance, is unclear in atypical teratoid rhaboid tumor. In order to determine the mRNA expression levels of Hippo pathway components, we performed quantitative real-time polymerase chain reaction on 3 ATRT cell lines (Figure 3). YAP, a transcriptional co-activator of the Hippo pathway, was overexpressed in 2 cell lines. The TEA domain family member proteins 1-4 (TEAD), the transcription factors that form a complex with YAP/TAZ and are thought to drive cellular proliferation, were overexpressed in all ATRT cell lines and in the H9 neural stem cell line.

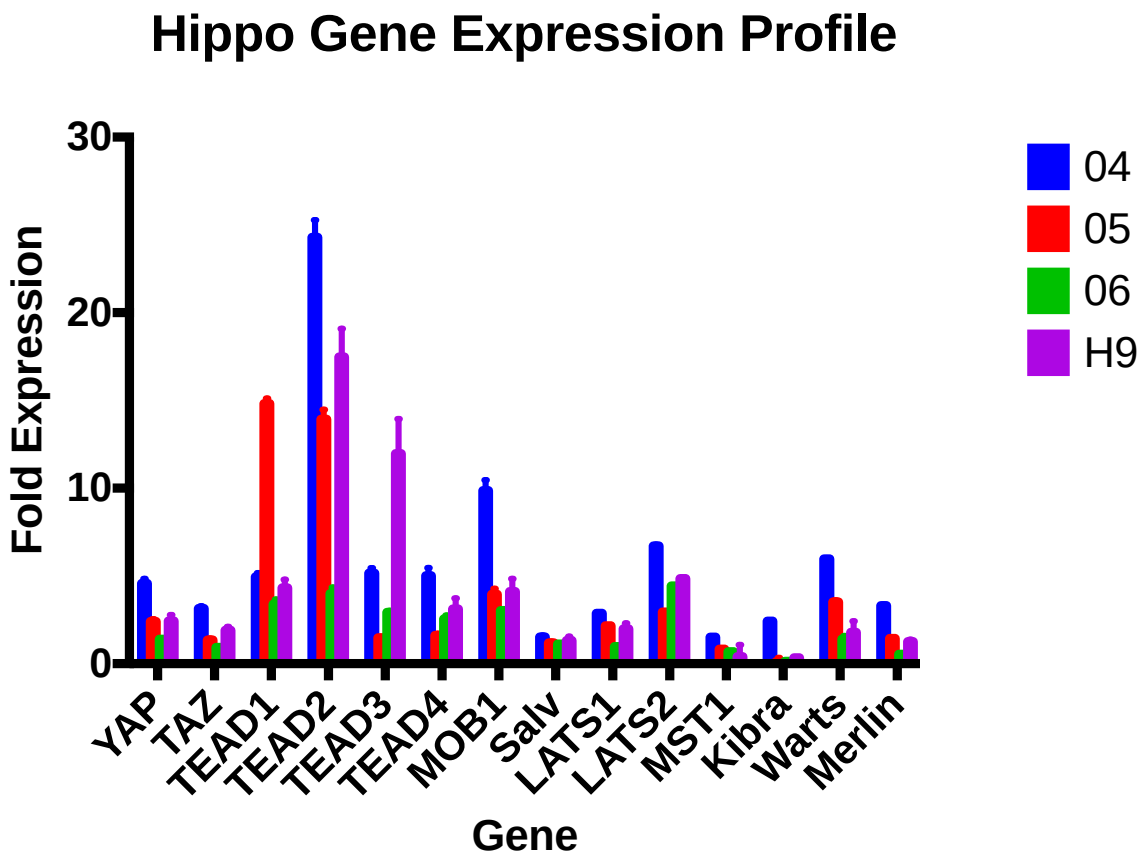

Figure 3. Quantitative RT-PCR analysis of Hippo pathway components in atypical teratoid rhabdoid tumor. qRT-PCR was performed on three ATRT cell lines (CHLA-04-ATRT, CHLA-05-ATRT, CHLA-06-ATRT) and a neural stem cell positive control (H9). Relative quantification of mRNA expression levels in the samples were compared to pediatric anoxic brain samples using the comparative Ct method and normalized to GAPDH.

Hippo pathway proteins are overexpressed in atypical teratoid rhabdoid tumor

Next, we investigated whether Hippo signaling pathway components were overexpressed at the protein level. Total YAP protein was overexpressed in ATRT cell lines, while no signal for phosphorylated YAP was seen by Western blot (Figure 4). The same pattern was seen in the neural stem cell line (H9) and malignant rhabdoid kidney cell line (G401). Similarly to the pattern in YAP/phospho-YAP, there was high protein expression of the regulatory MOB1 subunit but not its phosphorylated form (Figure 4). ATRT cell lines showed MST1 expression, but not MST2 (Figure 4).

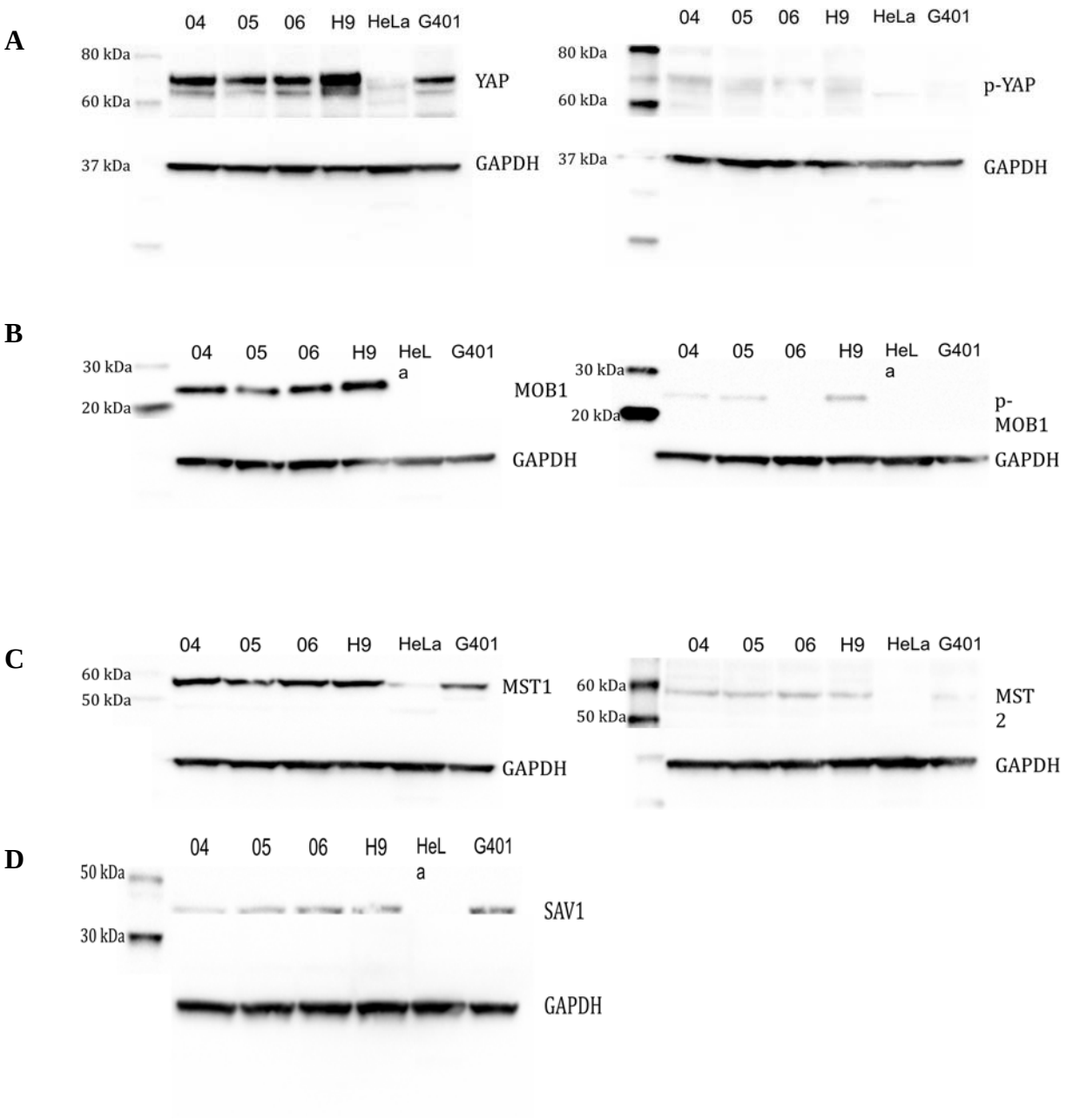

Figure 4. Canonical Hippo pathway protein expression levels by Western blot. (A) Protein expression levels of YAP/phospho-YAP. (B) Protein expression levels of regulatory protein MOB1/phospho-MOB1. (C) Protein expression levels of kinases MST1 and MST2. (D) Protein expression levels of scaffolding protein SAV1.

DISCUSSION

Our study demonstrates that the Hippo signaling pathway is dysregulated in atypical teratoid rhabdoid tumor. We showed that mRNA levels of canonical and non-canonical Hippo factors are elevated in ATRT cell lines. We also showed overexpression of canonical Hippo components by Western blot. These results are in agreement with a previous study that showed Hippo involvement in a *drosophila melanogaster* model system[1]. These results are further supported by this study's demonstrated efficacy of verteporfin in ATRT cells, a known YAP/TEAD inhibitor[1]. It remains to be seen whether verteporfin or other Hippo small molecule inhibitors are viable treatment modalities for ATRT in humans.

ATRT as a Stem-Cell Driven Tumor

There is growing evidence that some adult and pediatric brain tumors possess cancer stem-like cells[2,3]. In ATRT, pluripotent $CD133^+$ cells were isolated from tissue and shown to be more resistant to chemotherapy and radiation therapy than $CD133^-$ ATRT cells[4]. These cancer stem-like cells are thought to be responsible for both the regeneration of brain tumors after resection and their resistance to therapy. Our study demonstrated a strikingly similar mRNA expression profile between ATRT cell lines and the H9 neural stem cell line. This suggests that ATRT has a high population of brain tumor cancer-like stem cells. This would explain the poor prognosis of ATRT and its resistance to both radiotherapy and chemotherapy. Recently, TAZ has been implicated in the ability of cancer stem-like cells property of self-renewal[5].

The Importance of Hippo in ATRT

The Hippo signaling pathway has been shown to interact numerous pathways, including Wnt, Sonic Hedgehog, TGF-β, JAK-STAT, and Notch. In ATRT, Hippo has been shown to interact with both Wnt and Sonic Hedgehog[6]. These growth-signaling pathways are known to interact, but the importance of each in relation to one another is poorly understood. However, it has been hypothesized that the Hippo pathway is of crucial importance in these poorly understood

cross-talk mechanisms[7]. TAZ, a downstream Hippo transcriptional co-activator, has been shown to mediate Wnt/β-catenin activity[8,9].

FUTURE DIRECTION

There has been extremely limited research regarding the connection between Hippo and ATRT. This study has connected this tumor-suppressing pathway to the potential pathogenesis of the disease. The next step is to successfully knockdown YAP and TAZ and determine the genetic profile of YAP^- ATRT cells by qRT-PCR and Western blot. There also should be investigations into the potential for therapeutic targets. Verteporfin, a YAP/TEAD inhibitor, should be tested in ATRT cells to determine its efficacy. This includes an IC-50 and a cell proliferation assay. Investigation into the connection between Hippo and ATRT could potentially yield a new therapeutic strategy to a traditionally difficult disease.

CONCLUSION

The Hippo pathway has emerged over the past several years as a new target for a variety of tumors. While investigations on Hippo and its relation to oncogenesis are in their infancy, there is increasing evidence that the pathway is involved in the pathogenesis of numerous cancers. This study suggests that the tumor-suppressing Hippo pathway is shut down in ATRT. Currently, there is no universal or effective therapy for this disease. The results of this study suggest that the Hippo pathway is a potential therapeutic target in the treatment of ATRT.

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