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Rapamycin targeting mTOR and hedgehog signaling pathways blocks human rhabdomyosarcoma growth in xenograft murine model

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ABSTRACT

Rhabdomyosarcomas (RMS) represent the most common childhood soft-tissue sarcoma. Over the past few decades outcomes for low and intermediate risk RMS patients have slowly improved while patients with metastatic or relapsed RMS still face a grim prognosis. New chemotherapeutic agents or combinations of chemotherapies have largely failed to improve the outcome. Based on the identification of novel molecular targets, potential therapeutic approaches in RMS may offer a decreased reliance on conventional chemotherapy. Thus, identification of effective therapeutic agents that specifically target relevant pathways may be particularly beneficial for patients with metastatic and refractory RMS. The PI3K/AKT/mTOR pathway has been found to be a potentially attractive target in RMS therapy. In this study, we provide evidence that rapamycin (sirolimus) abrogates growth of RMS development in a RMS xenograft mouse model. As compared to a vehicle-treated control group, more than 95% inhibition in tumor growth was observed in mice receiving parenteral administration of rapamycin. The residual tumors in rapamycin-treated group showed significant reduction in the expression of biomarkers indicative of proliferation and tumor invasiveness. These tumors also showed enhanced apoptosis. Interestingly, the mechanism by which rapamycin diminished RMS tumor growth involved simultaneous inhibition of mTOR and hedgehog (Hh) pathways. Diminution in these pathways in this model of RMS also inhibited epithelial mesenchymal transition (EMT) which then dampened the invasiveness of these tumors. Our data provide bases for using rapamycin either alone or in combination with traditional chemotherapeutic drugs to block the pathogenesis of high risk RMS.

1. Introduction

Sarcomas represent the 5th most common type of solid childhood cancers, with rhabdomyosarcomas (RMS) being the most common form accounting for 40% of these tumors. The yearly incidence of RMS is 4.5 cases/million children/year [1,2]. RMS can be broadly classified based on histological and genetic findings into alveolar RMS (ARMS) and embroynal RMS (ERMS). ERMS tumors are more common and generally associated with a better outcome even in patients with metastatic disease. Studies of survival rates in ERMS versus ARMS show a 5 year survival rate for ERMS in patients with metastatic disease at 42% for ERMS vs. 18% for ARMS [3]. The chromosomal translocations t(2:13)(q36;q14) and t(1;13)(q35;q14) are specific molecular events found in ARMS [4,5]. Recently, ARMS tumors have been further categorized as fusion positive and negative tumors with implications for important biological and clinical distinctions [6]. Hedgehog (Hh) signaling has been previously demonstrated to be involved in the pathogenesis of a subset of ERMS [7].

Recent advances in the understanding of molecular basis of refractory and metastatic RMS provide opportunities to test novel therapeutic options. In this study, we investigated the effects of rapamycin, a well-known mTOR inhibitor [8] on the growth of poorly differentiated human RMS in a murine xenograft model. Rapamycin (sirolimus) is a macrolide antibiotics with immunosuppressive and antifungal effects [9]. It had been used as an immunosuppressant post organ transplant [9]. Rapamycin binds to cytoplasmic receptor KF506-binding protein 12 (KFBP12). This complex helps in inhibiting mammalian target of rapamycin (mTOR) which is a downstream element in the (PI3K/AKT)

Abbreviations: RMS, rhabdomyosarcoma; ERMS, embroynal RMS; ARMS, alveolar RMS; VEGF, vascular endothelial growth factor; H&E, hematoxylin & eosin; PCNA, proliferating cell nuclear antigen.

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signaling pathway and is involved in cell cycle regulation and proliferation [9–12].

Our findings demonstrate that rapamycin effectively inhibits the growth of poorly differentiated human RMS xenograft tumors in nude mice. The tumor growth inhibition is associated with the reduction in proliferation and induction of apoptosis. The tumor growth inhibitory effects are associated with the diminution of Hh and mTOR signaling pathways. These pathways have been implicated in the pathogenesis of RMS [7,13]. Here, we also show that targeting of Hh/mTOR pathways by rapamycin dampens the epithelial mesenchymal transition (EMT) progression which is known to play an important role in the invasiveness and metastatic potential of these and other solid tumors [14]. Thus, rapamycin-treated residual xenograft tumors show a decrease in mesenchymal marker proteins.

2. Materials and methods

2.1. Cell culture

Human poorly differentiated RMS cell line A204 was obtained from the American Type Culture Corporation (Manassas, VA, USA). Cells were cultured in McCoy's 5A media (Hyclone) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 incubators.

2.2. Tumor xenograft study

Female Nu/Nu mice were purchased from NCI-Frederick Animal Production Program (Frederick, MD, USA). Mice used for experiments were 6–8 weeks of age. All animal procedures were performed according to guidelines and approvals of the Institute Animal Care and Use Committee of the University of Alabama at Birmingham. For this study, A204 cells were detached by trypsinization, washed, and re-suspended in PBS. Animals were divided into two groups of five mice each. Each animal received subcutaneously 2×10^6 cells/200 μL PBS in each flank. Twenty-four hours after the inoculation of RMS cells, group-1 mice received an intraperitoneal (i.p.) injection of vehicle, whereas groups-2 received i.p. injections of rapamycin (40 μg/mouse, daily) respectively for five weeks. Tumors were measured by digital calibers and tumor volumes calculated using the formula volume = length × width × height/2 plotted as a function of days on test.

2.3. Real-time PCR

RNA was extracted using Trizol, and reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, NY, USA). Quantitative PCR was performed using Taqman Fast Advanced Master Mix Product Insert (Applied Biosystems, NY, USA). Experiments were performed in triplicate for both the target and the endogenous housekeeping gene. Relative quantification of the target gene expression was calculated by the comparative threshold cycle (Ct) method: $2^{-\Delta\Delta Ct}$ where $\Delta Ct = Ct_{target}$ gene $- Ct_{housekeeping}$ gene and $\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{vehicle}$. List of primers used in this study is provided as in Supplementary Table S1.

2.4. Western blotting

Briefly, 60 μg of total protein from tumor cell lysate was separated on a SDS–PAGE gel (10%) and blotted onto PVDF membrane. Western blot assay was performed as described previously [15]. List of primary antibodies used in this study is provided as Supplementary Table S2.

2.5. Immunofluorescent staining

Immuno-staining was done on paraffin-embedded tumor sections as previously described [15] employing primary antibodies (Supplemental Table S2). The processed sections were visualized on an Olympus BX51 microscope.

3. Results

3.1. Rapamycin treatment inhibits gross tumor growth in xenograft tumors

A204 cells following inoculation in nude mice form stable xenograft tumors [15]. Rapamycin treatment of nude mice bearing these RMS reduces tumor growth significantly. The differences in the growth of tumors in vehicle-treated control and rapamycin-treated groups were apparent from the third week of tumor cell inoculation, however, at weeks 4 and 5 these differences were highly significant ($p < 0.05$). At the termination of the experiment at week 5, there was an inhibition of more than 95% in tumor growth (Fig. 1A). The histology of these tumors as shown in Fig. 1B confirmed a poorly differentiated tumor phenotype with the diffuse cell growth in the control group whereas, rapamycin-treated tumors were more differentiated and highly organized. Rapamycin-treated tumors also showed areas with marked necrosis which were largely absent in tumors from vehicle-treated control mice.

3.2. Rapamycin treatment inhibits proliferation and induces apoptosis in xenograft tumors

To define the mechanism by which rapamycin inhibits growth of A204 tumors we first typed them for biomarkers of proliferation.

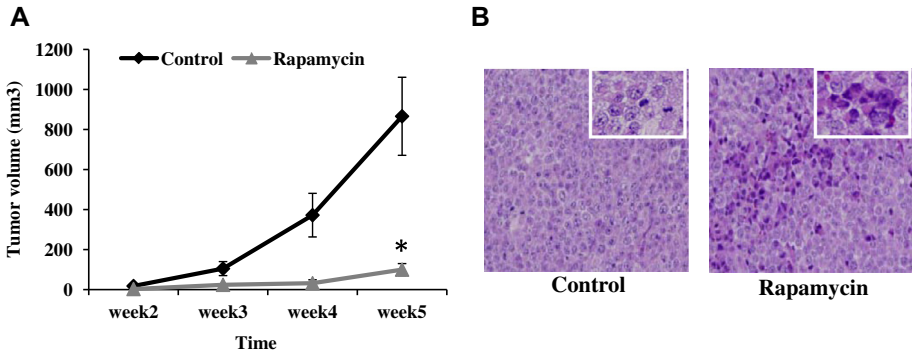

Fig. 1. Effects of rapamycin treatment on the xenograft RMS tumor growth. (A) The effect of rapamycin on tumor volume. This figure shows rapamycin's inhibitory effect on tumor growth. Marked suppression is seen in comparison to the vehicle-treated group ($p < 0.05$). (B) H&E staining of *rapamycin*-treated vs. vehicle-treated control tumors showing more differentiated and highly organized tissue in the rapamycin-treated group with a decrease in viable cells as compared to the control group.

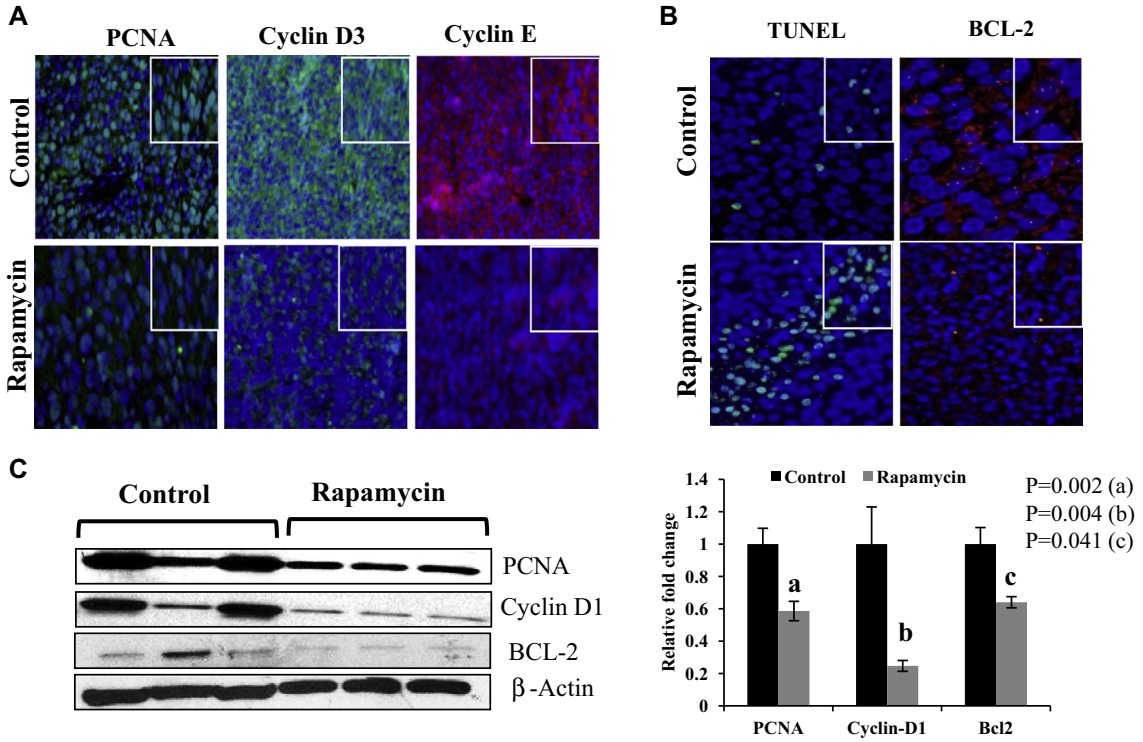

Fig. 2. Effect of rapamycin treatment on the biomarkers representing proliferation and apoptosis in RMS xenograft tumors. (A) PCNA, TUNEL, and BCL-2 immune histochemical staining. This figure shows the decreased proliferation associated with reduced cell cycle progression. (B) Staining for pro-apoptotic effect in the control versus rapamycin group shows increased apoptosis in the rapamycin group in comparison to the control as seen in TUNEL green positive cell staining. BCL-2 expression as an anti-apoptotic protein is seen more in the control group in comparison to the treatment groups. (C) Western blot analysis: showing inhibition of PCNA and cyclins D1 and decreased anti-apoptosis BCL-2 expression ($p = 0.002$, 0.004 and 0.041 respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We observed an intense nuclear staining for PCNA in vehicle-treated control tumors whereas only few cells positive for PCNA staining could be visualized in rapamycin-treated tumors (Fig. 2A). Lower PCNA expression may correlate with a better prognosis [16]. Similarly, cyclin D3 and E which are important regulators of cell cycle in G1/S phase [17], were found to be highly expressed in control tumors and showed significant reduction in their expression following rapamycin treatment (Fig. 2A). Similar effect of rapamycin was noted on the expression of cyclin D1 (Fig. 2C). After ascertaining that rapamycin inhibits proliferation, we also measured the apoptotic effects of rapamycin in A204 tumors. As expected, we observed an enhanced number of TUNEL-positive cells in rapamycin-treated tumors whereas vehicle-treated tumors showed only a few cells positive for TUNEL (Fig. 2B). Consistently, the expression of anti-apoptotic protein BCL-2 [15], was diminished significantly ($p < 0.05$) in rapamycin-treated group as compared to control (Fig. 2B and C).

3.3. Rapamycin targets Hh and mTOR signaling pathways in RMS xenograft tumors

Hh signaling pathway is known to be induced in ERMS sarcomas [18]. In addition, mTOR/AKT is also known to be involved in the pathogenesis of RMS and other sarcomas [13]. Therefore, we employed A204 xenograft tumors to study whether these pathways are important in terms of pathogenesis of the neoplasm and whether a known inhibitor of mTOR, rapamycin can abrogate the activation of these pathways in this model of poorly differentiated RMS. In this regard, previously rapamycin was shown to inhibit a downstream target of Hh signaling, GLI1 [19]. As shown in Fig. 3A, rapamycin treatment decreased significantly the expression of Hh effecter genes, GLI1 and GLI2 as well as PTCH1 and PTCH2 that are typically upregulated via a regulatory feedback loop. This reduction in the expression of these proteins was also confirmed by immune-fluorescence staining (Fig. 3B). In addition, as expected, rapamycin significantly reduced mTOR signaling pathway together with the expression of p-AKT (Fig. 3B and C). These data suggest that rapamycin under certain experimental conditions can reduce not only mTOR signaling pathway but also Hh pathway in the same experimental setting. The decrease in these two pathways by rapamycin was accompanied by a decrease in mRNA expression of cell cycle regulatory proteins including cyclin D1 which was reduced by more than 80% (data not shown). Cyclin D1 is a downstream transcription target for both Hh and mTOR signaling pathways [20,21].

3.4. Rapamycin treatment inhibits epithelial mesenchymal transition

Both Hh and mTOR signaling pathways regulate epithelial–mesenchymal transition (EMT) as well as angiogenesis [22,23]. We therefore, tested whether treatment with rapamycin can modulate the expression of proteins that regulate EMT and angiogenesis. As shown in Fig. 4A, we found a significant decrease in the expression of EMT regulating proteins fibronectin, twist and vimentin. Similarly, angiogenesis related protein VEGF was also reduced. The transcription factors twist and slug, known to be involved in the regulation of EMT, show 64% and 72% inhibition, respectively, while N-Cadherin which also reflects mesenchymal characteristics was reduced by 53% (Fig. 3B).

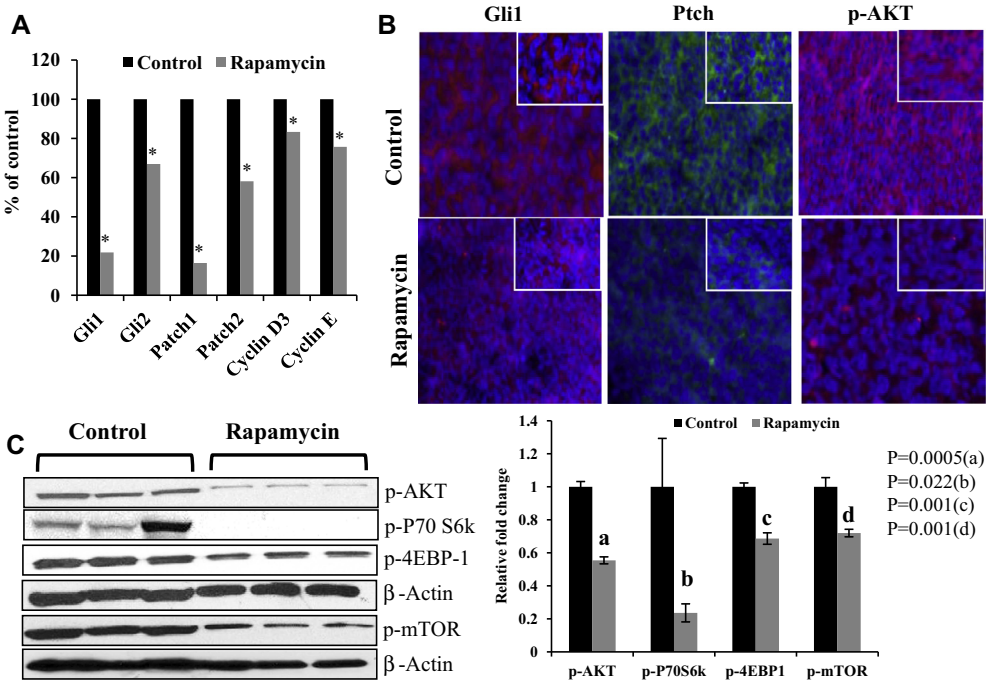

Fig. 3. Effects of rapamycin on the mTOR and Hh signaling pathways. (A) Shows RT-PCR mRNA expression for the rapamycin-treated group versus the control group. Major inhibitory effect is seen in the rapamycin-treated group in GLI-1,Gli-2,Ptch-1 and Ptch-2. (B) Immuno-staining showing inhibition of GLI-1 and Ptch-1. Decreased AKT activation is seen in the form of decreased pAKT staining. (C) Western blot analysis showing effector molecules in the AKT/mTOR pathway and the inhibition seen AKT,mTOR,4EBP-1 and P70S6 in the rapamycin-treated group ($p = 0.0005$, 0.022, 0.001 and 0.001 respectively).

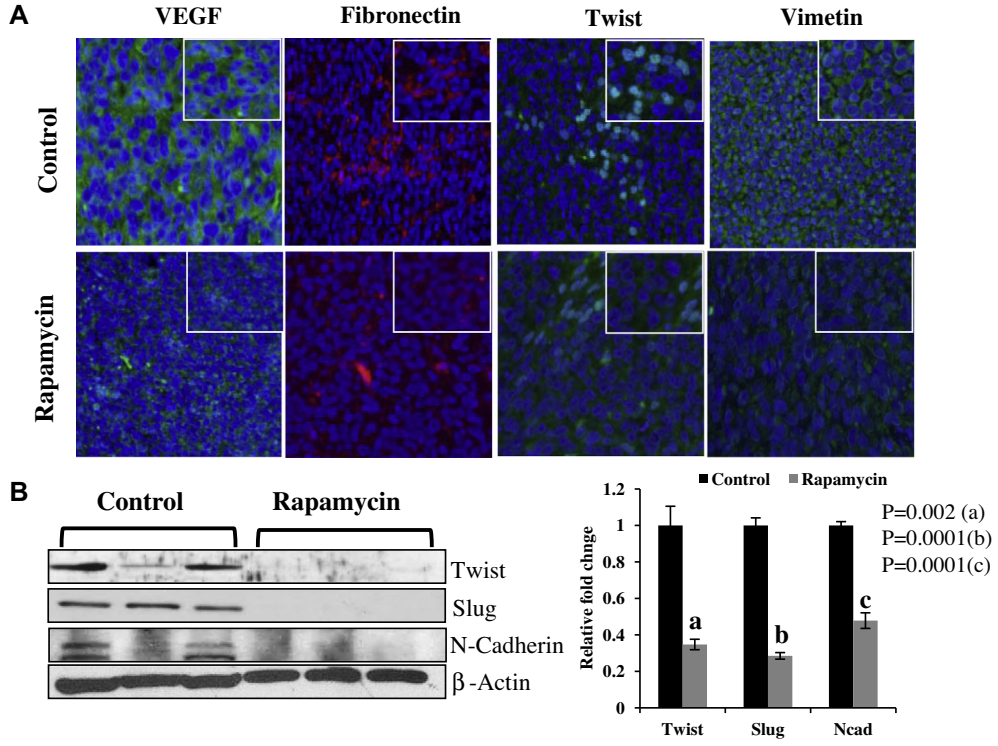

Fig. 4. Effects of rapamycin treatment on the expression of biomarkers representing epithelial-mesenchymal transition (EMT). (A): VEGF, fibronectin, twist and vimentin immune florescence staining. Inhibition in the expression of VEGF (vascular endothelial growth factor), twist, fibronectin and vimentin is seen in the rapamycin-treated group. (B) EMT markers twist, slug and N-Cadherin are also decreased significantly ($p = 0.002$, 0.0001 and 0.0001 respectively).

4. Discussion

RMS represents the most common soft-tissue sarcoma in children [1,2]. Current modalities of therapy are unsatisfactory particularly for metastatic and recurrent RMS [2]. This study provides novel observations that blocking the driver oncogenic Hh and mTOR signaling pathways by the administration of rapamycin abrogates the tumor growth and aggressiveness of fast growing

human RMS xenograft tumors. In previous studies employing RMS cell lines (A204, RH1 and RH30) the efficacy of rapamycin to inhibit proliferation and to induce apoptosis was demonstrated [24,25]. The use of mTOR mutant cell lines conferred resistance to the drug therapy indicating a direct effect through its binding to the mTOR protein using the FKBP-12 receptor. The pro-apoptotic effect of rapamycin in this study was thought to be independent of p53 [25]. In an independent study, Rh10, Rh28, Rh30, Rh30r, Rh41 and Rh18 RMS cell lines were used to test the efficiency of rapamycin. Rapamycin showed variable levels of inhibition in the different RMS cell lines used [26]. Similarly, temsirolimus (CCI-779), a rapamycin ester analogue showed an inhibitory effect on tumor growth which was accompanied by the inhibition of mTOR and its downstream effectors S6 and 4E-BP1. This inhibitory effect was considered to be due to an anti-angiogenic effect of mTOR inhibition affecting HIF-1α and VEGF [23]. However, our study provides additional evidence that rapamycin may be highly efficacious in tumors expressing both mTOR and Hh signaling pathways as tumor driving oncogenic signals. Earlier we also showed that enhancing the levels of wild-type p53 either by stabilizing this protein or by converting mutated p53 to its wild-type conformation may kill RMS [15].

Clinical studies using temsirolimus in patients with recurrent/refractory solid tumors have been completed [24]. Generally, mTOR inhibitors found to be well tolerated in children. Thus, rapamycin and other mTOR inhibiting analogues represent attractive agents for use in patients with RMS. Although, we observed an impressive therapeutic effect of rapamycin as a single drug, its further exploration in combination with well-established chemotherapeutic regimens or in combination with other biological agents may be a promising approach to RMS therapy. In summary, this study provides evidence that rapamycin usage serve as a potential therapeutic intervention for refractory and recurrent RMS either as single agent or in combination with other chemotherapeutic agents. Further, our data provide evidence that rapamycin may attenuate metastatic potential of these tumors by targeting mTOR/Hh signaling pathways concomitantly.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.001.

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