mTOR inhibition reduces growth and adhesion of hepatocellular carcinoma cells *in vitro*

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Abstract. Mechanistic target of rapamycin (mTOR) signaling is typically increased in hepatocellular carcinoma (HCC). A panel of HCC cell lines (HepG2, Hep3B and HuH6) was exposed to various concentrations of the mTOR inhibitors, everolimus and temsirolimus, in order to investigate their effects on cell growth, clonal formation, cell cycle progression, and adhesion and chemotactic migration using MTT and clonal cell growth assays, fluorometric detection of cell cycle phases and a Boyden chamber assay. In addition, integrin α and β adhesion receptors were analyzed by flow cytometry and blocking studies using function blocking monoclonal antibodies were conducted to explore functional relevance. The results demonstrated that everolimus and temsirolimus significantly suppressed HCC cell growth and clonal formation, at 0.1 or 1 nM (depending on the cell line). In addition, the number of cells in G_0/G_1 phase was increased in response to drug treatment, whereas the number of G_2/M phase cells was decreased. Drug treatment also considerably suppressed HCC cell adhesion to immobilized collagen. Integrin profiling revealed strong expression of integrin α1, α2, α6 and β1 subtypes; and integrin α1 was upregulated in response to mTOR inhibition. Suppression of integrin α1 did not affect cell growth; however, it did significantly decrease adhesion and chemotaxis, with the influence on adhesion being greater than that on motility. Due to a positive association between integrin α1 expression and the extent of adhesion, whereby reduced receptor expression was correlated to decreased cell adhesion, it may be hypothesized that the adhesion-blocking effects of mTOR inhibitors are not associated with mechanical contact inhibition of the α1 receptor but with integrin α1-dependent suppression of oncogenic signaling, thus preventing tumor cell-matrix interaction.

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Introduction

Hepatocellular carcinoma (HCC) represents the fifth most common type of cancer in males and the ninth most common in females (1). The prognosis of HCC is poor due to the highly aggressive nature of the disease, particularly for HCC patients with recurrent and/or metastatic tumors (2,3).

Increasing knowledge regarding the molecular alterations that initiate malignancy has led to the development of novel compounds targeting pathways aberrantly activated in cancer. Of these, the multiple receptor tyrosine kinase inhibitor sorafenib, which primarily targets platelet-derived growth factor receptor and vascular endothelial growth factor receptor, is the worldwide standard therapy for the treatment of advanced HCC. However, although sorafenib has significantly improved the treatment protocol, the overall response rate is disappointingly low and resistance inevitably develops, further limiting the efficacy of the drug (4).

Recent studies have provided strong evidence to suggest that phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) signaling is upregulated in HCC, and activation of this pathway triggers rapid HCC development and progression (5,6). In clinical practice, mTOR hyperactivity has been associated with high-grade tumors and tumors with poor prognosis (7). Therefore, mTOR inhibition may be considered a plausible strategy in mitigating HCC. The present study aimed to investigate the antitumor effects of two mTOR inhibitors, everolimus and temsirolimus. Temsirolimus has been approved for first-line treatment of patients with poor prognosis renal cell carcinoma (RCC), whereas the oral mTOR inhibitor, everolimus, has been recommended for patients with advanced progressive RCC or for patients with failed vascular endothelial growth factor-targeted therapy (8). In the present study, experiments were conducted on a panel of HCC cell lines, and aimed to determine the effects of mTOR inhibitors on tumor growth and invasion. In addition, integrin α and β subtype expression was investigated, since these receptors are not only involved in tumor growth and invasion, but also in cellular differentiation.

Materials and methods

Cell culture. HepG2 and HuH7 cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). Hep3B cells were purchased from DSMZ (Braunschweig, Germany). HepG2 cells were grown and subcultured in Dulbecco's modified Eagle's medium:Nutrient Mixture F-12 medium (Thermo Fisher Scientific GmbH, Dreieich, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific GmbH), 20 mM Hepes-buffer and gentamicin (0.5 ml/l). HuH7 and Hep3B cells were incubated in RPMI-1640 medium, supplemented with 10% FBS, 20 mM HEPES-buffer, 1% glutamax, and 1% penicillin/streptomycin (all Gibco; Thermo Fisher Scientific GmbH) at 37˚C in a humidified incubator containing 5% CO_2.

mTOR inhibitors. Everolimus (Novartis Pharma AG, Basel, Switzerland) and temsirolimus (LC Laboratories, Woburn, MA, USA) were dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution and stored in aliquots at -20˚C. Prior to use, the compounds were diluted in cell culture medium. The effects of 0.1-100 nM everolimus or temsirolimus were determined on cell growth to evaluate dose dependency. All further experiments were conducted with 1 nM everolimus or temsirolimus. Cells treated with culture medium alone (supplemented with DMSO, diluted $1:10^5$-$1:10^8$) served as controls. To evaluate the toxic effects of the drugs, following a 72 h incubation at 37˚C in a humidified incubator containing 5% CO_2, cell viability was determined by trypan blue staining (Gibco; Thermo Fisher Scientific GmbH). A Zeiss ID 03 light microscope (Zeiss AG, Oberkochen, Germany) was used.

Measurement of tumor cell growth. Cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Roche Diagnostics GmbH, Penzberg, Germany). HepG2, Hep3B and HuH6 cells, (50 μl, $1x10^5$ cells/ml) were seeded onto 96-well tissue culture plates. After 24, 48 and 72 h incubation at 37˚C MTT (0.5 mg/ml) was added for an additional 4 h. Thereafter, cells were lysed in a buffer containing 10% SDS in 0.01 M HCl. The plates were incubated overnight at 37˚C in an atmosphere containing 5% CO_2. Absorbance was measured at 550 nm using a microplate ELISA reader. Each experiment was conducted in triplicate. After subtracting background absorbance, results were expressed as percentage difference, related to a control set to 100%.

Clonogenic growth. HepG2, HuH7, or Hep3B cells, treated with 1 nM everolimus or temsirolimus, were transferred to 6-well plates at 2,000 cells/well. Following 10 days of incubation, without changing the cell culture medium (everolimus and temsirolimus remained in culture), colonies were fixed with 1% glutaraldehyde for 10 min at room temperature and counted. Colonies containing ≥50 cells were counted using a Zeiss ID 03 light microscope (Zeiss AG). Non-treated cells served as controls.

Cell cycle analysis. Cell cycle analysis was performed once the tumor cell cultures had grown to sub-confluency, and after 24 h of drug treatment. Tumor cell populations were stained with propidium iodide using a Cycleyest Plus DNA Reagent kit (BD Biosciences, Heidelberg, Germany) and were then subjected to flow cytometry using a FACScan flow cytometer (BD Biosciences). A total of 10,000 events were collected for each sample. Data acquisition was conducted using CellQuest version 6.0 software (BD Biosciences) and cell cycle distribution was calculated using ModFit version 3.0 software (BD Biosciences). The number of gated cells in the G_1-, S-, or G_2/M-phases was expressed as a percentage of the total cell population.

Western blot analysis. To investigate the expression levels of cell cycle-regulating proteins, tumor cell lysates (50 μg; protein concentration was quantified by Bradford assay) were loaded onto a 7% polyacrylamide gel and were electrophoresed for 90 min at 100 V. The lysis buffer consisted of Tris-NaCl, 10% Tergitol, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 2 mM NaF, 2 mM Na_3VO_4 and 2 mM PMSF. Protein was then transferred to nitrocellulose membranes for 1 h at 100 V. After blocking with non-fat dry milk for 1 h at room temperature, the membranes were incubated overnight at 4˚C with monoclonal antibodies directed against the following cell cycle-associated proteins: Phosphorylated (p)-Akt [clone 104A282, mouse immunoglobulin (Ig)G1; dilution 1:500; cat. no. 550747; BD Biosciences], p-mTOR (clone D9C2; IgG, Ser2448; dilution 1:1,000; cat. no. 5536S) and p-Raptor (IgG, Ser792; dilution 1:1,000; cat. no. 2083S; all Cell Signaling Technology Europe, B.V., Leiden, The Netherlands). Horseradish peroxidase-conjugated goat-anti-mouse IgG (dilution, 1:5,000; cat. no. 161-0380; Bio-Rad Laboratories, Inc., Hercules, CA, USA) served as the secondary antibody (30 min incubation at room temperature). The membranes were briefly incubated at room temperature with an enhanced chemiluminescence (ECL) detection reagent (ECL™; Merck KGaA, Darmstadt, Germany; cat. no. WBKLS0100) to visualize the proteins and were then analyzed using the Fusion FX7 system (Peqlab Biotechnologie GmbH, Erlangen, Germany). β-actin (1:1,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA) served as an internal control.

Tumor cell adhesion assay. For the tumor cell adhesion assay, 6-well plates were coated with collagen G (extracted from calfskin, consisting of 90% collagen type I and 10% collagen type III; diluted to 400 μg/ml in PBS; Thermo Fisher Scientific GmbH) overnight. Plastic dishes served as the background control. Plates were washed with 1% bovine serum albumin (BSA; Sigma Aldrich; Merck KGaA) in PBS to block nonspecific cell adhesion. HCC tumor cells ($0.5x10^6$) were then added to each well for 60 min. Subsequently, non-adherent tumor cells were washed off, and the remaining adherent cells were fixed for 10 min at room temperature with 1% glutaraldehyde and counted under a Zeiss ID 03 light microscope (Zeiss AG). Mean cellular adhesion, defined as adherent $cells_{coated\ well}$-adherent $cells_{background}$, was calculated from five different observation fields (5x0.25 mm^2).

Tumor cell chemotaxis. Serum-induced chemotaxis was investigated using 6-well Transwell chambers (Greiner Bio-One GmbH, Frickenhausen, Germany) with 8-μm pores. HCC tumor cells ($0.5x10^6$/ml) were placed in the upper chamber in

serum-free medium. The lower chamber contained complete cell culture medium including 10% serum (Sigma Aldrich; Merck KGaA). After 20 h at 37˚C, the upper surface of the Transwell membrane was gently wiped with a cotton swab to remove cells that had not migrated. Cells that had moved to the lower surface of the membrane were stained using hematoxylin and counted under a light microscope (Zeiss ID 03; Zeiss AG). Mean chemotaxis was calculated from five different observation fields (5x0.25 mm^2).

Integrin surface expression. HepG2, HuH7 or Hep3B cells were detached from their culture flasks by Accutase (PAA Laboratories; GE Healthcare) and washed in blocking solution (PBS, 0.5% BSA). Cells were then incubated for 60 min at 4˚C with phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) directed against the following integrin subtypes: Anti-α1 (mouse IgG1, clone SR84; cat. no. 742362), anti-α2 (mouse IgG2a, clone 12 F1; cat. no. 555669), anti-α3 (mouse IgG1, clone C3 II.1; cat. no. 746157), anti-α4 (mouse IgG1, clone 9F10; cat. no. 658332), anti-α5 (mouse IgG1, clone IIA1; cat. no. 555615), anti-α6 (rat IgG2a, clone GoH3; cat. no. 555734), anti-β1 (mouse IgG1, clone MAR4; cat. no. 557332), anti-β3 (mouse IgG1, clone VI-PL2; cat. no. 555752), and anti-β4 (rat IgG2a; clone 439-9B; cat. no. 555720; all 20 μl/test; using the manufacturing dilution; all BD Biosciences). Tumor cell integrin expression was then measured using a FACScan [BD Biosciences; FL-2H (log) channel histogram analysis; 1x10^4 cells per scan] and was expressed as mean fluorescence units. A mouse IgG1-PE (MOPC-21) or IgG2a-PE (G155-178) (BD Biosciences) was used as an isotype control.

Integrin suppression. To determine whether integrin α1 impacts tumor growth, HepG2 cells were incubated for 60 min at 37˚C with 10 μg/ml function-blocking anti-integrin α1 (clone FB12) mAb (Merck KGaA; cat. no. MAB1973Z). Controls were incubated with cell culture medium alone. Subsequently, tumor cell growth was analyzed using the MTT assay, as aforementioned. To evaluate whether integrin α1 acts on tumor cell motility, adhesion and migration experiments were conducted using HepG2 cells following integrin α1 suppression. To evaluate tumor cell binding to matrix proteins, 24-well plates coated with fibronectin (BD Biosciences) were used. Plastic dishes served as the background control. Plates were washed with 1% BSA in PBS to block nonspecific cell adhesion. Subsequently, 0.5x10^6 tumor cells were added to each well and incubated for 60 min at 37˚C. Non-adherent tumor cells were washed off and the remaining adherent cells were fixed with 1% glutaraldehyde for 10 min at room temperature and counted by a light microscope (Zeiss ID 03; Zeiss AG). The mean cellular adhesion rate, defined as adherent $cells_{coated\ well}$-adherent $cells_{background}$, was calculated from five different observation fields. To evaluate tumor cell migration, serum-induced chemotaxis was examined using 6-well Transwell chambers (Greiner Bio-One GmbH) with 8-μm pores preformed as described above (*Tumor cell chemotaxis* subsection), however, only 0.5x10^6 HepG2 cells were applied.

Statistical analysis. Statistical analysis was performed using BiAS software version 11.06 (http://www.bias-online.de/). All experiments were performed between three and six times. Data are presented as the mean ± standard deviation. Statistical significance between groups was determined using the Mann-Whitney U test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exposure to everolimus or temsirolimus reduces HCC cell growth. Ascending concentrations of everolimus or temsirolimus induced a dose-dependent significant reduction in the number of HCC cells. Everolimus exerted a growth inhibitory effect at 0.1 nM in Hep3B and HuH7 cells, where temsirolimus exerted an inhibitory effect at 1 nM in HepG2 and HuH7 cells; the most obvious effect was apparent at 100 nM compared with the untreated controls (Fig. 1). No signs of toxicity were apparent, as determined by a trypan blue exclusion assay (data not shown).

Clonogenic growth was significantly reduced when all three tumor cell lines were treated with 1 nM everolimus or temsirolimus (Fig. 2A-C). In addition, everolimus (1 nM) and temsirolimus (1 nM) modulated cell cycle progression. The number of HepG2 cells in the G_2/M- and S-phases was reduced, whereas the number of tumor cells in the G_0/G1-phase was increased compared with the controls (Fig. 2D).

Since everolimus and temsirolimus target the mTOR signaling pathway, the Akt-mTOR axis was also evaluated. p-mTOR and p-Raptor expression was suppressed following treatment of all cell lines with both compounds (Fig. 2E). However, as total protein content was not detected in the present study, the activation status of the proteins cannot be confirmed. In addition, p-Akt expression was reduced in the HepG2 and Hep3B cell lines, but was undetectable in HuH7 cells.

Everolimus and temsirolimus modify integrin α1 expression. The integrin subtypes α1, α2, α6 and β1 were strongly expressed on HepG2 cells, whereas α3, α4, α5, β3 and β4 were not detected (Fig. 3A). Integrin expression was also detected on Hep3B and HuH7 cells; it was demonstrated that α1, α2, α6 and β1 were expressed on these cell lines (Fig. 3B). Treatment of HepG2 cells with everolimus or temsirolimus significantly elevated the expression of integrin α1 on the tumor cell surface, without acting on α2, α6 or β1 expression (Fig. 3C).

Everolimus and temsirolimus suppress HCC cell adhesion but not migration. Treatment with everolimus or temsirolimus (1 nM) significantly downregulated adhesion of HepG2, HuH7 and Hep3B cells to immobilized collagen (Fig. 4A). The most prominent effects were evoked in HepG2 and Hep3B cells. Control values were 61.4±11.2 cells/mm^2 (HepG2), 66.8±14.2 cells/mm^2 (HuH7) and 79.4±14.8 cells/mm^2 (Hep3B). A chemotaxis assay revealed that HepG2 and Hep3B motility was enhanced following exposure to temsirolimus or everolimus exposure (Fig. 4B). However, the control values were low (HepG2: 16.2±4.2 cells/mm^2, Hep3B: 6.8±3.5 cells/mm^2). HuH7 cells did not migrate through the filter membrane.

Integrin α1 suppression. Since everolimus and temsirolimus induced a distinct upregulation in integrin α1 on HepG2 cells, subsequent experiments aimed to determine the functional

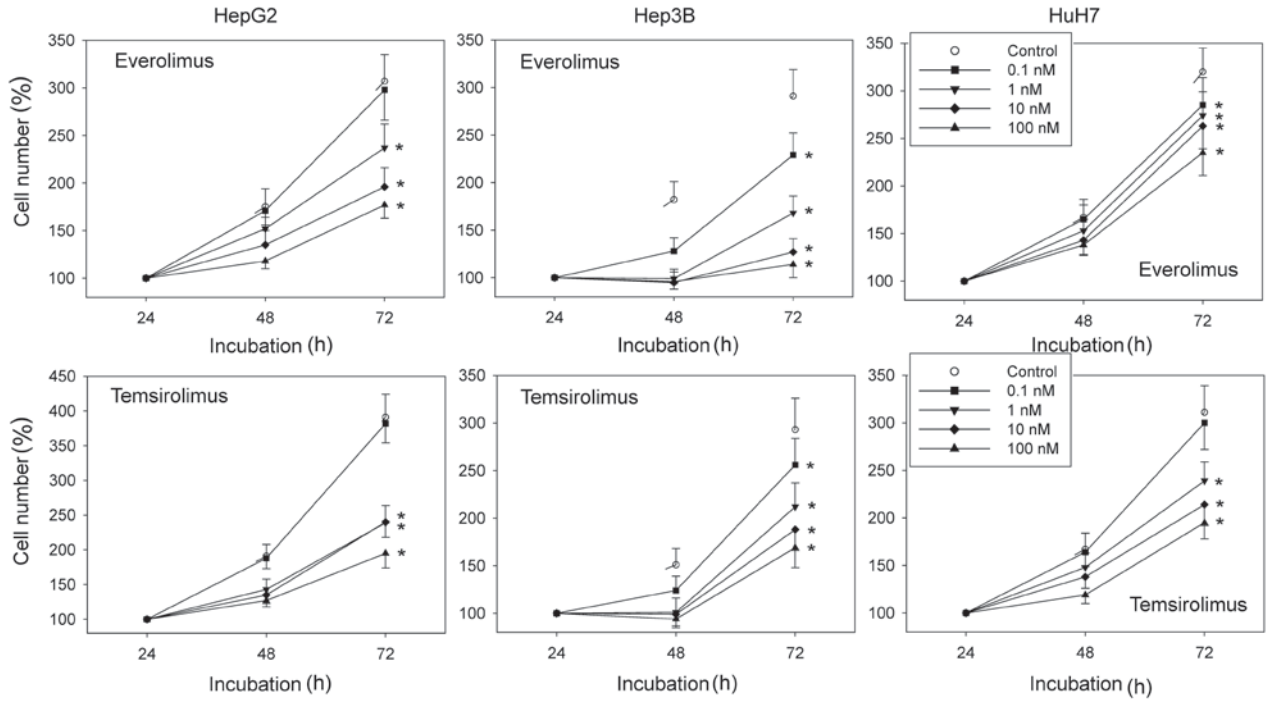

Figure 1. Growth inhibitory effects of everolimus or temsirolimus on HepG2, Hep3B and HuH7 cells at 24-72 h. The results of one of the six independent experiments performed are presented. *P<0.05 vs. the control group, set to 100% (n=6).

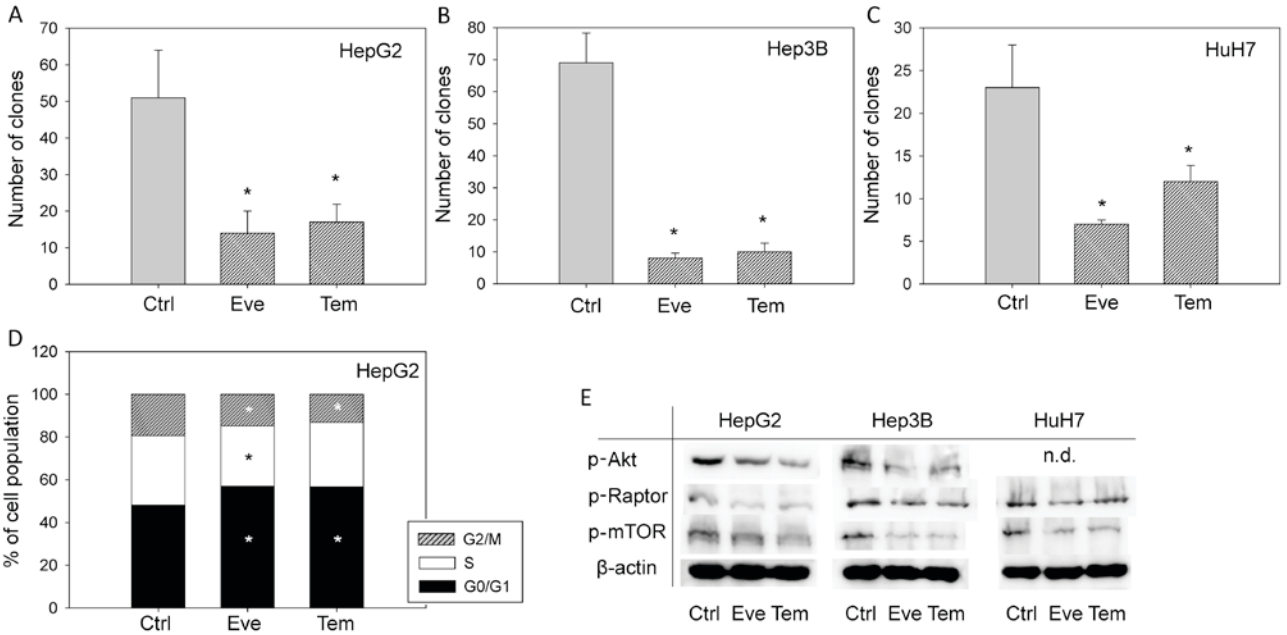

Figure 2. Clonogenic growth of (A) HepG2, (B) Hep3B and (C) HuH7 cells with or without Eve or Tem treatment for 10 days. (D) Cell cycle analysis of HepG2 cells after 24 h Eve or Tem exposure compared with the Ctrl cells. The proportion of cells in each phase is expressed as a percentage of total cells. Experiments were conducted in triplicate and repeated five times. *P<0.05 vs. the Ctrl group. (E) Western blot analysis of mTOR-associated proteins in HepG2, Hep3B and HuH7 cells. β-actin served as an internal control. A representative image of three independent experiments is presented. Ctrl, control; Eve, everolimus; mTOR, mechanistic target of rapamycin; n.d., not detected; p-phosphorylated; Tem, temsirolimus.

relevance of this receptor. Suppression of integrin α1 slightly decreased HepG2 cell growth; however, this was not significant (Fig. 4C). The strongest effects were observed with respect to tumor cell adhesion, which was reduced by >40% following integrin α1 suppression (Fig. 4D). Chemotactic activity was also significantly decreased in the presence of anti-integrin α1 compared with the control group (30% decrease; Fig. 4D).

Discussion

Better understanding of tumor cell biology has led to the development of numerous targeted therapeutic agents. The PI3K/Akt/mTOR pathway has been identified as a pivotal key regulator of cell growth, cell proliferation and cell survival. Targeting the mTOR pathway has, therefore, been proposed

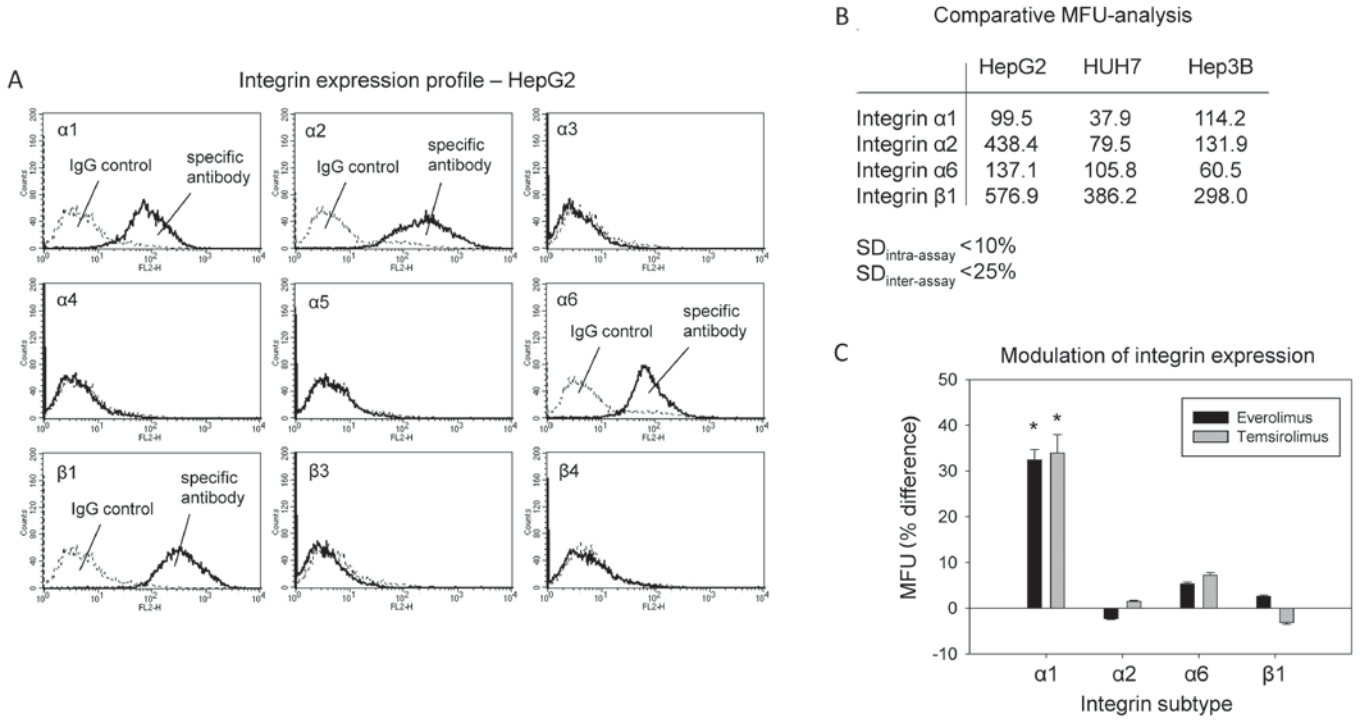

Figure 3. (A) Integrin expression in untreated HCC cells. The dotted line indicates background fluorescence (IgG control) and the solid line indicates specific fluorescence (specific antibody). (B) Comparative analysis of integrin subtype expression on HepG2, Hep3B and HuH7 cells. (C) Integrin subtype expression on HepG2 cells following 24 h exposure to everolimus or temsirolimus, compared with untreated controls set at 100%. *P<0.05 vs. the control group. IgG, immunoglobulin G; MFU, mean fluorescent units; SD, standard deviation.

as an innovative strategy to treat cancer, and mTOR inhibitors have been approved for RCC treatment. However, the clinical relevance of mTOR inhibition in treating HCC remains unclear. Everolimus has been reported to delay tumor progression in patients with sorafenib-refractory HCC (9); however, a recent investigation did not demonstrate improved patient survival in a second-line setting or in sorafenib-intolerant patients (10). Yeo *et al* (11) and Cho *et al* (12) hypothesized that only selected patients, depending on the expression of relevant target proteins, may benefit from an mTOR-based treatment regimen. The benefit of an everolimus-sorafenib combination is controversial, since although a dose-finding study on patients with advanced HCC yielded encouraging results (13), a randomized multicenter, multinational phase II trial did not indicate a synergistic effect (14).

The present study demonstrated the growth inhibitory effects of everolimus and temsirolimus on numerous HCC cell lines; these agents exhibited effects at 0.1 or 1 nM, respectively. The effects observed may, at least in part, be induced by the downregulation of p-mTOR and particularly p-Raptor. Notably, p-Akt was not detected in HUH7, which indicates that this cell type may be associated with an Akt independent pathway with very low (undetectable) Akt activity or a less sensitive antibody may have been used in the present study.

In accordance with the present study, HepG2 cells have been reported to respond to 1 nM everolimus *in vitro* (15). In addition, the half maximal inhibitory concentration values of everolimus or temsirolimus in HepG2 cells have been reported to range between 0.9 nM (16) and 9 μM (17). Similar sensitivity to mTOR inhibition has been reported for Hep3B and HuH7 cells (18,19), indicating that mTOR inhibition may be of clinical relevance in treating HCC. When discussing the behavior of the cell lines used in the present study, it must be emphasized that HepG2 cells were isolated from a liver biopsy with primary hepatoblastoma and HCC characteristics (20). Although HepG2 cells possess tumor-specific characteristics, they do not form tumors when injected into athymic mice and are histologically similar to the hepatoblastoma from which the cell line was derived (20). Therefore, HepG2 should be considered a hepatoblastoma, rather than a carcinoma, cell line.

Based on the results of a cell cycle analysis, cell growth inhibition in the presence of everolimus and temsirolimus may be caused by driving the tumor cells from G_2/M- and S-phases into G_0/G_1. The differences between the number of G_2/M- and S-phase cells in the treated and non-treated tumor cells were moderate; however, similar moderate differences have also recently been observed, when HepG2 cells were exposed to the mTOR inhibitor sirolimus. Suppression of tumor cell proliferation by this drug was also associated with only a moderate G_0/G_1 increase and S-phase decrease (21). In addition, it has been speculated that a G_0/G_1 phase arrest, but not apoptosis, may be the predominant mechanism responsible for the antiproliferative activity of sirolimus (22). Everolimus has been reported to suppress cyclins A, B1 and D1 in an *in vivo* HCC xenograft model (22). Although the role of these cyclins in cell cycle progression has yet to be elucidated in detail, there is evidence that loss of cyclin A, B1 and/or D1 may at least partially contribute to HCC cell accumulation in G_0/G_1-phase (23,24). Notably, exposing Hep3B and HuH7 cells to everolimus also induced a G_0/G_1-phase arrest, indicating a common mechanism for this class of drugs (15).

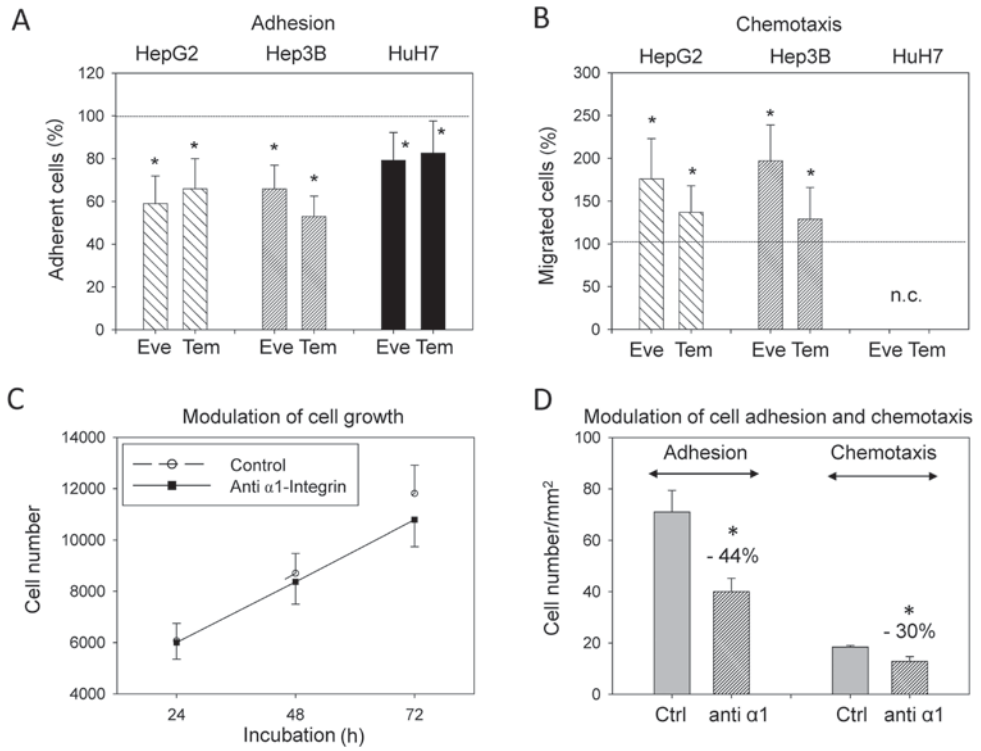

Figure 4. Effects of Eve or Tem on (A) adhesion to immobilized collagen and (B) chemotaxis of HepG2, Hep3B and HuH7 cells. Chemotaxis was assessed using a Transwell chamber assay. (A and B) Mean values from five experiments are presented compared to the untreated Ctrl cells (cell culture medium supplemented with dimethyl sulfoxide, diluted 1:10^7) set at 100%. *P<0.05 vs. the Ctrl group. (C) Effects of integrin α1 suppression on HepG2 cell growth. (D) Effects of integrin α1 suppression on HepG2 cell adhesion and chemotaxis. Tumor cells were preincubated for 60 min with a function-blocking anti-integrin α1 antibody. Ctrl cells were untreated (0.25 M NaCl containing 0.1% sodium azide, diluted 1:100 in cell culture medium). *P<0.05 vs. the Ctrl group (n=6). Ctrl, control; Eve, everolimus; Tem, temsirolimus.

Suppression of adhesion by everolimus and temsirolimus was determined in the present study. Maximum effects were exerted on HepG2 and Hep3B cells, with a >40 and 50% reduction, respectively. This is important, since tumor cell-matrix interaction is critical for malignant tumor progression and metastatic spreading. The results of the chemotaxis assay are difficult to interpret in this context, since the percentage of migrating cells increased in the presence of mTOR inhibitors. However, it must be noted that only a few cells migrated across the filter membrane in the control group. Therefore, slight differences in the absolute number of motile cells following everolimus or temsirolimus treatment are associated with large percentage changes. An elevation of just 3 single Hep3B cells following treatment was associated with a percentage difference >40% compared with the control group. Whether the moderate elevation in cell migration, based on absolute cell number count, is clinically relevant or unspecific remains to be elucidated. Based on a wound-healing assay, the motility of HepG2, HuH7 and Hep3B cells has recently been reported to be significantly reduced by everolimus, whereas a Transwell migration assay, which was conducted in parallel, demonstrated a slight elevation in HepG2 and HuH7 migration (18). These data suggested a partial assay-dependent effect. To determine the effects of mTOR inhibitors on tumor cell migration, further investigation is required. Since everolimus and temsirolimus markedly suppressed HCC cell adhesion in the present *in vitro* model, it is hypothesized that this property is the dominant mechanism underlying the effects of these compounds.

Integrins serve a significant role in tumorigenesis and progression, including tumor growth, adhesion and invasion. The integrin profile analysis conducted in the present study detected a strong surface expression of the α1, α2, α6, and β1 integrin subtypes. Integrin α5 was not detected on HepG2 cells, which is contradictory to the findings of an earlier study, which revealed a moderate integrin α5 surface level on HepG2 cells (24). However, in the previous investigation, integrin α5 was not detected by western blotting, thus suggesting that integrin α5 does not serve a crucial role in HepG2 cell behavior. In the present study, integrin α1 was the only integrin significantly increased in response to everolimus or temsirolimus treatment. The relevance of this increase is not yet clear. Based on integrin blocking studies, integrin α1 does not appear involved in cell growth regulation. However, suppression of integrin α1 was strongly associated with reduced adhesion and migration, with a stronger effect on adhesion.

The role of integrin α1 in HCC metastasis is currently unclear. Liu *et al* (25) detected a positive correlation between integrin α1 and migration and invasion, which is in accordance with the present study. However, compared with the present investigation, adhesion assays were not conducted. The integrin-blocking model used in the present study explored the mechanical contact of α1 to its substrate. Since everolimus- or temsirolimus-induced suppression of HCC cell adhesion was accompanied by elevated integrin α1 expression, the positive association between α1 and migration cannot be transferred to the adhesion process. Another mode of action must, therefore, be assumed, which is not associated with mechanical contact inhibition of α1 and its receptor

in the collagen matrix. Notably, previous studies have indicated that integrin α1 may indirectly influence HCC progression by deactivating oncogenic signaling (26,27). Chen *et al* (28) hypothesized that integrin α1 may initiate mesenchymal to epithelial transition, with a high expression level associated with a low tumor cell adhesion capacity. This scenario may also hold true in the present model system, where this particular integrin may control cellular re-differentiation, leading to a less adhesive phenotype. However, this hypothesis is speculative and requires further confirmation. In addition, integrin α1 expression has been reported to be reduced in patients with metastatic breast cancer (29), and a high integrin α1 level has been revealed to correlate with a beneficial therapeutic response in a patient with melanoma (30).

In conclusion, in the present study, the *in vitro* malignancy of HCC was reduced following treatment with the mTOR inhibitors, everolimus and temsirolimus, thus indicating that the mTOR pathway may be a potential target in the treatment of HCC. Both drugs suppressed HCC cell growth and adhesion, and were demonstrated to regulate integrin α1 expression, which is a novel finding. In addition, tumor-matrix interaction was blocked and, consequently, migration may decrease. However, the effects of mTOR inhibitors on invasive processes require further evaluation. At present, clinical reports remain ambivalent regarding the value of mTOR inhibition (31,32). Possibly, identification of a predictive biomarker may aid in selecting patients most likely to respond to everolimus or temsirolimus. In addition to optimizing the mTOR inhibitor-based protocol, which includes dual targeting of the mTOR complexes (mTORCs), mTORC1 and mTORC2 (18,33), further studies should focus on combination strategies that interfere not only with the mTOR signaling cascade, but with different tumor targets.

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