Therapeutic Effect of Rapamycin on Gallbladder Cancer in a Transgenic Mouse Model

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Abstract

The macrolide fungicide rapamycin has shown significant antiproliferative action toward a variety of tumor types. In this study, we used BK5.erbB2 transgenic mice as an animal model to examine the therapeutic effect of rapamycin as a potential treatment for gallbladder cancer. Homozygous BK5.erbB2 mice overexpressing the wild-type rat erbB2 gene in basal epithelial cells of the gallbladder have an ~70% incidence of gallbladder adenocarcinoma by 2 to 3 months of age. Groups of mice (~2–3 months of age) were treated with rapamycin by i.p. injection (once daily for 14 days) and then sacrificed 24 h after the last treatment. Rapamycin significantly reduced the incidence and severity of gallbladder carcinoma in BK5.erbB2 mice in a dose-dependent manner. Tumors responsive to treatment exhibited a higher number of apoptotic cells. Furthermore, rapamycin treatment led to decreased levels of phosphorylated p70 S6 kinase (Thr389) in gallbladder tissue as assessed by both Western blot and immunofluorescence analyses. Finally, immunofluorescence staining revealed elevated phosphorylated Akt (Ser473) and phosphorylated mammalian target of rapamycin (mTOR; Ser2448) in human gallbladder cancer compared with normal gallbladder tissue. Based on our results using a novel genetically engineered mouse model and the fact that the Akt/mTOR pathway is activated in human gallbladder cancer, rapamycin and related drugs may be effective therapeutic agents for the treatment of human gallbladder cancer.

Introduction

In the United States alone, ~7,500 new cases of biliary tract cancers are diagnosed per year, and 5,000 of those cases are diagnosed as gallbladder cancer (1). Gallbladder cancer is a relatively aggressive and frequently lethal disease, with a 5-year survival rate of <5% (2). Many gallbladder carcinomas are at an advanced stage at the time of diagnosis, and metastases to the liver and regional lymph nodes are common. Chemotherapeutic agents currently in use for treatment of gallbladder cancer include mitomycin C, 5-fluorouracil, gemcitabine, and platinum analogues. However, the reported response rates to these drugs are only 10% to 24% (3), which in part contributes to the low survival rate of patients afflicted with gallbladder cancer. Thus, more effective strategies for the treatment of gallbladder cancer are essential to improve the prognosis for gallbladder cancer patients.

Transgenic mouse models offer useful tools for studying the effect of potential therapies for many types of cancer, including gallbladder cancer. Previously, we showed that overexpression of erbB2 in the basal layer of the biliary tract epithelium led to the development of gallbladder adenocarcinoma in ~70% of the transgenic mice by 2 to 3 months of age (4, 5). The gallbladder adenocarcinomas that develop in BK5.erbB2 mice have molecular alterations similar to those reported in human gallbladder cancers, such as overexpression and/or activation of erbB2, EGFR, c-MET, and COX2 genes (6–9). Recently, we reported the therapeutic efficacy of orally active tyrosine kinase inhibitors (TKI) directed at the epidermal growth factor receptor (EGFR) or to both EGFR and erbB2 against gallbladder tumors that develop in this novel mouse model (10). It is well documented that activation of growth factor receptor tyrosine kinases can lead to the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling, including the EGFR (11, 12). Mammalian target of rapamycin (mTOR), also known as FRAP, RAFT1, and RAPT1, is a serine/threonine kinase regulated by Akt. mTOR is a central regulator of cell growth and proliferation and functions as a biological switch between life and death that senses changes in the cellular environment and helps cells respond to these changes (13). Although the signal transduction pathway is not fully understood, mTOR activation by PI3K/Akt plays a key role in regulating protein translation through modulation of p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) function (14–16).

Rapamycin, an inhibitor of mTOR, binds the immunophilin FK506 binding protein (FKBP12) to form the FKBP12-rapamycin complex, which then interacts with and inhibits the activity of mTOR (17). To date, studies have revealed that rapamycin can potently arrest growth of cells derived from a broad spectrum of cancers, including rhabdomyosarcoma, neuroblastoma, glioblastoma, small cell lung cancer, osteosarcoma, pancreatic cancer, breast cancer, prostate cancer, murine melanoma, leukemia, and B-cell lymphoma (18–25). In addition, rapamycin and its derivatives suppress growth of several human and murine tumors in vivo (e.g., small lung cancer, pancreatic cancer, colon cancer, and breast cancer; refs. 26–31). In the current study, we show that rapamycin inhibits the growth of primary gallbladder adenocarcinoma that develops in BK5.erbB2 mice by inhibition of mTOR function. This signaling pathway is up-regulated in gallbladder tumors from BK5.erbB2 mice as well as in advanced human gallbladder tumors.
These results suggest that rapamycin and related drugs may be useful therapeutic agents for the treatment of human gallbladder cancer.

Materials and Methods

Animals and experimental design. Homozygous BK5.erbB2 transgenic mice (ICR genetic background) at 2 to 3 months of age were used for all animal studies and were randomized according to age and sex between vehicle-treated and rapamycin-treated groups to prevent potential bias. Rapamycin (A.G. Scientific, San Diego, CA) was dissolved in 4% ethanol, 5.2% PEG400, and 5.2% Tween 80 and was given by i.p. injection once daily for 14 days. Body weight was monitored daily. Mice were sacrificed 24 h after the last injection of rapamycin.

Histologic analysis of gallbladder tissue. After the mice were sacrificed, gallbladders were removed and fixed in formalin and embedded in paraffin before sagittal sectioning. Sections of 5 μm were cut and stained with H&E. Paraffin-embedded sections of human gallbladder cancer were obtained from M.D. Anderson Cancer Center.

Western blot analysis. For Western blot analysis, pooled epithelial cell lysates were prepared from 50 gallbladders from nontransgenic mice, 5 gallbladder tumors from BK5.erbB2 mice, and 5 gallbladders from BK5.erbB2 mice treated with rapamycin. Gallbladder cell lysates were electrophoresed through 7% SDS/polyacrylamide gels as previously described (5, 10). Separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with 5% bovine serum albumin for 1 h at room temperature in TBS [0.5 mol/L NaCl, 20 mmol/L Tris (pH 7.5)], protein levels of Akt, phosphorylated Akt (p-Akt), mTOR, phosphorylated mTOR (p-mTOR), p70S6K, and phosphorylated p70S6K (p-p70S6K) were detected by incubating the membrane with the corresponding primary rabbit polyclonal antibody against Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), p70S6K, and p-p70S6K (Thr389; Cell Signaling Technology, Beverly, MA). FITC-conjugated, affinity-purified anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) was used as the secondary antibody. Quantitation of Western blot data was done using a densitometer.

Immunofluorescence staining. The expression and localization of p-Akt, p-mTOR, and p-p70S6K were determined using immunofluorescence on sections of gallbladders as described previously (5). The primary and secondary antibodies used are described above. The sections were analyzed using an Olympus laser confocal microscope and an Olympus BX60 microscope.

Terminal deoxynucleotidyl transferase–mediated nick-end labeling assay. Terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay was done according to the manufacturer’s instructions using an in situ cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN).

Statistical analysis. χ² tests were used to compare the incidence of adenocarcinoma between vehicle-treated and rapamycin-treated groups. The incidence of adenocarcinoma was then estimated as a binary outcome from a logistic-regression model, using maximum-likelihood, to determine if it was correlated to the dose of rapamycin.

Results

Evaluation of therapeutic efficacy of rapamycin. Previous studies, using other human tumor xenograft models (e.g., prostate), have administered rapamycin in the dose range of 0.1 to 40 mg/kg in mice (26–31). We tested the tolerance of BK5.erbB2 transgenic mice to 0, 1, 2.5, 5, and 10 mg/kg rapamycin administrated by i.p. injection once daily for 14 days. Inflammation caused by injection, histologic abnormalities of liver sections, and significant loss of body weight were not observed in rapamycin-treated mice, indicating that rapamycin treatment at these doses was well tolerated in BK5.erbB2 mice. To evaluate the therapeutic efficacy of rapamycin on gallbladder carcinoma in BK5.erbB2 mice, we used 72 homozygous BK5.erbB2 transgenic mice at ~2 to 3 months of age and divided them into two age- and sex-matched groups. One group of the mice received vehicle, and the other group received 2.5 mg/kg rapamycin by i.p. injection once daily for 14 days. All the mice were sacrificed 24 h after the last treatment. Gallbladder...
adenocarcinoma incidence was calculated based on histologic analyses of gallbladders from each group of mice. The gallbladder adenocarcinoma incidence in the vehicle-treated group was 56%. Twenty-eight percent of mice in this group had normal gallbladders, and 17% had hyperplastic gallbladders. As shown in Fig. 1A, 2.5 mg/kg rapamycin treatment significantly reduced the gallbladder adenocarcinoma incidence to 29% ($P < 0.05$, $\chi^2$ test). The percentage of mice that had noncancerous gallbladders was increased to 71% (40% normal gallbladders and 31% hyperplastic gallbladders). We also examined the effect of 1.25 and 5 mg/kg rapamycin on gallbladder adenocarcinoma in a separate experiment. For this additional experiment, a total of 70 mice were used. The mice were divided into three groups as follows: vehicle, 1.25 mg/kg rapamycin, and 5 mg/kg rapamycin. The treatment protocol was identical to that used to evaluate the 2.5 mg/kg dose of rapamycin. The results of this experiment are shown in Fig. 1B. The gallbladder adenocarcinoma incidence in the vehicle-treated group was 67%. Twelve percent of mice in the vehicle-treated group had normal gallbladders, and 21% had hyperplastic gallbladders. Administration of 1.25 mg/kg rapamycin reduced the gallbladder adenocarcinoma incidence to 57% and increased the incidence of noncancerous gallbladders to 43% (26% normal and 17% hyperplastic gallbladder). Administration of 5 mg/kg rapamycin significantly reduced the gallbladder adenocarcinoma incidence to 39% and increased the noncancerous gallbladder incidence to 61% (26% normal and 35% hyperplastic gallbladders, respectively; Fig. 1B; $P < 0.05$, $\chi^2$ test). As shown in Fig. 2, probit analysis indicated that the reduced incidence of gallbladder carcinoma correlated with the dose of rapamycin administered ($P = 0.014$, logit).

To further evaluate the effect of rapamycin on gallbladder tumors in BK5.erbB2 mice, TUNEL assays were done on gallbladder tissue from vehicle-treated and rapamycin-treated mice. For these analyses, we did TUNEL staining on four sets of tissues, representatives of which are shown in Fig. 3A. TUNEL staining in DNase I treated normal gallbladder epithelium is shown (top left) as a control for TUNEL-positive cells. An adenocarcinoma from a vehicle-treated mouse shows very few apoptotic cells (top right). Only a small number of TUNEL-positive cells were present in a gallbladder tumor that was apparently refractory to rapamycin treatment (bottom left). Finally, Fig. 3A (bottom right) shows the more typical findings observed in gallbladder tissue from rapamycin-treated mice. In this regard, we observed a reversion to a milder, hyperplastic phenotype with the presence of a significant number of TUNEL-positive cells. Fifty percent of the hyperplastic gallbladders from mice treated with rapamycin...
exhibited this high level of apoptotic cells. Figure 3B shows the actual percentage of TUNEL-positive cells in multiple gallbladder sections from vehicle-treated or rapamycin-treated mice. The average percentage of TUNEL-positive cells was increased in hyperplastic gallbladders treated with rapamycin compared with hyperplastic gallbladders from vehicle-treated mice. Interestingly, the analysis of TUNEL staining in hyperplastic gallbladders from the rapamycin-treated mice revealed two distinct types of staining patterns. As noted above, 50% of the rapamycin-treated hyperplastic gallbladders had a significantly higher percentage of apoptotic cells (15.3 ± 3.3%) compared with that in the vehicle-treated hyperplastic gallbladders (0 ± 0%; P < 0.05). The other 50% of rapamycin-treated hyperplastic gallbladders had few apoptotic cells (0.9 ± 1.6%) similar to the vehicle-treated hyperplastic gallbladders. The combination of these two different types of samples contributed to the high SD seen in Fig. 3B. Because the gallbladder adenocarcinoma incidence in the rapamycin-treated group was significantly reduced compared with that in the vehicle-treated group, we believe that at least a portion of the hyperplastic gallbladders with high apoptotic index were from adenocarcinomas susceptible to rapamycin treatment. Although further work will be necessary to prove it conclusively, many of the tumors that responded to rapamycin treatment underwent apoptosis during the process of reversion to a milder phenotype.

Analysis of Akt, mTOR, and p70S6K status in gallbladder tissue from BK5.erbB2 mice. To further explore the therapeutic efficacy of rapamycin in the BK5.erbB2 mouse model, we examined the status of its target mTOR and signaling molecules both immediately upstream (Akt) and downstream (p70S6K). As shown in Fig. 4, this signaling pathway is up-regulated in gallbladder tissue of BK5.erbB2 mice compared with corresponding tissue from nontransgenic mice. In this regard, Western blot analyses (Fig. 4A) show elevated levels of p-Akt (Ser473), p-mTOR (Ser2448), and p-p70S6K (Thr389) in gallbladder tissue from transgenic mice. This was confirmed by immunofluorescence staining of gallbladder tissue using phosphospecific antibodies as shown in Fig. 4B.

Effect of rapamycin on phosphorylation of mTOR and p70S6K in gallbladder tissue from BK5.erbB2 mice. To determine whether rapamycin treatment affected the incidence of gallbladder cancer by inhibition of its target mTOR, we examined the level of p-p70S6K (Thr389) by both Western blot analysis and immunohistochemical staining of gallbladder tissues. As shown in Fig. 5A, after quantitation, Western blot analyses confirmed a reduced relative level of p-p70S6K in gallbladder tissue from rapamycin-treated (5 mg/kg) mice compared with vehicle-treated transgenic mice. In addition, this observation was confirmed by immunofluorescence analysis of tissue sections from rapamycin-treated mice as shown in Fig. 5C. We also examined the level of p-mTOR (Ser2448) in gallbladders from vehicle-treated or rapamycin-treated mice by Western blot analysis. As shown in Fig. 5B, the relative level of p-mTOR in gallbladders was not significantly different between the vehicle-treated or rapamycin-treated groups.

Analysis of Akt and mTOR status in human gallbladder cancer. As part of the current study, we analyzed the status of Akt and mTOR in human gallbladder cancer compared with normal gallbladder tissue. Up-regulation of this signaling pathway would provide a rationale for considering inhibition of mTOR as a therapeutic strategy for human gallbladder cancer. Immunofluorescence staining of p-Akt and p-mTOR was done in 27 human gallbladder adenocarcinoma (stage IIB diagnosed by the criteria of the American Joint Committee on Cancer) and 7 normal human gallbladders. The results showed that elevated levels of p-Akt were observed in 74.1% (20 of 27) human gallbladder cancer specimens and in only 28.6% (2 of 7) normal human gallbladder specimens. Elevated levels of p-mTOR also were observed in 92.6% (25 of 27) human gallbladder cancer specimens and in only 28.6% (2 of 7) normal human gallbladder specimens. These data reveal that human gallbladder adenocarcinoma has elevated p-Akt and p-mTOR levels compared with noncancerous tissue. Representative images of immunofluorescence staining are shown in Fig. 6.
Discussion
The current study was designed to evaluate the therapeutic efficacy of rapamycin against gallbladder carcinoma that develop in a novel transgenic mouse model for this human cancer. We found that the Akt/mTOR/p70S6K pathway was activated in gallbladder tissue from transgenic mice, and that the incidence of gallbladder carcinoma in BK5.erbB2 mice was significantly reduced in a dose-dependent manner following treatment with rapamycin. This effect of rapamycin correlated with a reduction in phosphorylation of p70S6K at Thr389 (Fig. 5). The effect of rapamycin was seen as a reduction of cancer incidence with reversion to a milder phenotype, either hyperplasia or a relatively normal epithelium, after 14 days of treatment. In addition, evidence was obtained that tumors responsive to rapamycin treatment displayed a significant increase in apoptotic cells. This was seen primarily as an increase in apoptotic cells in hyperplastic gallbladder epithelium of rapamycin-treated mice. Finally, we found evidence that the Akt/mTOR pathway is activated in human gallbladder cancers. Overall, the current results suggest that targeting this signaling pathway either alone or in combination with other agents may be an effective therapeutic strategy for this disease.

As noted in the Introduction, we have shown that the adenocarcinomas that develop in BK5.erbB2 transgenic mice have constitutively activated EGFR and increased EGFR/erbB2 heterodimer formation (5, 10). In addition, our previous studies have shown that the mitogen-activated protein kinase signaling pathway is activated, and that cyclooxygenase-2 (COX-2) expression is elevated in gallbladder tissue and tumors obtained from these mice (5, 10). As shown in Figs. 4 and 5, the Akt/mTOR/p70S6K pathway is also activated in gallbladder tumors that develop in these mice. The activation of Akt is known to occur downstream of growth factor receptors such as the EGFR via activation of PI3K (11, 12, 32–35). Thus, the activation of this pathway in gallbladder tissue and tumors of BK5.erbB2 mice is likely due to up-regulation of erbB signaling. Furthermore, we provide evidence that this signaling pathway is activated in human gallbladder cancer as well (see Fig. 6). In the latter case, activation of Akt/mTOR signaling pathway could also result from elevated erbB activation as a significant percentage of human gallbladder tumors have been shown to overexpress erbB2, EGFR, or both (6, 7, 36–42). In addition, overexpression of transforming growth factor-α has also been reported in human gallbladder carcinoma (43). Interestingly, mRNA levels of two important downstream mediators of Akt/mTOR signaling (p70S6K and 4E-BP1) were found to be up-regulated in human biliary tract cancer (44). These latter data further support a potential role for mTOR signaling in human biliary tract cancer.

mTOR is a central signaling molecule downstream of Akt (13). It is known that mTOR integrates signaling from both growth factors and nutrients, and that it can regulate cell growth and cell cycle progression (reviewed in refs. 13, 15, 45–47). In addition, mTOR signaling is up-regulated in a significant number of human tumors either through up-regulation of Akt or other regulatory pathways (reviewed in refs. 13–16, 45–47). Increasing evidence supports a critical role for mTOR as a regulator of protein synthesis and translation initiation (13–16, 45–47). mTOR can phosphorylate a number of substrates, including p70S6K and 4E-BP1 (14–16, 45–47). Phosphorylation of these two substrates seems to be essential for protein synthesis and translation initiation in response to nutrients.
and growth factors. As noted in the Introduction, blockade of this pathway using either pharmacologic or genetic approaches has been shown to inhibit growth of a number of tumors/tumor cell lines (18–29, 48, 49). Rapamycin inhibits mTOR by inhibiting its kinase activity through binding to FKBP12 (17). As shown in Fig. 5, rapamycin treatment significantly reduced the level of p-p70S6K (Thr389) in gallbladder tissue of BK5.erbB2 mice. This effect is consistent with its ability to inhibit mTOR and correlated with the reduction in carcinoma incidence.

At the cellular level, the exact mechanism for the effect of rapamycin on gallbladder tumors of BK5.erbB2 mice will require further investigation. However, our initial analyses suggest that rapamycin treatment induced apoptosis in cells of responsive tumors, causing a reversion to a less severe phenotype (i.e., hyperplasia). This could have occurred through inhibition of mTOR phosphorylation and decreased protein synthesis. Alternatively or perhaps in addition, rapamycin may have altered the levels and/or activity of antiapoptotic molecules such as Bcl2 and Stat3 (13). The presence of a small number of tumors that seemed resistant to rapamycin treatment in this model is interesting. Recently, we reported a similar finding when examining the therapeutic efficacy of TKIs against gallbladder tumors in BK5.erbB2 mice (10). Several possible mechanisms could explain refractoriness to rapamycin in human tumors (48). It is clear from our previous and current work that there is some heterogeneity in the tumors that develop in BK5.erbB2 mice. Tumor heterogeneity could be the basis of this resistance. For example, some tumors might have acquired additional genetic alterations that overcame the blockade of mTOR by rapamycin. Further work with these tumors may help to shed light on resistance mechanisms and other pathways that could be targeted pharmacologically in combination with rapamycin to effectively treat these tumors.

In summary, the current data suggest that rapamycin can reduce the incidence of gallbladder carcinoma in a relevant mouse model for human gallbladder cancer. This drug and other drugs that target mTOR may be a useful chemotherapeutic approach for treating human gallbladder cancer given that this pathway seems to be activated in human gallbladder tumors. Furthermore, targeting mTOR using rapamycin in combination with other agents may also be an effective strategy for treating biliary tract cancers. For example, in addition to TKIs, which we have shown recently to be effective therapeutic agents in this model (10), COX-2 inhibitors have also shown effectiveness in this model (50). Future work in the BK5.erbB2 mouse model of biliary tract cancer will explore these and other combinations for their effectiveness. The long-term goal is to translate these findings into human clinical trials.

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