High prevalence of mTOR complex activity can be targeted using Torin2 in papillary thyroid carcinoma

Maqbool Ahmed1,†, Azhar R.Hussain1,†, Prashant Bavi1, Saeeda O.Ahmed1, Saif S.AlSohbi2, Fouad Al-Dayel3, Shahab Uddin1,† and Khawla S.Al-Kuraya1,4,‡

1Human Cancer Genomic Research, 2Department of Endocrinology and 3Department of Pathology, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Saudi Arabia and 4Department of Life Science, College of Science and College of Medicine, Al-Faisal University, Riyadh 11533, Saudi Arabia

†To whom correspondence should be addressed. Human Cancer Genomic Research, King Faisal Specialist Hospital and Research Center, MBC#98-16, PO Box 3354, Riyadh 11211, Saudi Arabia. Tel: +966-1-205-5167; Fax: +966-1-205-5170; Email: kkuraya@kfshrc.edu.sa

The mammalian target of rapamycin (mTOR) signaling cascade is a key regulatory pathway controlling initiation of messenger RNA in mammalian cells. Although dysregulation of mTOR signaling has been reported earlier in cancers, there is paucity of data about mTOR expression in papillary thyroid carcinoma (PTC). Therefore, in this study, we investigated the presence of mTORC2 and mTORC1 complexes in a large cohort of >500 PTC samples. Our clinical data showed the presence of active mTORC1 and mTORC2 complexes in 81 and 39% of PTC samples, respectively. Interestingly, coexpression of mTORC1 and mTORC2 activity was seen in a 32.5% (164/504) of the PTC studied and this association was statistically significant (P = 0.0244). The mTOR signaling complex was also found to be associated with activated AKT and 4E-BP1. In vitro, using Torin2, a second-generation mTOR inhibitor or gene silencing of mTOR expression prevented mTORC1 and mTORC2 activity leading to inactivation of P70S6, 4E-BP1, AKT and Bad. Inhibition of mTOR activity led to downregulation of cyclin D1, a gene regulated by messenger RNA translation via phosphorylation of 4E-BP1. Torin2 treatment also inhibited cell viability and induced caspase-dependent apoptosis via activation of mitochondrial apoptotic pathway in PTC cells. Finally, Torin2 treatment induces anticancer effect on PTC xenograft tumor growth in nude mice via inhibition of mTORC1 and mTORC2 and its associated pathways. Our results suggest that coexpression of mTORC1 and mTORC2 is seen frequently in the clinical PTC samples and dual targeting of mTORC1 and mTORC2 activity may be an attractive therapeutic target for treatment of PTC.

Materials and methods

**Patient selection and tissue microarray construction**

Five hundred and thirty-six patients with PTC diagnosed between 1988 and 2004 were selected from King Faisal Specialist Hospital and Research Center. All PTC were analyzed in a tissue microarray (TMA) format. Clinical and histopathological data were available for all the patients. Long-term follow-up data were available for most of the patients. TAMs were constructed with 2-fold redundancy from formalin-fixed, paraffin-embedded PTC specimens as described previously (29). Tumor regions were mapped by a pathologist for coring. The TMA was constructed with 0.6 mm diameter cores spaced 0.8 mm apart using a modified tissue microarrayer (Beecher Instruments, Sun Prairie, WI). The TMA block was cut into 5.0 µm sections, adhered to the slide by an adhesive tape-transfer method (Instrumedics, Hackensack, NJ) and UV cross-linked. The Institutional Review Board of the King Faisal Specialist Hospital and Research Center approved the study under the Project RAC# 2060008 on PTC archival clinical samples.

**Immunohistochemistry**

Primary antibodies used, their dilutions and incidences are listed in Supplementary Table 1, available at Carcinogenesis Online.

Abbreviations: mTOR, mammalian target of rapamycin; PARP, poly (ADP ribose) polymerase; PBS, phosphate-buffered saline; PTC, papillary thyroid carcinoma; siRNA, small interfering RNA; TMA, tissue microarray.

†These authors contributed equally to this work.

© The Author 2014. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
retrieval, Dako Target Retrieval Solution pH 9.0 (Catalog number S2368) was used, and the slides were microwaved at 750 W for 5 min and then at 250 W for 20 min. The Dako EnVision Plus System kit was used as the secondary detection system with 3,3′-diaminobenzidine as chromogen. All slides were counterstained with hematoxylin, dehydrated, cleared and mounted. Negative controls included omission of the primary antibody. Only fresh cut slides were stained simultaneously to minimize the influence of slide aging and maximize reproducibility of the experiment. Each TMA spot was assigned an intensity score from 0 to 3 (0, 1, 2, and 3) and proportion of the tumor staining for that intensity was recorded as % increments from a range of 0–100 (P0, P1, P2, P3). A final H score (range 0–300) was obtained by adding the sum of scores obtained for each intensity and proportion of area stained (H score = I0 × P0 + I1 × P1 + I2 × P2 + I3 × P3).

Using H score and X-tile version 3.6.1.1 (30), scoring was done as described previously with a cutoff of 120, 130, 50 and 0 considered as high p-mTOR Ser2481, p-mTOR Ser2481, p-p70S6 and p-4E-BP1 expression, respectively. Other immunohistochemistry markers such as p-AKT Ser473 and PIK3CA (p-110x) were scored as described previously (21,31).

Statistical analysis

Contingency table analysis and chi-square tests were used to study the relationship between different variables. Survival curves were generated using the Kaplan–Meier method, with significance evaluated using the Mantel–Cox log-rank test. The limit of significance for all analyses was defined as a P-value of 0.05; two-sided tests were used in all calculations. The IMP 9.0 (SAS Institute, Cary, NC) software package was used for data analyses. For in vitro analysis, standard deviation and Student’s t-test have been used for statistical purposes.

Cell culture

PTC cell line BCPAP was purchased from DSMZ (Braunschweig, Germany), and TPC-1 was kindly provided by Dr Bryan McIver (Department of Endocrinology, Mayo Clinic, Rochester, MN) as a gift and cultured as described (32). Briefly, both cell lines were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in humidified atmosphere containing 5% CO2. All in vitro experiments were performed in media supplemented with 5% fetal bovine serum. Both the cell lines were fingerprinted in-house using short tandem repeat sets PCR and the results were confirmed with published data (33).

Reagents and antibodies

Torin2 was purchased from Toceis Bioscience (Ellisville, MO), zVAD-fmk was purchased from Calbiochem (San Diego, CA). Antibodies against p-mTOR (Ser2448), p-mTOR (Ser2481), p-p70S6 kinase (Ser389), p70S6 kinase, p-4E-BP1 (Thr37/40), p-AKT (Ser473), caspase-9, cytochrome c, poly (ADP ribose) polymerase (PARP), caspase-3 and cleaved caspase-3 were purchased from Cell Signaling Technologies (Beverly, MA). Bax clone 6A7 was purchased from Sigma (St Louis, MO). Survivin antibody was purchased from R&D. Beta-actin and XIAP antibodies were purchased from Abcam (Cambridge, UK). Annexin V was purchased from Molecular Probes (Eugene, OR).

Cell lysis and immunoblotting

Cells were treated with indicated doses of Torin2 as described in the legend and lysed as described previously (21). Immunoblotting was performed with different antibodies and visualized by an enhanced chemiluminescence (Amersham, Arlington Heights IL) system.

Cell growth studies by MTT assay

Cells (104) were incubated in triplicate in a 96 well plate in the presence or absence of indicated test doses of Torin2 in a final volume of 0.2 ml for 48 h. The ability of Torin2 to inhibit cell growth was determined by MTT cell viability assays, as described earlier (22). Replicates of six wells for each dosing included vehicle control were analyzed for each experiment.

Live Dead assay

To measure apoptosis, Live Dead assay (Invitrogen, Eugene, OR) was used as described by the manufacturer. Briefly, 1 × 106 cells were treated with indicated doses of Torin2 for 48 h. Following incubation, cells were resuspended in 1 ml phosphate-buffered saline (PBS) containing 50 μM calcein AM and 8 μM ethidium homodimer and cells were incubated in the dark for 20 min. Fifty microliters of suspension was transferred on slides and visualized under an Olympus fluorescent microscope using a long pass filter.

Cell cycle analysis, Annexin V staining and DNA laddering

PTC cell lines were treated with different concentrations of Torin2 as described in the legends. For cell cycle analysis, cells were washed once with PBS and resuspended in 500 μl hypotonic staining buffer and analyzed by flow cytometry as described previously (29). For detection of apoptosis, cells were harvested and percentage apoptosis was measured by flow cytometry after staining with fluorescein-conjugated Annexin V and propidium iodide (Molecular Probes).

Assay for cytochrome c release

Cytochrome c release from mitochondria was assayed as described earlier (21). Twenty micrograms of proteins from cytosolic and mitochondrial fraction of each samples were analyzed by immunoblotting using an anti-cytochrome c antibody.

Measurement of mitochondrial potential

Cells (1 × 106) were treated with indicated doses of Torin2 for 48 h. Cells were washed twice with PBS, suspended in mitochondrial incubation buffer (Alexis Corp., Farmingdale, NY). JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was added to a final concentration of 10 μM and incubated at 37°C in dark for 30 min. Cells were washed twice with PBS and resuspended in 500 μl of mitochondrial incubation buffer and mitochondrial membrane potential (percentage of green and red aggregate) was measured by flow cytometry as described earlier (21).

Gene silencing using small interfering RNA

mTOR small interfering RNA (siRNA) and scrambled control siRNA were purchased from Cell Signaling Technologies. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h following which the lipid and siRNA complex was removed and fresh growth medium was added. Cells were lysed 48 h after transfection and specific protein levels were determined by western blot analysis with specific antibodies.

Animals and xenograft study

Six-week-old nude mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a pathogen-free animal facility for at least 1 week before use. All animal studies were done in accordance with institutional guidelines. For the xenograft study, mice were inoculated subcutaneously into the right abdominal quadrant with 10 million TPC-1 cells in 200 μl PBS. After 1 week, mice were randomly assigned into three groups (n = 5): the first group received 10% dimethyl sulfoxide, and two groups received 2 and 10 mg/kg body weight intraperitoneally. Mice were injected these drugs twice weekly. The body weight and tumor volume of each mouse were monitored weekly. The tumor volume was measured as previously (29). After 4 weeks of treatment, mice were killed and individual tumors were weighed, and then snap frozen in liquid nitrogen for storage.

Results

Expression/activation of mTORC1 and mTORC2 in PTC samples

We first sought to determine the expression of mTORC1 and mTORC2 using a TMA cohort of 536 PTC samples. Using immunohistochemistry, we initially examined the phosphorylation of mTOR on Ser2448, a marker of mTORC1 activity (34), as well as the phosphorylation of mTOR on Ser2481, a marker for mTORC2 activity (35). Constitutive phosphorylation of mTOR at Ser2448 and Ser2481 was observed in 81 and 39% of PTC samples, respectively.

Correlation of mTOR pathway with various clinicopathological features and molecular markers in PTC

Activation of both mTOR proteins was significantly more common in younger age group as shown in Table I and Supplementary Table 2, available at Carcinogenesis Online. As expected, p-mTORC1 expression was significantly associated with activated 4E-BP1 expression (P = 0.0290) and p-mTORC2 with p-AKT (P = 0.0006) and PIK-3CA (P = 0.0235) (Figure 1); however, p-MTORC2 expression was also found to be associated with activated 4E-BP1 (P = 0.0002) (Supplementary Table 2, available at Carcinogenesis Online). Similarly, p-p70S6 Thr37/40 expression in PTC significantly correlated with p-AKT (P = 0.0021) and PIK3CA 110 subunit protein (P = 0.0116) (data not shown).

Interestingly, coexpression of mTORC2 and mTORC1 activity was seen in a 32.5% (164/504) of the PTC studied and this association was statistically significant (P = 0.0244). Although p-mTORC1 expression showed a significant association with early stage (P = 0.0286), no correlation was observed with gender, histology subtype and extra thyroidal extension (Table I). Expression of both mTOR proteins did not show any association with disease-free survival.

Taking into consideration, these findings as well as previously reported presence of active mTORC1 and mTORC2 complexes in
various cancers (36), we performed functional assays on PTC cell lines using Torin2, a second-generation ATP-competitive inhibitor that is potent and selective for mTOR (37) to determine whether inhibition of mTOR pathways can induced growth inhibition and apoptotic cell death in vitro and in vivo.

**Constitutive expression of mTORC1 and mTORC2 complexes and its associated pathways in PTC cell lines**

High levels of phosphorylated (activated) mTOR have been reported in a variety of solid tumors (38,39). mTOR has also been shown to regulate many molecules that are involved in protein translational pathways such as activation of p70S6 kinase and 4EBP in many cancer cells (14,15). In the next series of experiments, we sought to determine whether functional mTORC1 and mTORC2 are present in PTC cell lines and their inhibition causes inactivation of its associated signaling partners. As shown in Figure 2A, both PTC cell lines showed constitutively activated mTOR at Ser2448 (mTORC1) and treatment of PTC cells with Torin2 blocked mTORC1 activity in a dose-dependent manner. Torin2 treatment also dephosphorylated p70S6 and 4E-BP1 in PTC cells. It has been shown that mTORC1 regulates expression of cyclin D1 via phosphorylation of 4E-BP1 (40). Therefore, we sought to determine the expression of cyclin D1 following treatment with Torin2. As shown in Figure 2A, expression of cyclin D1 was downregulated in both cell lines following Torin2 treatment suggesting that inactivation of 4E-BP1 also controls expression of cyclin D1 in PTC cells (40). In addition to activation of mTORC1, Torin2 treatment of PTC cell lines also dephosphorylated mTOR at Ser2481 that is the marker of mTORC2 activity (Figure 2B). As AKT activity has been shown to be controlled by mTORC2 activation (19,20), we also directly examined the activation of AKT and its downstream target, Bad, in PTC cells following treatment with Torin2. As shown in Figure 2B, there was inactivation of AKT and Bad in PTC cells treated with Torin2. In order to confirm the specificity of Torin2 on activation of mTORC1 and mTORC2 and their downstream targets, we also performed transfection studies with siRNA specifically targeted against mTOR to determine the status of these proteins in PTC cells. BCPAP and TPC-1 cells were transfected with mTOR-specific siRNA and like Torin2, the siRNA-targeting mTOR inactivated p70S6, 4E-BP1, AKT and downregulated expression of cyclin D1 (Figure 2C). These data suggest that mTOR regulates the activity of p70S6 leading to inactivation of 4E-BP1 and downregulation of cyclin D1 on one hand and inactivates AKT and its downstream target, Bad, that play a critical role in cell growth and survival.

**mTOR kinase inhibition causes inhibition of cell viability and induces apoptosis in PTC cell lines**

We next sought to determine whether treatment of PTC cell lines with Torin2 caused inhibition of cell viability. PTC cell lines were treated with various doses ranging from 50 to 800 nM Torin2 for 48 h and cell proliferation was determined by MTT assays. Figure 3A shows that as the dose of Torin2 increased from 50 to 800 nm, cell growth inhibition significantly increased in a dose-dependent manner in both the cell lines. IC$_{50}$ was calculated using CalcuSyn software using the above-mentioned doses. As shown in Supplementary Figure 1, available at *Carcinogenesis* Online, the IC$_{50}$ for BCPAP and TPC-1 cell lines were 347.6 and 380.5 nM.
mTOR signaling in PTC

Inhibition of mTOR activity in PTC cells induces apoptosis via mitochondrial and caspase-mediated pathway

The event required for the activation of the mitochondrial apoptotic pathway is inactivation of Bad protein that translocates to mitochondrial and results in conformational changes of proapoptotic protein Bax at the amino terminus, exposing the epitope required for homodimerization (42). Inactivation of Bad secondary to inhibition of mTOR led to activation of Bax protein in BCPAP cell line that was detected using Bax 6A7 antibody that only recognizes N-terminal Bax epitope that is exposed only upon Bax activation (43). This activation was detected within 4 h after Torin2 treatment and continued up to 16 h (Figure 4A). There was also downregulation of Bcl-2 and Bcl-XL in PTC cells treated with Torin2 (data not shown). We then tested the effect of Torin2 on the mitochondrial membrane potential and release of cytochrome c in these cells. Cells were treated with Torin2 for 48 h and labeled with JC1 dye, and mitochondrial membrane potential was measured by flow cytometry. As shown in Figure 4B, treatment of cells with Torin2 resulted in loss of mitochondrial membrane potential in PTC cells as measured by JC1-stained green fluorescence depicting apoptotic cells. We next sought to determine whether cytochrome c was released from PTC cells after treatment with 200 nm and 400 nM Torin2. Expression of cytochrome c increased in cytosolic fractions treated with Torin2 compared with untreated cells in PTC cells (Figure 4C). Finally, we also examined the expression of inhibitors of apoptosis proteins following treatment of PTC cells with Torin2. As shown in Figure 4D, there was downregulation of XIAP, cIAP1 and Survivin in PTC cells treated with Torin2. These data suggest that Torin2 treatment of PTC cell lines leads to activation of the mitochondrial apoptotic pathway.

Once cytochrome c is released, it causes activation and cleavage of caspases that leads to apoptosis in cells (21). We therefore sought to determine whether Torin2-induced release of cytochrome c is capable of activation of caspase-9, caspase-3 and PARP in PTC cells. As shown in Supplementary Figure 2A, available at Carcinogenesis Online, there was activation and cleavage of caspases-9, -3 and PARP following treatment with 200 and 400 nM Torin2 for 48 h in PTC cells. In order to confirm whether Torin2 treatment actually induces apoptosis via activation of caspases, we pretreated PTC cells with 80 µM zVAD-fmk, a universal inhibitor of caspases for 2 h followed by treatment with 400 nM for 48 h. As shown in Supplementary Figure 2B, available at Carcinogenesis Online, there was activation and cleavage of caspase-3 and PARP in PTC cells treated with Torin2; however, pretreatment of PTC cells with zVAD-fmk inhibited Torin2-induced activation and cleavage of caspase-3 and PARP. We also found that pretreatment of PTC cells with zVAD-fmk inhibited Torin2-induced apoptosis (Supplementary Figure 2C and D, available at Carcinogenesis Online) clearly indicating that Torin2-induced apoptosis is caspase dependent.

Torin2-mediated inhibition of mTOR activation suppresses PTC tumorigenesis in nude mice xenograft

Our in vitro data showed that Torin2 treatment had the ability to prevent phosphorylation of p70S6, 4E-BP1, AKT and downregulated fractions. There was an increase in G1 fraction of cells following treatment with Torin2 for 24 h (data not shown) followed by increase in sub-G1 (Apo) fraction of cells from 8.47% in untreated control sample to 33.54% after 200 nM of Torin2 treatment and 41.16% after 400 nM of Torin2 treatment for 48 h in BCPAP cell line. Similar results were obtained in TPC-1 cell line where the sub-G1 population increased from 12.44% in untreated control sample to 31.50% after 200 nM of Torin2 treatment and 44.08% after 400 nM of Torin2 treatment for 48 h suggesting that these cells were actually dying of apoptosis. Finally, we also stained the cells with fluorescein-conjugated Annexin V/propidium iodide and determined apoptosis by flow cytometry. As shown in Figure 2D, there was increased apoptosis in cells treated with 200 and 400 nM Torin2 in both the cell lines tested. These data are in concordance with another report that showed that combined inactivation of mTORC1 and mTORC2 leads to apoptosis in thyroid cancer (41).

In this study, we sought to determine the contribution of mTOR signaling in thyroid cancer including papillary thyroid cancer (PTC). We sought to determine the contribution of mTOR signaling in PTC. mTOR signaling is activated in PTC (6-10). However, whether this activation is a cause or consequence of PTC development has not been defined. Among the factors that can influence mTOR signaling, growth factors, oncogenic factors, and cytokines are most often considered. However, we recently found that mTORC2 is activated in thyroid cancer (11). In this study, we focused on the activation of mTOR in PTC and show its contribution to PTC tumorigenesis.

Fig. 1. TMA-based immunohistochemical analysis of p-mTOR (Ser2481), p-4E-BP1, p-p70S6 and p-AKT (Ser473) in PTC patients. PTC array spots showing high expression of p-mTOR (Ser2481) (A), p-4E-BP1 (C), p-p70S6 (E) and p-AKT (Ser473) (G). In contrast, another set of PTC tissue array spots showing low of p-mTOR (Ser2481) (B), p-4E-BP1 (D) p-p70S6 (F) and p-AKT (Ser473) (H). Magnification (×20) with the inset showing a ×100 magnified view of the same.

respectively. Therefore, we used 200 and 400 nM doses of Torin2 for treatment of PTC cell lines. We next wanted to determine whether PTC cells treated with Torin2 underwent cell growth inhibition or cell death. We therefore treated BCPAP and TPC-1 cells with 200 and 400 nM Torin2 for 48 h and then stained the cells with 50 µM calcein AM and 8 µM ethidium homodimer to detect cell death. We therefore treated BCPAP and TPC-1 cells with 200 and 400 nM Torin2 for 48 h and then stained the cells with 50 µM calcein AM and 8 µM ethidium homodimer to detect cell death. As shown in Figure 3B, there was increase in red cells following treatment with 200 and 400 nM Torin2 suggesting that PTC cells treated with Torin2 were dying rather than undergoing cell cycle arrest. To confirm whether these cells were dying of apoptosis, we stained the cells with propidium iodide following treatment under similar conditions and examined the cells by flow cytometry for different cell cycle
expression of cyclin D1 in PTC cells. In addition, our *in vitro* data also showed that treatment of PTC cells with Torin2 induced caspase-dependent apoptosis via the mitochondrial apoptotic pathway. These data imply that targeting the mTOR pathway in PTC cells may be beneficial as a therapeutic modality. Therefore, we expanded our study to nude mice xenograft of PTC cells followed by treatment with Torin2 to determine the effect of mTOR inhibition on tumor growth and volume. Nude mice were inoculated subcutaneously in the right abdominal quadrant with 10 million TPC-1 cells. Mice were then treated with either two doses of Torin2 in treatment group (2 and 10 mg/kg/dose), or vehicle dimethyl sulfoxide-treated control groups (*n* = 5). After 4 weeks of treatment, mice were killed and tumors were collected. As shown in Figure 5A, Torin2 treatment causes a time-dependent regression of TPC-1 xenograft tumors in mice compared with vehicle-treated mice that reached significance (*P* < 0.0500) at the end of fourth week of treatment at the dose of 10 mg/kg/dose Torin2. A significant reduction in tumor weight (Figure 5B) was also observed at this treatment condition. Additionally, images of tumor after necropsy showed that Torin2 treatment resulted in shrinkage of tumor size with minimal necrosis (Figure 5C). Finally, proteins extracted from tumors post-necropsy were immunoblotted with antibodies against mTOR, p-4E-BP1, cyclin D1, Bcl-Xl and caspase-3. As shown in Figure 5D, decrease in tumor volume and size was due to inactivation of mTOR, AKT, 4E-BP1 and downregulation of expression of cyclin D1 and Bcl-Xl. Reduction of tumor size also correlated with activation of caspase-3. These data suggest that Torin2 is also effective against PTC xenograft in shrinking the tumor size via inhibition of mTOR-dependent pathways and induction of caspase-mediated apoptosis.

**Discussion**

The mTOR protein is a serine/threonine kinase that forms two functionally unique complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 function is mediated...
mTOR signaling in PTC

through phosphorylation of S6K1 and 4E-BP1, which stimulate messenger RNA translation and growth (14,15), whereas mTORC2 has been shown to phosphorylate a very important member of the PI3-kinase pathway, AKT (19,20). Our clinical data showed that both the complexes of mTOR, mTORC1 and mTORC2, were activated in 81 and 39% of PTC samples, respectively. This is in agreement to an earlier pilot study that has investigated the mTOR pathway in a small cohort of 30 thyroid cancers and reported mTORC1 expression in 100% of the PTC histology subtype samples studied (44). In addition, we also found that mTORC1 was significantly associated with mTORC2 and 4E-BP1 and mTORC2 was associated with activated AKT. Our data also found a significant association between high expression of P70S6 and BRAF-mutated PTC (P = 0.0080) compared with PTC with wild-type BRAF, which is in concordance with a previous study reported by Faustino et al. (45). However, unlike Faustino et al. study, we did not find any association with activated mTOR (2448) in BRAF mutated PTC samples (unpublished data).

The United States Food and Drug Administration approved two mTOR inhibitors, temsirolimus and everolimus, for the treatment of renal cell carcinoma, which have shown promising results (19). However, recent studies have shown that these results are short lasting and do not cure the disease (26,27,46). One major problem with these FDA-approved drugs is that their target was mTORC1 only and not mTORC2 and therefore resistance quickly developed against these agents. The presence of coexpression both the complexes of mTOR in our cohort of clinical samples clearly indicates the importance of targeting both the mTOR complexes simultaneously to avoid resistance to therapy. Therefore, in this study, we have examined in detail the role of Torin2, a second-generation selective inhibitor of mTOR complexes that has been shown to be effective against activation of mTORC1 and mTORC2 on PTC cell lines and PTC xenograft model. We found that Torin2 inactivated mTORC1 and mTORC2 at dose of 400 nM in both the cell lines used. Not only did the activity of mTORC1 and mTORC2 decreased, the downstream targets of mTOR complexes, P70S6, 4E-BP1 and AKT, were also inactivated following Torin2 treatment. 4E-BP1 and

Fig. 3. Torin2 treatment inhibits cell viability and induces apoptosis in PTC cells. (A) Torin2 treatment inhibits cell viability in a dose-dependent manner in PTC cells. BCPAP and TPC-1 cells were incubated with 0, 50, 100, 200, 400 and 800 nM Torin2 for 48 h. Cell proliferation assays were performed using MTT as described in Materials and methods. The graph displays the mean ± SD of three independent experiments with replicates of six wells for all the doses and vehicle control for each experiment *P < 0.01, statistically significant (Student’s t-test). (B) PTC cells (1 x 10^6) were treated with 200 and 400 nM Torin2 for 48 h. Following incubation, cells were resuspended in 1 mL PBS containing 50 μM calcein AM and 8 μM ethidium homodimer and cells were incubated in the dark for 20 min. Fifty microliters of suspension was transferred on slides and visualized under an Olympus fluorescent microscope using a long pass filter and cell death was measured as described in Materials and methods. (C) Cell cycle analysis of thyroid cell lines following Torin2 treatment for 48 h. BCPAP and TPC-1 cells were treated with 200 and 400 nM Torin2 for 48 h. Thereafter, the cells were washed, fixed and stained with propidium iodide and analyzed for cell cycle fractions by flow cytometry as described in Materials and methods. Apo fractions also known as sub-G1 population denote cells undergoing apoptosis. The bar graph is an average of three independent experiments. (D) Torin2-induced apoptosis detected by Annexin V/propidium iodide dual staining. BCPAP and TPC-1 cells were treated with 200 and 400 nM Torin2 for 48 h and cells were subsequently stained with fluorescein-conjugated Annexin V and propidium iodide and apoptotic cells were analyzed by flow cytometry. The bar graph is an average of four independent experiments performed on different days.
M.Ahmed et al.

P70S6, once phosphorylated, lead to formation of the eIF4F translation initiation complex (47) that is necessary for the maintenance of malignant phenotype of cancer cells (48). In addition, 4E-BP1 overexpression is also associated with a poor survival in melanoma (49) and controls the expression of cell cycle regulator, cyclin D1 (40). Therefore, by inactivating 4E-BP1, the eIF4F complex fails to develop and cells are allowed to go into a cell cycle arrest due to downregulation of cyclin D1.

Interestingly, Torin2 treatment of PTC cells also inactivated an important survival molecule, AKT, that has been shown to play a major role in cell survival, growth of malignant cells (50) and has been found to promote a poor survival in various cancers (21,23). By inactivating AKT, the downstream target of AKT Bad, a proapoptotic member of the Bcl-2 family, is also dephosphorylated. Native Bad interacts with antiapoptotic proteins, Bcl-2 and Bcl-Xl negating their effect thereby allowing Bax to exert its proapoptotic action at the level of mitochondria (42). Our data indicate that Torin2 treatment causes activation of Bax protein, probably leading to conformational changes of the protein thereby allowing the membrane potential of mitochondria to change leading to release of cytochrome c into the cytosol.

For efficient apoptosis to occur, the mitochondrial apoptotic pathway needs to be activated for the caspases to exert their proapoptotic effects. However, inhibition of the inhibitors of apoptosis is also necessary for this pathway to be completely active. Torin2 treatment downregulates the expression of key members of the inhibitor of apoptosis family, XIAP, cIAP1 and Survivin, indicating the effectiveness of this inhibitor in successfully activating the apoptotic machinery. Caspases are the final effectors of both extrinsic and intrinsic apoptotic pathway and are present as inactive zymogens that need to be activated and cleaved to exert their apoptotic function (51). In our study, we found that Torin2 treatment activates and cleaves caspases-9 and caspase-3 via activation of the mitochondrial apoptotic pathway leading to efficient apoptosis in PTC cells thereby highlighting the importance of Torin2 in the management of PTC.

Our in vitro data were validated by in vivo studies where PTC xenografts treated with Torin2 showed regression of tumor size and volume secondary to inactivation of mTORC1 and mTORC2. In addition, we also detected inactivation of downstream targets of mTOR complexes, 4E-BP1 and AKT, as well as downregulation of indirect targets such as cyclin D1, Bcl-Xl and caspase-3. These findings clearly indicate the effectiveness of Torin2 in successfully inactivating the mTOR complexes leading to regression of PTC xenografts in vivo.

In summary, our clinical data provide ample evidence that mTOR1 and mTOR2 complexes are active in a significant number of PTC samples. Our in vitro data showed that inhibition of mTORC1 and mTORC2 activity by Torin2, a second-generation mTOR inhibitor, can induce mitochondrial-mediated apoptosis in PTC cells. Finally, Torin2-mediated anticancer effects were seen in PTC generated xenografts in mice via inhibition of mTORC1 and mTORC2 activity. These

Fig. 4. Torin2 treatment activates mitochondrial apoptotic pathway in PTC cells. (A) TPC-1 cells were treated with 400nM Torin2 for indicated time points. Following treatment, cells were lysed and immunoprecipitated with Bax monoclonal antibody (clone 6A7) and probed with polyclonal Bax antibody (Sc493). (B) TPC-1 and BCPAP cell lines were treated with 200 and 400nM Torin2 for 48h. Following incubation, cells were washed twice with PBS and stained with 10µM JC1 dye in 500µl mitochondrial incubation buffer and incubated at 37°C in the dark for 30min. After the incubation, cells were washed in PBS and resuspended in mitochondrial incubation buffer and change in mitochondrial membrane potential was detected by flow cytometry. (C) Torin2 treatment causes release of cytochrome c from mitochondria into cytosol. TPC-1 and BCPAP cells were treated with 200 and 400nM Torin2 for 48h. Mitochondrial-free cytosolic fractions were isolated and immunoblotted with antibodies against cytochrome c and beta-actin. (D) Torin2 treatment downregulates expression of inhibitors of apoptosis in PTC cells. TPC-1 and BCPAP cells were treated with 200 and 400nM Torin2 for 48h. Following treatment, cells were lysed and immunoblotted with antibodies against XIAP, cIAP1, Survivin and beta-actin.
data provide evidence for the development of clinical-translational efforts using mTORC1/mTORC2 inhibitors such as Torin2 for therapeutic intervention of PTC.

Funding

King Abdulaziz City for Science and Technology (KACST); National Science Technology and Innovation Plan (NSTIP) from the NSTIP project: NSTIP 11-BIO2219-20.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Fig. 5. Anticancer effect of Torin2 on TPC-1 xenograft in vivo. Nude mice at 6 weeks of age were injected with 10 million TPC-1 cells. After 1 week, the animals were divided into three groups, group 1 received no treatment, the second group received Torin2 (2 mg/kg) and the third group received Torin2 (10 mg/kg) injected intraperitoneally twice weekly. (A) Effect of Torin2 on TPC-1 xenograft. The volume of each tumor was measured every week. The average (n = 5) tumor volumes in vehicle-treated mice (blue line) and mice treated with indicated two doses of Torin2 (2 mg/kg; red line and 10 mg/kg; green line) were plotted. (B) After 4 weeks of treatment, mice were killed and tumor weights were measured. (C) Representative tumor images of vehicle-treated mice and mice treated with two doses of Torin2 after necropsy. (D) Whole-cell homogenates from mice treated with vehicle, and two doses of Torin2 were immunoblotted with antibodies against mTOR phosphorylated at Ser2448 and 2481, p4E-BP1, pAKT, cyclin D1, Bcl-Xl and caspase-3. Beta-actin was used as a control for equal loading.

Acknowledgements

We would like to acknowledge the efforts of Sally Al Abdulmohsen, Saravanan Thangavel, Sarita Prabhakaran and Valorie Balde for technical assistance and Zeeshan Quadri for statistical analysis.

Conflict of Interest Statement: None declared.

References

M.Ahmed et al.


Received July 2, 2013; revised January 30, 2014; accepted February 22, 2014