mTOR and P70 S6 Kinase Expression in Primary Liver Neoplasms

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ABSTRACT

Purpose: mTOR and P70 S6 kinase (S6K) play a key role in regulating protein translation. The role of mTOR and S6K in hepatocellular carcinoma has not been investigated, but this pathway is of particular interest because an effective inhibitor, rapamycin, is available. This study was undertaken to determine the prevalence and clinicopathological correlates of mTOR pathway activation in hepatocellular carcinoma and to determine whether rapamycin inhibits the pathway in cell culture.

Experimental Design: Total and phosphorylated mTOR and S6K protein expression were studied by immunohistochemistry in hepatocellular carcinomas (n = 73), fibrolamellar carcinomas (n = 13), and hepatic adenomas (n = 15). Results were correlated with tumor growth pattern as defined by the WHO (trabecular, pseudoglandular/acinar, compact, and scirrhus), tumor size, Ki-67 proliferation index, and the modified Edmondson nuclear grade, which has a scale of 1 to 4. HepG2 and Hep3B cell lines were treated with rapamycin to see the effect on proliferation and S6K phosphorylation.

Results: Increased expression of total mTOR was seen in 5% of hepatocellular carcinoma, whereas overexpression of phospho-mTOR was evident in 15% of hepatocellular carcinoma. Phospho-mTOR positivity correlated with increased expression of total S6K, which was found in 45% of cases. Total S6K overexpression was positively correlated with tumor nuclear grade, inversely with tumor size, and was unassociated with the proliferation index or WHO growth pattern. Rapamycin treatment of HepG2 and Hep3B cell lines markedly inhibited cell proliferation and reduced S6K phosphorylation in both cell lines.

Conclusions: The mTOR pathway is activated in a subset of hepatocellular carcinoma. Rapamycin can inhibit proliferation of neoplastic hepatocytes in cell culture.

INTRODUCTION

Hepatocellular carcinomas are one of the 10 most frequent carcinomas worldwide, and the incidence is increasing (1). The overall prognosis for most individuals with hepatocellular carcinoma is dismal with few effective treatment options (2). However, in the past decade, new therapies for malignancies have been developed that specifically target critical signaling and metabolic pathways, e.g., the epidermal growth factor receptor signaling pathway (3). Such treatments hold promise as effective therapies for malignancies, including hepatocellular carcinomas.

The mTOR pathway is an attractive target for cancer therapeutics as effective inhibitors such as rapamycin block mTOR phosphorylation of S6K. Protein translation is highly regulated in cells, in part by the mTOR pathway, which senses nutrient and energy availability and phosphorylates p70 S6 kinase (S6K) to stimulate protein translation (4, 5). S6K in turn phosphorylates the 40S ribosomal subunit of protein S6 leading to increased translation of mRNAs containing 5’-terminal oligopyrimidine tracts (6). mRNA transcripts containing 5’-terminal oligopyrimidine tracts code for ribosomal proteins and elongation factors important in protein translation.

The mTOR signaling pathway is known to be up-regulated in various carcinoma cell lines, as well as in human ovarian (7) and breast carcinomas (8). In breast carcinoma, overexpression of S6K predicts locoregional recurrence in a subset of patients (8). mTOR and S6K protein expression have not been examined in hepatocellular carcinomas. Herein, we report that this pathway is up-regulated in a proportion of hepatocellular carcinoma and that rapamycin inhibits cell proliferation and blocks S6K phosphorylation.

MATERIALS AND METHODS

Primary Liver Tumors. This study was performed with appropriate Institutional Review Board approval. To study protein expression in a large number of hepatocellular tumors, tissue arrays were constructed from formalin-fixed, paraffin-embedded tissues on all hepatocellular neoplasms in the Johns Hopkins Surgical pathology files with available tissues from 1985 to 2001, including 73 typical hepatocellular carcinomas, 15 hepatic adenomas, and 13 fibrolamellar carcinomas. The arrays included paired nonneoplastic and neoplastic liver tissue from each patient. Each case had at least four 1.5-mm cores of tumor and four 1.5-mm cores of nonneoplastic liver. Control tissues from diverse organs were included. The arrays were also constructed with four cases present twice on two different blocks to serve as an internal control for reproducibility of staining.

Immunohistochemistry. Immunostains were performed for total and phosphorylated mTOR and for total and phosphorylated p70 S6 Kinase. All mTOR and S6K antibodies were from Cell Signaling Technology (Beverly, MA): total mTOR, dilution 1:20; phospho-mTOR(Ser2448), dilution 1:5; total S6K, dilution 1:5; and phospho-S6K(Thr389), dilution 1:5. The phos-
pho-mTOR antibody recognizes mTOR when phosphorylated at Ser2448, which is a nutrient regulated phosphorylation site in the catalytic domain (9). The phoso-S6K antibody recognizes S6K when phosphorylated at Thr389, which most closely correlates with S6K activity in vivo (10). The Ki-67 antibody (Dako, Carpinteria, CA) was used at a 1:1000 dilution.

Five-micron sections were incubated with the primary antibody for 1 hour at room temperature. After the primary antibody, the sections were incubated for 30 minutes in Dako EnVision Peroxidase, a labeled-dextran polymer, followed by incubation with 3,3′-diaminobenzidine for 5 minutes. Prostate adenocarcinoma tissues served as the positive control for all antibodies. Mitotic figures were also routinely positive for total and phoso-S6K. No staining was seen when primary antibody was replaced by normal rabbit serum IgG.

Immunostaining results were evaluated by comparing the intensity of cytoplasmic staining in the tumor to the intensity of staining in the paired nonneoplastic tissue. Positive cases were subjectively scored as showing mild, moderate, or marked staining intensity. Nuclear positivity was estimated to the nearest 10%. Results were correlated with tumor growth pattern as defined by the WHO (trabecular, pseudoglandular/acinar, compact, and scirrhus), with tumor size and with the modified Edmondson nuclear grade, which has a scale of 1 to 4. For Ki-67 scoring, 200 hepatocyte nuclei were counted, and the percentage of positive cells was calculated.

Rapamycin-treated Cell Lines. The effect of rapamycin (Cell Signaling Technology, Beverly, MA) was studied on HepG2 and Hep3B cell lines (American Type Culture Collection, Manassas, VA). The cells were grown in MEM with 10% fetal bovine serum until confluence. Untreated cells were formalin fixed and paraffin embedded for immunohistochemistry. Additional cells were washed and subjected to serum deprivation by replacing the MEM with 0.5% fetal bovine serum for 48 hours. This was followed by serum stimulation with 10% fetal bovine serum for 1 hour. Subsequently, the cell lines were treated with three different concentration of rapamycin (0, 1, and 20 nmol/L) and incubated for 30 minutes. Proteins were extracted with the Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Equal amounts of extracted proteins were immunoprecipitated, separated on an SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membranes were blocked by 5% nonfat milk powder for 30 minutes, followed by 1% BSA for another 30 minutes and treated with primary antibody for total and phosphorylated S6K as detailed above for 3 hours. Subsequently, the membranes were washed and incubated with antirabbit IgG conjugate coupled with horseradish peroxidase for 1 hour. The membranes were again washed, and the protein-antibody-conjugate complex was detected with the Supersignal West Pico Chemiluminescent Substrate (Pierce).

Cell Proliferation Assay with Alamar Blue. HepG2 and Hep3B cell lines were grown in tissue culture plates in MEM with 10% fetal bovine serum. The cells were trypsinized after reaching 80% confluence, and equal numbers of cells were plated in wells of 96 microtiter plates. The plates were incubated at 37°C for 1 hour and then three different concentrations of rapamycin (0, 1, and 20 nmol/L) were added along with Alamar Blue, followed by re-incubation at 37°C. The percentage of dye uptake, a measure of the number of viable cells, was determined spectrophotometrically at 570 and 600 nm wavelength.

Statistical Analysis. The student t test was used to compare metric variables and χ² tests to compare frequencies. The Mann-Whitney test was used to compare medians for analysis of tumor size, which included several larger tumors that were outliers for tumor size. SYSTAT version 10 (SPSS 2000) was used for data analysis.

Fig. 1 All images are from the same case. A, original magnification, ×100. The normal liver is moderately positive for total mTOR. Benign bile ducts are also positive for mTOR. B, original magnification, ×100. The hepatocellular carcinoma overexpresses total mTOR. C, original magnification, ×100. The normal liver is negative for total S6 Kinase. D, The hepatocellular carcinoma is strongly positive for total S6 kinase.
RESULTS

Demographics. For the tissue arrays, hepatocellular carcinomas were studied from 54 males and 19 females, with an average age of 54 ± 18 years at the time of surgical resection. The underlying liver diseases were available in 44 cases and included hepatitis C (n = 17), hepatitis B (7), hepatitis C and B co-infection (2), ethanol cirrhosis (5), nonalcoholic fatty liver disease (1), primary sclerosing cholangitis (1), autoimmune cirrhosis (1), cryptogenic cirrhosis, or no known liver disease (10). The average tumor size was 6.4 ± 4.1 cm and median was 5.0 cm. The 13 fibrolamellar carcinomas were from 10 females and 3 males with a median age of 27 years. Nine cases were primary tumors with an average size of 10 ± 3 cm and median was 9 cm, whereas the remaining cases were from metastatic disease. The hepatic adenomas were from 15 women and have been previously characterized in greater detail (11).

mTOR Protein Expression in Hepatocellular Carcinoma. For all of the antibodies, the immunostaining was reproducible with all four cases that were included twice on the arrays demonstrating similar staining patterns. The nonneoplastic liver was diffusely and weakly to moderately positive for total mTOR in all cases (Fig. 1). In addition to hepatocytes, bile ducts were also positive. No differences in intensity of hepatocyte staining were noted in the nonneoplastic livers for the different underlying disease or between those with no fibrosis versus cirrhosis. Increased cytoplasmic positivity was seen in 4 of 73 (5%) of hepatocellular carcinomas (Table 1). A greater proportion of hepatocellular carcinomas, 11 of 73 (15%), were positive for phosphorylated mTOR with mild (n = 3) and moderate (n = 8) degrees of immunolabeling. Phospho-mTOR positivity was associated with increased total S6K (P = 0.001) but not nuclear S6K positivity (P = 0.18). Phospho-mTOR was negative in nonneoplastic liver tissues. No nuclear staining for either total or phosphorylated mTOR was observed. There was no difference in the average the Ki-67 proliferation index for those cases with phospho- or total mTOR expression compared with those without positivity.

S6K Protein Expression in Hepatocellular Carcinomas. The normal liver was generally negative for total S6K with a few cases weakly positive. Hepatocellular carcinomas showed increased cytoplasmic S6K in 33 of 73 (45%) of cases (Fig. 1) and positivity correlated with phospho-mTOR as discussed above but also correlated negatively with tumor size (P = 0.01; Mann-Whitney test), with a mean tumor size in positive cases of 5.0 ± 3 versus 7.7 ± 5 cm in negative cases. Nuclear positivity for total S6K was present in 38 of 73 (52%) of hepatocellular carcinomas. The median percentage of positive nuclei in positive cases was 30% for hepatocellular carcinoma, 40% for fibrolamellar carcinoma, and 20% for hepatic adenoma. In hepatocellular carcinomas, nuclear positivity for total S6K was associated with nuclear grade (Table 2; P = 0.01) but not with tumor size, underlying liver disease, proliferation index, or WHO growth pattern (all P > 0.05). No difference was see in the average proliferation index for those cases with phospho or total mTOR expression compared with those without positivity.

mTOR and S6K Expression in Hepatic Adenomas and Fibrolamellar Carcinomas. Four fibrolamellar carcinomas showed overexpression of total mTOR, and three of these four were also positive for phospho-mTOR. Despite the presence of four cases with increased total mTOR, no fibrolamellar carcinoma showed overexpression of cytoplasmic S6K. No hepatic adenomas had increased expression of total mTOR, but 5 of 15 showed overexpression of phospho-mTOR. Similarly to the fibrolamellar carcinoma, however, no hepatic adenoma had increased total S6K expression.

Table 1 Immunostaining results for total mTOR and S6K organized by predominant morphological growth pattern of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Total (N)</th>
<th>Total mTOR overexpression (N)</th>
<th>Phospho mTOR overexpression (N)</th>
<th>Total S6K overexpression (cytoplasm) (N)</th>
<th>Total S6K nuclear positive (N)</th>
<th>Phospho-S6K nuclear positive (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular/macrotrabecular</td>
<td>32</td>
<td>2</td>
<td>2</td>
<td>18 (56%)</td>
<td>16 (50%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Compact/fatty</td>
<td>33</td>
<td>2</td>
<td>8</td>
<td>12 (36%)</td>
<td>18 (55%)</td>
<td>0</td>
</tr>
<tr>
<td>Pseudoglandular/acinar</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scirrhous</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
<td>0</td>
</tr>
<tr>
<td>Clear cell/other</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2 (40%)</td>
<td>3 (40%)</td>
<td>0</td>
</tr>
<tr>
<td>Fibrolamellar</td>
<td>13</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>6 (47%)</td>
<td>2 (15%)</td>
</tr>
<tr>
<td>Hepatic adenoma</td>
<td>15</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>3 (20%)</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. Hepatic adenomas are also listed.

Table 2 Frequency of mTOR and S6K positivity by nuclear grade for nonfibrolamellar carcinoma hepatocellular carcinomas

<table>
<thead>
<tr>
<th>Modified Edmonson grade</th>
<th>Total</th>
<th>Total mTOR overexpression (N)</th>
<th>Phospho-mTOR overexpression (N)</th>
<th>S6K increased cytoplasm staining (N)</th>
<th>S6K nuclear positive (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>1 (8%)</td>
<td>1 (8%)</td>
<td>4 (33%)</td>
<td>5 (42%)</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>1 (4%)</td>
<td>5 (19%)</td>
<td>14 (52%)</td>
<td>10 (37%)</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>2 (7%)</td>
<td>4 (14%)</td>
<td>11 (38%)</td>
<td>19 (66%)</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
<td>1 (20%)</td>
<td>4 (80%)</td>
<td>4 (30%)</td>
</tr>
</tbody>
</table>
Rapamycin Inhibits HepG2 and Hep3B Hepatoblastoma Cell Lines. To determine the immunophenotype at baseline, formalin-fixed, paraffin-embedded cells were immunostained, and both HepG2 and Hep3B cell lines were total mTOR positive, phospho-mTOR positive, total S6K negative, and phospho-S6K positive in proliferating cells. Treatment of cell lines HepG2 and Hep3B with rapamycin led to a marked reduction in proliferation by 3 hours after treatment (Fig. 2). In addition, decreased expression of phospho-S6K was seen by Western blot analysis, whereas the amount of total S6K remained unchanged (Fig. 3).

DISCUSSION

The results of this study demonstrate that the mTOR pathway is up-regulated in a subset of hepatocellular carcinomas and 45% demonstrated increased S6K expression. Our findings also show that phospho-mTOR overexpression is associated with increased total cytoplasmic S6K, which in turn is associated with the degree of tumor differentiation. Furthermore, treatment of cell lines HepG2 and Hep3B with rapamycin inhibits proliferation and decreases phosphorylation of S6K. On the basis of the results of this study, inhibitors of the mTOR pathway may be effective in treatment of hepatocellular carcinomas, although additional evaluation is needed. In contrast and underscoring the fundamental biological differences between hepatocellular carcinomas and fibrolamellar carcinomas or hepatic adenomas, no fibrolamellar carcinomas and no hepatic adenomas had increased S6K cytoplasmic expression, despite increased mTOR in a proportion of both fibrolamellar carcinomas and hepatic adenomas.

Only four hepatocellular carcinoma (5%) showed increased total expression of mTOR, whereas eleven (15%) were positive for phospho-mTOR. These results suggest that examination of total mTOR in isolation will miss cases where the pathway is activated. In contrast to the small proportion of cases with overexpression of mTOR, 45% of hepatocellular carcinoma showed increased expression of total S6K. Although our study does not address the reasons for increased total S6K expression, it is of interest to note that Zimonjic et al. (12) have reported frequent gains of chromosome 17q23 in hepatocellular carcinoma cell lines, the chromosomal location that includes the RPS6KB1 gene. Similar findings of increased total S6K expression have been reported in breast carcinoma, where RPS6KB1 gene amplification is found in 10% of primary tumors (8).

Nonetheless other factors that we did not study may play a role in the overexpression of total S6K, including transcriptional regulation, as well as protein degradation of S6K.

Somewhat surprisingly, we found total S6K expression was associated with a smaller tumor size. Although this was statistically significant, the biological relevance is unclear.

Fig. 2. Alamar Blue assay. Treatment of HepG2 (A) and Hep3B (B) cell lines with rapamycin reduced cell proliferation by 3 hours after treatment.

Fig. 3. Western blot analysis. After treatment with rapamycin, there was a reduction in phosphorylated S6K in HepG2 (A) and Hep3B (B) cell lines. No change is seen in total S6K.
Although translation control and thus mTOR and S6K undoubt-
edly influences a cell’s ability to cycle, we found no association be-
 tween S6K and mTOR expression and increased proliferation as mea-
sured by Ki-67–labeling index, raising the possibility that the rate of cell cycling per se may not be as strongly influenced.

In addition to the cytoplasmic staining for S6K, we also
found an increase in nuclear staining in a significant proportion
of cases. The function of S6K in the nucleus is unclear, but
hepatocellular carcinomas, fibrolamellar carcinomas, and he-
patic adenomas all had a proportion of tumors that demonstrated
nuclear positivity. Kim et al. (13) have demonstrated that inhi-
bition of crm-1, a nuclear export protein, can lead to accumu-
lation of S6K in the nucleus. However, the function of S6K in
the nucleus is not clear.

This study is limited by a number of factors that require
future clarification. First, hepatocellular carcinomas frequently
vary in their molecular profile depending on the underlying
cause of liver disease. For example, the FHIT gene is much
more commonly dysregulated in hepatocellular carcinomas
from China than those from the United States (14). Although we
did not observe differences in mTOR pathway expression
among different underlying liver diseases, additional studies
with larger samples will be needed to clarify this potential
source of variation. In particular, we had a few HBV-related
hepatocellular carcinomas. At the molecular level, the mTOR
pathway interacts with and is affected by numerous additional
pathways such as AKT, and additional dissection of these path-
ways will be needed to clarify the precise mechanism for inhi-
bition of cell proliferation.

Although we found that rapamycin inhibited cell prolifer-
ation and phosphorylation of S6K in liver carcinoma cell lines
and is potentially an attractive therapeutic candidate, a number
of important questions remain. For example, it is unclear if
immunohistochemistry positivity for mTOR or S6K would be a
reliable guide for potential rapamycin response and would be
helpful in selecting cases for treatment because we do not have
data on cell lines negative for mTOR. It is also unclear how
effective rapamycin will be in animal models, which more
closely reflect the complex physiology of primary human he-
patocellular carcinomas, and we are currently undertaking such
studies.

In conclusion, the mTOR pathway is active in hepatocel-
lular carcinoma and overexpression of S6K is found in 45% of
tumors. Rapamycin can directly inhibit cell proliferation and
phosphorylation of S6K in liver carcinoma cell lines and is
potentially an attractive candidate for therapeutic intervention.

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