Suppression of Peutz-Jeghers Polyposis by Targeting Mammalian Target of Rapamycin Signaling

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Abstract

Purpose: Peutz-Jeghers syndrome (PJS) is a unique disorder characterized by the development of hamartomas in the gastrointestinal tract as well as increased risks for variety of malignancies. Germ-line mutations of LKB1 cause PJS. We have generated Lkb1+/- mice, which model human PJS. Rapamycin and its analogues are promising preventive and therapeutic agents that specifically inhibit signaling from mammalian target of rapamycin (mTOR). Hyperactivation of mTOR signaling has been associated with PJS. The objective of the study is to investigate the efficacy of mTOR inhibition in suppressing Peutz-Jeghers polyposis in Lkb1+/- mice.

Experimental Design: We initiated a trial of rapamycin in Lkb1+/- mice at 9 months of age (after the onset of polyposis) at the dose of 2 mg/kg/d for a 2-month period. We assessed the efficacy of rapamycin by measuring polyp sizes and tumor burden. To examine the effect of rapamycin on mTOR signaling, phosphorylation levels of S6 were evaluated by immunostaining.

Results: We observed a significant decrease in mean tumor burden (Student's t test, P = 0.023) as well as total tumor burden in rapamycin-treated group compared with control group. Comparison of the polyp size observed in both rapamycin-treated and control groups showed that rapamycin efficiently decreased the tumor burden of large polyps (≥8 mm). This inhibition of rapamycin was associated with a decrease in phosphorylated S6 levels in the polyps.

Conclusions: Rapamycin effectively suppresses Peutz-Jeghers polyposis in a mouse model, suggesting that rapamycin or its analogues may represent a new targeted therapy for the treatment of PJS.

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder, characterized by mucocutaneous hyperpigmentation and multiple gastrointestinal hamartomatous polyps (1). Patients with PJS also have a dramatically increased incidence of various types of malignancies (2, 3). These hamartomas, although benign, may lead to significant complications such as bowel obstruction, necessitating multiple surgical resections of clinically significant polyps (4). There is no treatment for this disease aside from surgical resection and follow-up endoscopies (5). Until recently, no chemopreventive agents had been suggested to decrease tumor burden in individuals with PJS. However, a recent pilot study (6) suggested that cyclooxygenase-2 inhibitor (celecoxib) could be beneficial in the chemoprevention of PJS polyposis.

Cyclooxygenase-2 is not currently known to be directly influenced by LKB1, but it is up-regulated in some polyps and most cancers from patients with PJS (7–11).

Approximately 70% of clinically defined PJS families harbor pathogenic germ-line point mutations or larger deletions of LKB1 (12–14). LKB1 is a serine-threonine kinase. Numerous molecular and biochemical studies have indicated that LKB1 functions as a tumor suppressor that regulates multiple biological processes and signaling pathways (15–17). Although homozygous Lkb1 knockout mice die at midgestation (18), several mouse model studies (7, 19–22), including our own (19), have reported that heterozygous Lkb1 knockout mice develop severe gastrointestinal polyposis, thus modeling the polyposis of PJS. The polyps seen in these Lkb1+/- mice are due to LKB1 haploinsufficiency (7, 19–22).

LKB1 has been reported as the major upstream kinase of AMPK (23–26). Through phosphorylation of AMPK, which in turn phosphorylates and activates TSC2, LKB1 negatively regulates mammalian target of rapamycin (mTOR) signaling (27). mTOR, a serine/threonine kinase, monitors intracellular nutrients and energy availability and promotes cell growth and proliferation (28). The dysregulation of the mTOR signaling pathway has been reported in multiple types of tumors. S6 kinase and eukaryotic translation initiation factor 4E–binding protein 1 are two well-characterized downstream targets of mTOR. On phosphorylation by mTOR, S6 kinase phosphorylates ribosomal S6 protein in response to growth factors. Elevated levels of phospho-S6 (pS6) kinase and pS6 are

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Materials and Methods

Animals. Heterozygous Lkb1 knockout mice have been generated and described previously (17). All mice are on a C57BL/6j congenic background. Mice were housed under conventional specific pathogen-free conditions at the Department of Veterinary Medicine and Surgery, The University of Texas M. D. Anderson Cancer Center. All experiments described in this article were conducted according to an approved Institutional Animal Care and Use Committee protocol. To carry out the trial, we selected 36 healthy animals at 9 months of age and randomly assigned them to treatment and control groups. For each group, heterozygous Lkb1 mice were housed three to five mice per cage according to their sex.

Drug administration. Rapamycin (Wyeth Pharmaceuticals) was diluted in a solution containing 0.2% sodium-carboxymethylcellulose and 0.25% polysorbate-80 in distilled water and stored at 4°C protected from light. Thirty-six 9-month-old heterozygous Lkb1 mice were divided into two groups and injected i.p. with rapamycin at the dose of 2 mg/kg/d or the vehicle (the solution used to dilute the rapamycin) 5 days per week for 2 months.

Scoring of polyps. After 2 months of treatment, all mice were euthanized. The gastrointestinal tracts of the mice were dissected, fixed, coded, and scored blindly by two independent individuals (C.W. and A.R.) and graded on a scale from 1 to 3 (1, weak staining; 2, moderate staining; and 3, strong staining). There were no major discrepancies between the two observers.

Immunostaining for pS6. Mouse tissues were dissected, fixed with 10% formalin solution, embedded in paraffin, and cut into 4- to 5-μm sections. Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H2O2 for 10 min at room temperature. Sections were then placed in 10 mmol/L citrate buffer (pH 6.0) and microwaved for 15 min for antigen retrieval. The anti-pS6 antibodies (Cell Signaling Technology) were applied at 1:50 dilution and incubated for 1 h at room temperature. Immunodetection was done with an LSAB 2 system (DAKO). Hematoxylin was used as a counterstain. Immunostaining for pS6 expression was scored by two blinded observers (C.W. and A.R.) and graded on a scale from 1 to 3 (1, weak staining; 2, moderate staining; and 3, strong staining). There were no major discrepancies between the two observers.

Statistical analysis. The statistical significance of the differences in tumor burden of polyps for rapamycin-treated mice and control mice was determined with Student’s t test. Fisher’s exact test was used to compare the frequency distribution of tumor sizes in treated and control mice.
control groups and of immunostaining scores in treated and control groups. Spearman correlations were used to assess the correlation among tumor numbers in different size categories in different treatment groups, as well as the correlation among immunostaining scores in different treatment groups. We used Stata version 9 for all analysis. Data are presented as means ± SE.

Results

**Rapamycin strongly suppresses polyposis in Lkb1+/− mice.** To investigate whether the inhibition of mTOR activity was an effective means of suppressing polyposis, rapamycin was used to treat of Lkb1+/− mice at the dose of 2 mg/kg/d. This dose was derived form literature where rapamycin was effective as an immunosuppressant in mice (30, 31). As described in our previous study (19), polyposis is first observed in Lkb1+/− mice at 6 months of age. We initiated rapamycin treatment of Lkb1+/− mice at 9 months of age to examine the potential suppressive effects of rapamycin. After a 2-month period, the Lkb1+/− mice (n = 18) in the treatment group and the Lkb1+/− littermate in the control group (n = 18) were sacrificed. The numbers of polyps in different size categories (diameter in millimeters) were compared and the results are summarized in Table 1. There was a statistically significant difference in polyp distribution between control and rapamycin-treated groups (Fisher’s exact test, P = 0.017). Spearman correlation test (P = 0.028) revealed the presence of a trend in polyp distribution in treated versus control groups. Controls had fewer polyps in the smaller size category (none in <2 mm) and more polyps in the larger size category (18.4% in >8 mm), whereas treated animals had more polyps in the smaller size category (7% in <2 mm) and fewer polyps in the larger size category (1.9% in >8 mm). It is noteworthy that there were four small polyps (<2 mm) detected in the treated group whereas no polyps <2 mm were detected in the control group, suggesting that the presence of these small polyps in the rapamycin-treated mice was caused by shrinkage of the larger polyps. Furthermore, the number of polyps in the category of >8 mm was significantly decreased from 7 in the control group to 1 in the rapamycin-treated group (Table 1). A closer comparison of the size distribution of polyps (calculated as a polyp ratio of control/treated) showed that the ratio increased as the polyp size increased, suggesting a progressive reduction of larger polyps following rapamycin treatment (Fig. 1A).

Next, we compared the tumor burden of polyps detected in control and rapamycin-treated mice in each different size category and overall (Table 1). There was a 47% decrease in total tumor burden in the rapamycin-treated mice (Table 1). Correspondingly, the mean tumor burden was significantly decreased from 1,708 ± 346 mm³ (control mice, n = 18) to 804 ± 131 mm³ (rapamycin-treated mice, n = 18; Student’s t test, P = 0.023; Fig. 1B). Taken together, these data suggest that rapamycin treatment would prevent the polyps from forming an obstructive mass.

**Effect of rapamycin treatment on mTOR signaling in Lkb1+/− mice.** To test whether the tumor-suppressing effect of rapamycin was associated with inhibition of mTOR signaling, the polyps from control and rapamycin-treated Lkb1+/− mice were collected, paraffin embedded, and further analyzed. H&E staining revealed no significant changes in the morphology of the polyps (Fig. 2A and B). We evaluated mTOR signaling in these polyps by immunostaining. Currently, existing antibodies for mTOR are not useful for immunohistochemistry because they do not produce clean results. Elevated pS6 is a useful marker for detecting increased mTOR kinase activity; therefore, we examined pS6 in polyps of Lkb1+/− mice. pS6 positivity was observed in polyps from both untreated Lkb1+/− mice (Fig. 2C)
and Lkb1+/− mice treated with rapamycin (Fig. 2D), indicating that activation of mTOR signaling was associated with polyp development. However, the level of pS6 expression was different in control versus treated animals. We quantified pS6 immunostaining by scoring polyps on a scale from 1 to 3, with 3 being the most intense staining (summarized in Fig. 3A). There was a significant difference in pS6 immunostaining between the control and rapamycin-treated groups (Fisher’s exact test, \(P = 0.011\)). Spearman correlation test \(P = 0.0019\) revealed the presence of a trend in pS6 levels in these two different groups. We found that more of the polyps in the control group showed higher levels of pS6 staining, whereas more of the polyps from the rapamycin-treated group had lower levels of pS6 staining. As shown in Fig. 3B, the average pS6 levels in the polyps from rapamycin-treated Lkb1+/− mice (\(n = 11; 1.45 \pm 0.16\)) were clearly reduced compared with those from the untreated control group (\(n = 17; 2.29 \pm 0.17\); Student’s \(t\) test, \(P = 0.0019\)). This statistically significant reduction in pS6 levels with rapamycin treatment is consistent with a direct effect on the mTOR signaling pathway.

## Discussion

The present study evaluates the efficacy of rapamycin treatment to reduce Peutz-Jeghers polyposis in a mouse model. Our results revealed that the tumor burden of Lkb1+/− mice was significantly reduced after rapamycin treatment, suggesting that rapamycin is an effective candidate for the treatment of PJS.

The development of hamartomatous polyps constitutes the most significant clinical component of PJS. These hamartomas, although benign, lead to significant complications such as bowel obstruction, rectal prolapse, or severe gastrointestinal bleeding, necessitating multiple emergency laparotomies and bowel resections in PJS patients (4). The morbidity of the polyposis has been reviewed in a 78-year follow-up study of the original PJS family (32). In this family, 22 people were diagnosed as affected. Sixteen of them have been through at least one laparotomy, resulting in a total of 33 laparotomies due to bowel obstruction.

Considering the clinical situation of PJS in which the disease is typically noted through bowel obstruction due to the large polyps, we initiated a rapamycin treatment in a mouse model of PJS after the onset of polyposis (9 months of age). The objective of this study design was to mimic the clinical situation of PJS where patients have a significant tumor burden but often do not know that they have PJS until symptoms develop. Our data show that a 2-month rapamycin treatment led to a significant reduction in large polyps and tumor burden. Immunostaining for pS6 revealed that pS6 immunoreactivity was elevated in the control group, which is in agreement with a previous study (27), whereas the levels of pS6 dramatically decreased after the treatment. S6 phosphorylation levels serve as a good marker for mTOR kinase activity. Thus, our results suggest that polyposis regression via rapamycin is associated with down-regulation of mTOR signaling. These findings support the utilization of mTOR inhibitors as an option for PJS treatment.

In a recent pilot study, Udd et al. (6) tested a selective cyclooxygenase-2 inhibitor (celecoxib) in a PJS mouse model and in patients with PJS and observed a distinct reduction in polyp burden, suggesting that celecoxib could be beneficial in the chemoprevention of PJS polyposis. Cyclooxygenase-2 is not currently known to be directly influenced by LKB1, whereas mTOR is known to be down-regulated by LKB1 signaling to AMPK, which phosphorylates and activates TSC2, the gatekeeper for mTOR signaling. Rapamycin and its analogues, CCI-779 and RAD001, are being evaluated in clinical trials for the treatment of several types of cancer (33–36). Our data suggest that rapamycin or its analogues may represent a new targeted therapy for the treatment of PJS.

In conclusion, we have shown that targeted therapy with a mTOR inhibitor dramatically suppressed the hamartomatous polyposis in the Lkb1+/− Peutz-Jeghers mouse model. Our findings have important implications for future clinical trials in patients with PJS. It would be of interest to conduct a rapamycin treatment study before polyp formation in this PJS mouse model to determine if this drug is able to prevent the formation of polyps and prevent bowel obstruction at the point of tumor initiation.

## References

Targeting mTOR Signaling in Peutz-Jeghers Syndrome


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