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Chemopreventive efficacy of rapamycin on Peutz–Jeghers syndrome in a mouse model

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ABSTRACT

Germline mutations in *LKB1* cause Peutz–Jeghers syndrome (PJS), an autosomal dominant disorder with a predisposition to gastrointestinal polyposis and cancer. Hyperactivation of mTOR-signaling has been associated with PJS. We previously reported that rapamycin treatment of $Lkb1^{+/-}$ mice after the onset of polyposis reduced the polyp burden. Here we evaluated the preventive efficacy of rapamycin on Peutz–Jeghers polyposis. We found that rapamycin treatment of $Lkb1^{+/-}$ mice initiated before the onset of polyposis in $Lkb1^{+/-}$ mice led to a dramatic reduction in both polyp burden and polyp size and this reduction was associated with decreased phosphorylation levels of S6 and 4EBP1. Together, these findings support the use of rapamycin as an option for chemoprevention and treatment of PJS.

1. Introduction

Peutz–Jeghers syndrome (PJS) is a unique autosomal dominant disorder [1,2]. The causative gene *LKB1*, is located on chromosome 19p13.3 and encodes serine threonine kinase 11 [3–5]. Up to 80% of families with clinically defined PJS harbor germline mutations in the *LKB1* gene [6–8]. Patients with PJS are characterized by cutaneous hypermelanocytic macules and hamartomatous polyp development in the gastrointestinal tract. In one study, polyps were detected in 88% of patients affected with PJS [9]. These hamartomas, although benign, may lead to significant clinical complications such as intestinal obstruction, abdominal pain, gastrointestinal bleeding, and anal extrusion of polyps. In the past, treatment of PJS was limited to surgical resection of clinically significant polyps and follow-up endoscopies [10,11], which may yield only partial improvement. In addition to the polyposis, PJS patients are also at increased risk for gastrointestinal and extraintestinal cancers [9,12–19]. For example, in a meta-analysis of 210 patients with PJS, the relative risk of cancer of the stomach, pancreas, lung and breast were found to be 213-, 132-, 17- and 15.2-fold higher than the general population, respectively; and the cumulative risk for developing cancer at any site is 93% [13]. Thus, there is an urgent need to identify effective preventive and chemotherapeutic agents for the treatment of PJS and PJS-associated cancers.

Mouse models provide useful tools for studying the mechanism of many diseases as well as potential therapeutic and preventive strategies. Previously, we and several other groups [20–24] reported that $Lkb1^{+/-}$ knockout mice develop severe gastrointestinal polyposis, which model human PJS. Haploinsufficiency of LKB1 is responsible for the polyps seen in these $Lkb1^{+/-}$ mice [20–24]. LKB1 is the major upstream kinase of AMPK [25–28] and directly phosphorylates Thr172 of AMPK. Upon phosphorylation, AMPK

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phosphorylates and activates TSC2, the gatekeeper for mTOR-signaling. Through AMPK/TSC2, LKB1 negatively regulates mTOR [mammalian target of rapamycin] signaling [29]. mTOR, a serine/threonine kinase, is a central regulator of cell growth and proliferation [30]. The mTOR-signaling pathway network is important for driving cell growth and proliferation, and hyperactivation of the mTOR-signaling pathway has been detected in many types of cancers. It has been reported that a reduced level of expression or loss of function of the LKB1 protein causes hyperactivity of mTOR-signaling in hamartomatous polyps from *Lkb1*$^{+/-}$ mice and *Lkb1*-deficent cells [29,31], as evidenced by the elevated phosphorylation levels of mTOR downstream targets such as the ribosomal protein S6 (S6) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1). More recently, Katajisto et al. demonstrated that rapamycin effectively blocked phospho-S6, indicating inactivation of the mTOR pathway, in *Lkb1*$^{lox/-}$ myofibroblast-enriched cultures [32]. These observations suggested that mTOR inhibitors such as rapamycin and its analogues could be useful for Peutz–Jeghers polyposis treatment. In addition, in two small trials of tuberous sclerosis patients, treatment with rapamycin induced regression of the astrocytomas [33] and reduced facial angiofibroma [34].

Previously, we reported that 2 months of rapamycin treatment in *Lkb1*$^{+/-}$ mice initiated at 9 months of age, which is after the onset of polyposis, effectively reduced the polyp burden by 47% [35]. In the current study, we explored the potential preventive effect of rapamycin on polyposis in *Lkb1*$^{+/-}$ mice by initiating the treatment at 5 months of age, before the onset of polyposis. We found that early rapamycin treatment had a more pronounced effect on polyp burden reduction than such treatment initiated after the onset of polyposis. Together, our results suggest that rapamycin may be a useful therapeutic and preventive agent for Peutz–Jeghers polyposis.

2. Materials and methods

2.1. Animals

The animal study was performed in accordance with the guidelines for animal experiments of The University of Texas M.D. Anderson Cancer Center. Generation and genotyping of heterozygous *Lkb1* knockout mice were described previously [23]. All mice were in a C57BL/6J congenic background and housed in a conventional specific-pathogen-free facility. The mice had free access to food and water before and throughout the study.

2.2. Drug administration

Rapamycin (Wyeth Pharmaceuticals, St. Davids, PA, USA) solution was prepared and stored as described previously [35]. We selected 28 healthy mice at 5 months of age and randomly assigned them to treatment and control groups. The mice were then injected intraperitoneally with rapamycin (2 mg/kg) or vehicle alone (the solution used to dilute the rapamycin) 5 days per week for 6 months.

2.3. Polyp scoring

All mice were euthanized 1 h after the last intraperitoneal injection. The stomach and duodenum of the mice were dissected, coded, and scored. The polyp size was measured with a digital micrometer and the volume was calculated by using the formula: $V = \pi/6 \times a^3$, where a is the diameter. The total polyp burden in each mouse was the sum of all polyps from that individual mouse.

2.4. Western blot analysis

Western blot analysis was performed by standard methods. Briefly, snap-frozen polyps dissected from *Lkb1*$^{+/-}$ mice were homogenized in NP-40 lysis buffer [150 mM NaCl, 1%NP-40, 50 mM Tris–HCl, 1.5 mM EDTA (pH 7.5)] containing protease inhibitors (1.6 μg/ml benzamidine HCl, 1 μg/ml phenanthroline, 1 μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF), and the total proteins were extracted. Equivalent amounts of protein were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with TBS-T [10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20 (pH 7.5)] containing 5% nonfat dry milk at room temperature overnight and then blotted with relevant antibodies at room temperature for 1 h. HRP-conjugated secondary antibodies were detected by using the Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Antibodies for S6, phospho-S6 (Ser235/236), phospho-4EBP1 (Thr70), and phospho-4EBP1 (Ser65) were obtained from Cell Signaling Technology (Beverly, MA). Actin and/or S6 were used as an internal control to visualize the total protein loading, and anti-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Immunostaining for phosphorylated S6 (phospho-S6)

Tissues were fixed with 10% formalin solution and embedded in paraffin. Sections (4-μm thick) were deparaffinized in xylene and then rehydrated in decreasing concentrations of ethanol. Immunostaining for phospho-S6 expression was performed as described previously [35], using a 1:50 dilution of the anti-phospho-S6 antibody (S235/236) (Cell Signaling Technology).

2.6. Statistical analysis

We used Stata 10 statistical software (Stata, College Station, TX) for all analyses described here. Fisher's exact test was used to compare the frequency distribution of tumor sizes in the treated and control groups. Student's *t*-test was used to determine the statistical significance of the differences in polyp burden between the treatment and control groups. Spearman correlations were used to assess the correlation among polyp numbers in different size categories in the two groups. Data are presented as means ± standard error.