

Autophagy Induction with RAD001 Enhances Chemosensitivity and Radiosensitivity through Met Inhibition in Papillary Thyroid Cancer

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Abstract

Although autophagy is generally considered a prosurvival mechanism that preserves viability, there is evidence that it could drive an alternative programmed cell death pathway in cells with defects in apoptosis. Because the inhibition of autophagic activity promotes resistance to both chemotherapy and external beam radiation in papillary thyroid cancer (PTC), we determined if RAD001, a potent activator of autophagy, improves the efficacy of either therapy. We found that RAD001 increased the expression level of light chain 3-II, a marker for autophagy, as well as autophagosome formation in cell lines and in human PTC *ex vivo*. RAD001 sensitized PTC to doxorubicin and external beam radiation in a synergistic fashion, suggesting that combination therapy could improve therapeutic response at less toxic concentrations. The effects of RAD001 were abrogated by RNAi knockdown of the autophagy-related gene 5, suggesting that RAD001 acts, in part, by enhancing autophagy. Because the synergistic activity of RAD001 with doxorubicin and external radiation suggests distinct and complementary mechanisms of action, we characterized how autophagy modulates signaling pathways in PTC. To do so, we performed kinome profiling and discovered that autophagic activation resulted in Src phosphorylation and Met dephosphorylation. Src inhibition did not reverse the effects of RAD001, whereas Met inhibition reversed the effects of autophagy blockade on chemosensitivity. These results suggest that the anticancer effects of autophagic activation are mediated largely through Met. We conclude that RAD001 induces autophagy, which enhances the therapeutic response to cytotoxic chemotherapy and external beam radiation in PTC. *Mol Cancer Res*; 8(9): 1217–26. ©2010 AACR.

Introduction

Thyroid cancer is the most common endocrine malignancy and is responsible for the majority of deaths from endocrine cancer (1). Papillary thyroid cancer (PTC) accounts for approximately 80% of thyroid cancers. Although PTC is generally associated with favorable outcomes after surgical resection and radioactive iodine ablation, it is the most common histologic subtype in patients with locally advanced disease (2, 3). Doxorubicin is the only Food and Drug Administration–approved chemotherapy agent for

advanced PTC that does not trap radioactive iodine; however, intrinsic resistance to doxorubicin results in limited efficacy and it is rarely used for these patients (4).

RAD001 (everolimus, Afinitor), a rapamycin analogue, is an oral mammalian target of rapamycin (mTOR) inhibitor. mTOR is an intracellular protein that acts as a central regulator of multiple signaling pathways induced by insulin-like growth factor, epidermal growth factor, platelet-derived growth factor, vascular endothelial growth factor, and amino acids that mediate abnormal growth, proliferation, and angiogenesis (5). RAD001 was recently approved for the treatment of papillary renal carcinoma (6), a malignancy driven by an activating Met mutation (7). Although we are now testing the therapeutic efficacy of RAD001 in advanced PTC, its mechanism of action in this context is unknown.

RAD001 stimulates autophagy (8, 9), a highly conserved process that entails the degradation of intracellular components through the lysosomal machinery to regenerate metabolites for energy and growth (10, 11). In many contexts, autophagy promotes cell survival under stressful conditions such as nutrient and growth factor deprivation (12, 13). However, excess or prolonged autophagy could promote an alternative mechanism that leads to programmed cell death, which is particularly relevant in cancers that are defective in apoptosis (14, 15). Prior work has shown that

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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doi: 10.1158/1541-7786.MCR-10-0162

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PTC is intrinsically resistant to cytotoxic chemotherapies because of defects in apoptosis (16).

We previously observed that inhibition of autophagy decreased PTC sensitivity to doxorubicin and external beam radiation (17). This result led us to hypothesize that RAD001 could enhance cancer cell response to these therapies through the activation of autophagy. In the current study, we found that the combined effects of RAD001 and doxorubicin together exceeded the sum of their individual effects. To understand the complementary activity of RAD001, we used kinome-wide analysis to determine how RAD001 affects signaling pathways. We found that RAD001 and autophagic activation inhibit Met phosphorylation, an event that imparts the effects of RAD001 on chemosensitivity and radiosensitivity.

Materials and Methods

Materials

Mouse anti-human microtubule-associated protein 1 light chain 3 (LC3) antibody was purchased from MBL International Cooperation. Mouse anti-actin antibody, pan Ab-5 was purchased from Neomarker. Rabbit anti-human Atg-5, Src, phosphorylated Src Tyr⁴¹⁶, Met, phosphorylated Met Tyr¹²³⁴, cleaved caspase-3 Asp¹⁷⁵, phosphorylated histone H3 Ser¹⁰, antibodies were purchased from Cell Signaling. Mouse anti-human Ki-67 antibody was obtained from BD Transduction Laboratories. Rabbit anti-human p62 antibody was from Abgent. Secondary horseradish peroxidase-conjugated mouse and rabbit antibodies were obtained from Vector Laboratories. 3-Methyladenine (3-MA), a class III phosphoinositide-3-kinase inhibitor widely used as autophagy inhibitor reported to act specifically on the autophagic/lysosomal pathway of degradation (13), was purchased from Calbiochem. Doxorubicin was obtained from Fluka. RAD001, an mTOR inhibitor that induces autophagy (18), was obtained from Novartis Pharmaceutical. PHA665752, a selective small molecule inhibitor of Met (19), was obtained from Pfizer, Inc. Dasatinib obtained from Broad Institute was synthesized as previously described (20).

Cell culture and RNAi transfections

The human PTC cell lines TPC-1 and 8505-C were cultured as described previously (21). TPC-1 cells were obtained from Dr. Orlo H. Clark (University of California at San Francisco, San Francisco, CA) and 8505-C cells were obtained from Dr. Sareh Parangi (Massachusetts General Hospital, Boston, MA) within the last 5 years and tested within the last 6 months by morphology check by microscope and growth curve analysis according to the Cell Line Verification Test Recommendations (American Type Culture Collection, technical bulletin no. 8, 2008). TPC-1 and 8505-C cells were used throughout passages 12 to 18 and 28 to 35 in this study. Cells were subcultured at 80% confluency by trypsinization [in a 0.5% (v/v) trypsin solution supplemented with 0.2% (v/v) EDTA]. Cells were transfected with scrambled short interfering RNA

(siRNA) or Atg-5 prevalidated siRNA duplex (the target sequences were no. 1, AACCTTTGGCCTAAGAAGAAA; no. 2, AAGACTTACCGGACCACTGAA; Qiagen) using LipofectAMINE 2000 reagent (Invitrogen), according to the instructions of the manufacturer. All transfections were done in six-well plates and cells were processed 48 hours after transfection.

Autophagy assay

Autophagosome formation was also confirmed by the appearance of punctate fluorescence from exogenous GFP-fused LC3, a specific lysosomal/autophagic biochemical vacuole marker (10). Cells were transfected with EGFP-LC3 plasmid (Addgene) using LipofectAMINE 2000 reagent. Forty-eight hours after transfection, cells were treated with DMSO and RAD001 (20 nmol/L) for 24 hours. The fluorescence of EGFP-LC3 was observed under a fluorescence microscope.

Human thyroid tumor tissue specimens

Human PTC tissues and normal adjacent thyroid tissue were obtained from thyroidectomy specimens resected from surgery patients. The specimens were immediately frozen in liquid nitrogen upon removal from the patients and the samples were stored frozen until the time of protein extraction. Tissue lysates were prepared using radioimmunoprecipitation assay buffer containing protease inhibitor cocktail as described previously (22). The Institutional Review Board of Brigham & Women's Hospital in Boston, MA approved this protocol (protocol number: 1999-P-001331/5).

Western blotting

Cell lysates were prepared and analyzed by Western blotting as described previously (21).

Transmission electron microscopy and morphometric analysis

Cultured PTC tissue specimens were washed with serum-free DMEM (Invitrogen) followed by a brief wash in 1 mol/L of cacodylate buffer and then fixed with 2.5% glutaraldehyde with 0.1 mol/L of sodium cacodylate buffer for 1 hour. Fixed cells and PTC tissue specimens were processed at the Harvard Medical School Transmission Electron Microscopy core facility by using protocols described previously (23) using a JEOL-60kV microscope (JEOL). Morphometric analysis was done as previously described (24). For each control or treatment group, 100 cells were randomly chosen from the fields for quantification of autophagic features. Closed vacuoles with double membranes containing cytosol and/or organelles were considered autophagic vacuoles (11). Cells with ≥ 10 autophagic vacuoles were determined as autophagic cells.

Ex vivo PTC tumor specimen culture and immunohistochemistry

A piece of each human tissue specimen was excised using sterile techniques. Vital specimens (0.5 mm³) were cultured in Millicell cell culture inserts (Millipore) containing 600 μ L

of DMEM/F12 medium with 15 mmol/L of HEPES (Sigma-Aldrich) supplemented with GlutaMAX I (Invitrogen) at 37°C and 5% CO₂ for 8 hours in the absence or presence of doxorubicin (1 μmol/L). Immunohistochemical analysis of Ki-67, p62, and cleaved caspase-3 was done as described previously (25). p62, a ubiquitin-binding scaffold protein, is degraded by autophagy and is a marker of autophagy (26).

Clonogenic survival assay and IC₅₀ determination

For cell survival determination, standard clonogenic survival assays were done as previously described (27), with minor modifications. Eight hundred cells were plated and then coincubated with doxorubicin (final concentrations of 200, 100, 50, 20, 10, 5, or 1 nmol/L) or medium without doxorubicin and cultured in a humidified CO₂ incubator at 37°C for 10 to 14 days. Cells were fixed for 15 minutes with a 3:1 methanol/acetic acid solution and stained for 15 minutes with 0.5% crystal violet (Sigma-Aldrich) in methanol. After staining, colony numbers were counted. IC₅₀ (a concentration of the compound causing 50% inhibition) values were determined. The mean ± SEM of triplicate samples was determined for each treatment. The data was analyzed by plotting the clonogenic survival rate (number of colonies in treated plates/number of colonies in controls) versus the doxorubicin concentration.

MTS cell proliferation assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Celltiter96 AQ, Promega) was used to assess RAD001 and doxorubicin effects on cell viability as previously described (21).

Radiosensitivity assay

For radiation experiments, cells were trypsinized, replated, and incubated for an additional 24 hours under standard conditions. Eight hundred cells were plated and then irradiated with doses ranging from 0 to 6 Gy (Gamma-cell 1000 apparatus, 200 kV, 15 mA, 1 Gy/min; Atomic Energy of Canada, Ottawa, Canada). Forty-eight hours after irradiation, the medium was replaced with fresh medium and then subjected to clonogenic survival assay. The mean ± SD of triplicate samples was determined for each treatment. The data was analyzed by plotting the log of the surviving fraction (number of colonies) versus radiointensity.

Kinome array analysis

We did a bead-based profiling of tyrosine kinase phosphorylation using individual Luminex xMAP microspheres (Luminex) in an immunosandwich assay, as previously described (28). In brief, an antibody-based

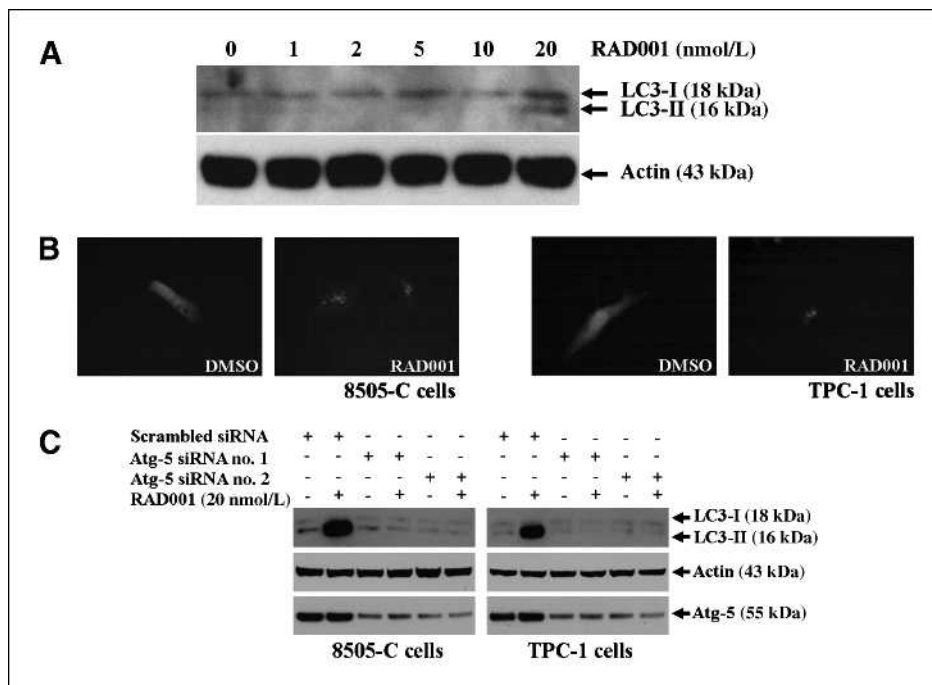


FIGURE 1. RAD001 induces LC3-II expression and punctate in 8505-C and TPC-1 cells. A, TPC-1 cells were treated with 0, 1, 2, 5, 10, and 20 nmol/L of RAD001 for 24 h. Cell lysates were prepared and LC3-I and LC3-II protein expression levels were monitored by Western blotting. Reprobing against actin was done to ensure equal protein loading. B, 8505-C or TPC-1 cells were transfected with EGFP-LC3 expression vector. Transfected cells were treated with DMSO or 20 nmol/L of RAD001 for 24 h as indicated. Treated cells were fixed and mounted, and the EGFP signals were further analyzed by fluorescence microscopy. Representative images from three independent experiments are shown. C, 8505-C and TPC-1 cells were transiently transfected with Atg-5 siRNA no. 1 and no. 2. Transfection with scrambled siRNA was used as a control. After transfection, cells with and without Atg-5 knockdown were exposed to DMSO or 20 nmol/L of RAD001 for 24 h. Cell lysates were prepared and LC3-I and LC3-II protein expression levels were monitored by Western blotting. Reprobing against Atg-5 was done to monitor Atg-5 knockdown efficiency. Reprobing against actin was done to ensure equal protein loading.

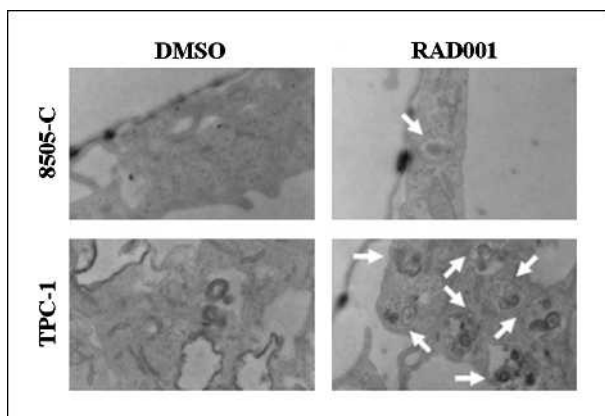


FIGURE 2. RAD001 stimulates autophagic vacuoles formation in 8505-C and TPC-1 cells. Representative electron microscopic images showing autophagic vacuoles with content (white arrows) following DMSO or RAD001 (20 nmol/L) treatment for 24 h in 8505-C and TPC-1 cells. Treated cells were subjected to transmission electron microscopy analysis (original magnification, $\times 15,000$).

kinase phosphorylation assay for a panel of 71 unique tyrosine kinases was used to comprehensively evaluate the effects of Atg-5 knockdown and RAD001 treatment. Scrambled or Atg-5 siRNA-transfected cells were washed in ice-cold PBS and harvested in $1\times$ cell lysis buffer (Cell Signaling) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich). The data was acquired with a Luminex FlexMAP 3D instrument (Luminex) according to the instructions of the manufacturer. Subtracting the sample and antibody background signals normalized raw values. Normalized values were considered positive if they were ≥ 10 . The preprocessed data were converted into .gct files and analyzed with GenePattern 3.0 (the Broad Institute, Cambridge, MA).

Statistical analysis

Differences between treatment groups were evaluated with a two-tailed independent Student's *t* test. Each assay was done in triplicate, and a value of $P < 0.05$ was considered statistically significant. We defined positive treatment as "synergistic" when agents had a combined effect on the inhibition of proliferation greater than the sum of their individual effects. To display such activity, we determined the minimum concentration of each agent necessary to achieve a significant effect (IC_{min}) and then determined the combined activity at the IC_{min} concentrations of both agents. The correlation between autophagic cell numbers in DMSO- and RAD001-treated cells was analyzed by χ^2 testing.

Results

RAD001 induces autophagy in PTC

To determine if RAD001 induces autophagy in PTC, we treated 8505-C and TPC-1 cells with RAD001 at doses previously shown to inhibit mTOR signaling in cell cultures (18). Then, we assayed the expression level of the LC3-II protein, which is generated when microtubule-associated

protein 1A/1B LC3 undergoes autosomal breakdown. Conjugation of ubiquitous cytosolic LC3-I to phosphatidylethanolamine produces the LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and is a reliable marker for autophagic activity. Immunoblotting revealed that 20 nmol/L of RAD001 was required to significantly increase the expression level of the LC3-II protein (Fig. 1A).

We next determined if the ectopic expression of LC3 affected the action of RAD001. PTC cells were transfected with a GFP-labeled LC3 plasmid; we then observed a significant increase in punctate fluorescence in RAD001-treated cells, which reflects the formation of autophagosomes (Fig. 1B). We also determined how siRNA knockdown of Atg-5, which is required for autophagy (29), affected RAD001 activity. Therefore, we used two distinct siRNAs that targeted nonoverlapping regions of the Atg-5 mRNA for this experiment. We found that Atg-5 depletion by the siRNAs, which are designated as nos. 1 and 2, abrogated the RAD001-mediated increase in LC3-II (Fig. 1C).

To further confirm the presence of autophagy, we did electron microscopy to visualize autophagosomes (Fig. 2). Morphometric analysis showed that RAD001 significantly increased the formation of autophagic vacuoles, which are characterized as double-membrane vacuoles containing cytoplasmic contents (24). This further shows that RAD001 induces autophagy in PTC (Table 1).

We next determined if RAD001 induces autophagic activity in human PTC specimens. To that end, we harvested thyroid specimens from patients undergoing resection of thyroid cancer and treated these with RAD001. Autophagy was evaluated by immunoblotting for the LC3-II protein and by electron microscopy. We found that RAD001 induced LC3-II protein expression in three consecutive tumors, demonstrating the ability of RAD001 to induce

Table 1. Normal and autophagic-positive 8505-C and TPC-1 cell numbers in response to DMSO and RAD001 (20 nmol/L) treatment were quantified according to standard morphometric analysis protocol

	DMSO	RAD001
8505-C cells		
Normal cells	97	16
Autophagic cells	3	84
Total cells	100	100
TPC-1 cells		
Normal cells	89	13
Autophagic cells	11	87
Total cells	100	100

NOTE: Autophagic cell numbers were significantly higher upon RAD001 compared with DMSO treatment (χ^2 test, $P < 0.001$).

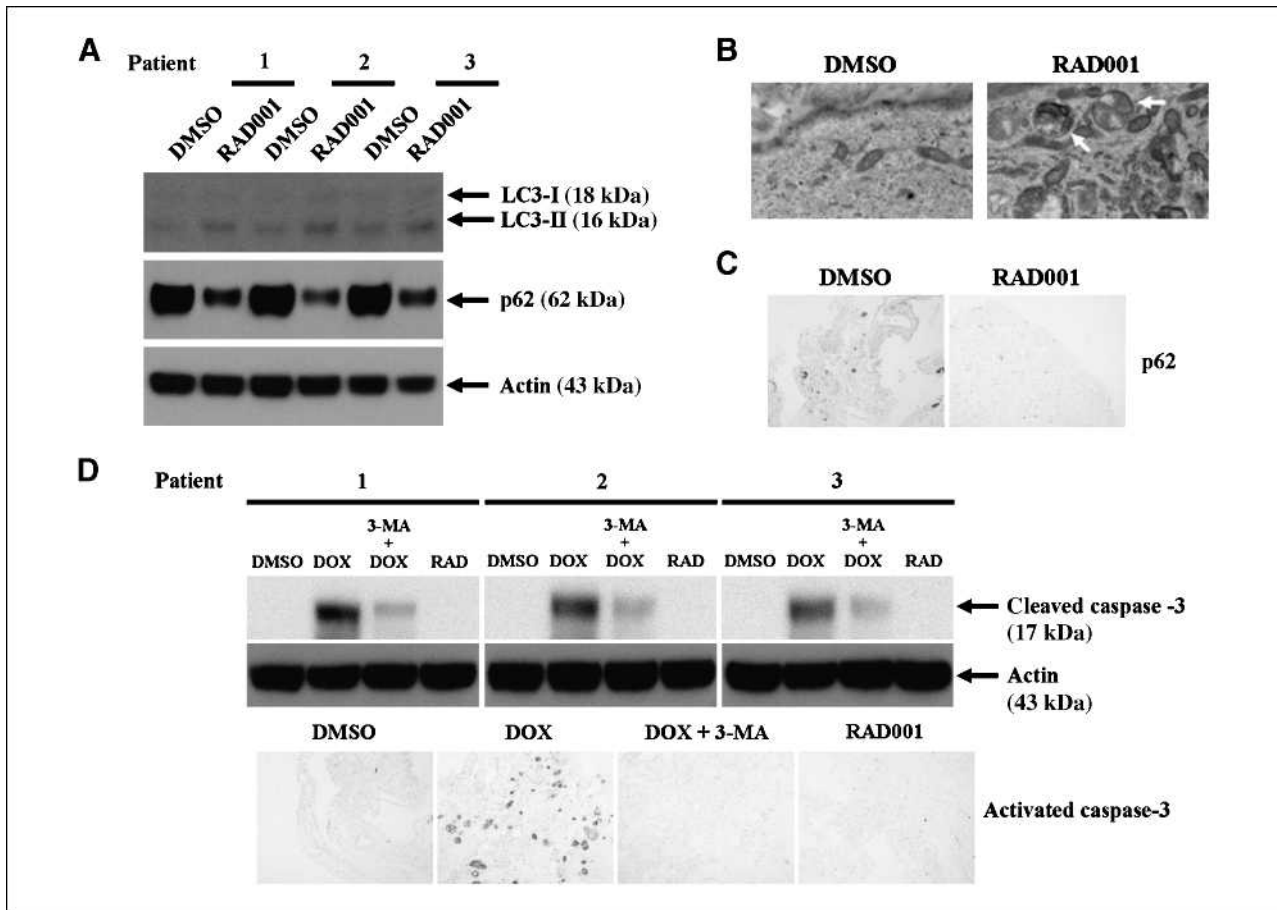


FIGURE 3. RAD001 induces autophagic activity in human PTC tissue specimens. A, fresh human PTC specimens from three consecutive patients were collected. *Ex vivo* specimens were maintained in culture media and exposed to DMSO or RAD001 (20 nmol/L) for 8 h. Tissue lysates were prepared. LC3-I, LC3-II, and p62 protein expression levels were monitored by Western blotting. Reprobing against actin was done to ensure equal protein loading. B, PTC tissue specimens were coincubated with DMSO or RAD001 (20 nmol/L) treatment for 8 h by *ex vivo* specimens culture model. Treated samples were subjected to transmission electron microscopy analysis (original magnification, $\times 15,000$). C, expression level of p62 in treated specimens was also assayed using immunohistochemistry analysis (all $\times 400$). D, fresh human PTC specimens were coincubated with DMSO, doxorubicin (1 $\mu\text{mol/L}$), doxorubicin (1 $\mu\text{mol/L}$) + 3-MA (10 $\mu\text{mol/L}$), or RAD001 (20 nmol/L). Cleaved caspase-3 protein level was monitored by Western blotting and immunohistochemistry analysis (all $\times 400$).

autophagy in human specimens (Fig. 3A). Likewise, RAD001-treated human *ex vivo* specimens displayed a substantial increase in autophagosomes that were visualized by electron microscopy, providing further evidence that autophagy is induced by RAD001 (Fig. 3B). RAD001-treated specimens showed a significant decrease in endogenous p62 expression, another marker for autophagy (26), further demonstrating the activation of autophagy in these human operative specimens (Fig. 3A and C). RAD001 did not change caspase-3 cleavage in PTC tissue, indicating that it had no significant effects on apoptosis (Fig. 3D). Together, our *in vitro* and *ex vivo* results indicate that RAD001 effectively activates autophagy in PTC.

RAD001 and doxorubicin have synergistic activity in PTC

To determine the effect of RAD001 on doxorubicin chemosensitivity, we first determined a baseline IC_{50} of doxorubicin of 98.61 and 52.97 nmol/L in 8505-C and

TPC-1 cells, respectively (Supplementary Fig. S1). Treatment with 20 nmol/L of RAD001 decreased the IC_{50} by 48% and 38% in 8505-C and TPC-1 cells, respectively. The effect of RAD001 on chemosensitivity was abrogated with Atg-5 siRNAs, suggesting that the effects of RAD001 on doxorubicin chemosensitivity are mediated through autophagy (Supplementary Fig. S1).

Using the MTS and clonogenic survival assays, we determined that the minimum inhibitory concentration (IC_{min}) of doxorubicin was 20 and 50 nmol/L in 8505-C and TPC-1 cells, respectively (Fig. 4A and C, left). The IC_{min} of RAD001 was 5 and 10 nmol/L in 8505-C and TPC-1 cells, respectively (Fig. 4A and C, right). In an MTS assay, the combination of doxorubicin and RAD001, at their IC_{min} , decreased cell survival by 293% and 299%, in comparison with doxorubicin alone in 8505-C and TPC-1 cells, respectively. The combination of both agents exceeded the additive effects of each individual agent (Fig. 4B). In a clonogenic assay, the combination

of doxorubicin with RAD001 at their IC_{min} decreased cell survival by 319% and 348%, in comparison with doxorubicin alone in 8505-C and TPC-1 cells, respectively (Fig. 4C). Consistent with synergistic activity, the efficacy of both agents exceeded the additive effects of the agents (Fig. 4D).

RAD001 improves radiosensitivity in PTC

Because external beam radiation is an important therapeutic modality for patients with locally advanced PTC, we determined if RAD001 could improve radiosensitivity in PTC cells. We treated 8505-C and TPC-1 cells with DMSO (control) or 20 nmol/L of RAD001 and then exposed them to radiation doses ranging from 0 to 6 Gy (Fig. 5A). PTC cells treated with RAD001 had a significant improvement in sensitivity to various doses of radiation. This effect was reversed when cells were treated with Atg-5 siRNA, suggesting that the radiosensitizing effects of RAD001 are mediated through autophagy.

In *ex vivo* experiments of human PTC, RAD001 activated autophagy, but did not seem to activate apoptosis (Fig. 5B). RAD001, in combination with doxorubicin or radiation, exhibited additive effects on the accumulation of LC3-II and the inhibition of histone H3 phosphorylation. No basal or additive caspase-3 cleavage signals were observed with RAD001 treatment, even when the specimens were treated with doxorubicin or external radiation. Consistent with our *in vitro* results, RAD001 activated autophagy and potentiated the autophagic activation caused by doxorubicin or external beam radiation in *ex vivo* specimens.

The effects of autophagic activation on chemosensitivity and radiosensitivity are mediated through Met

Because our results suggested that RAD001 has a distinct and complementary mechanism of action with doxorubicin, we determined the effects of RAD001 on cell signaling

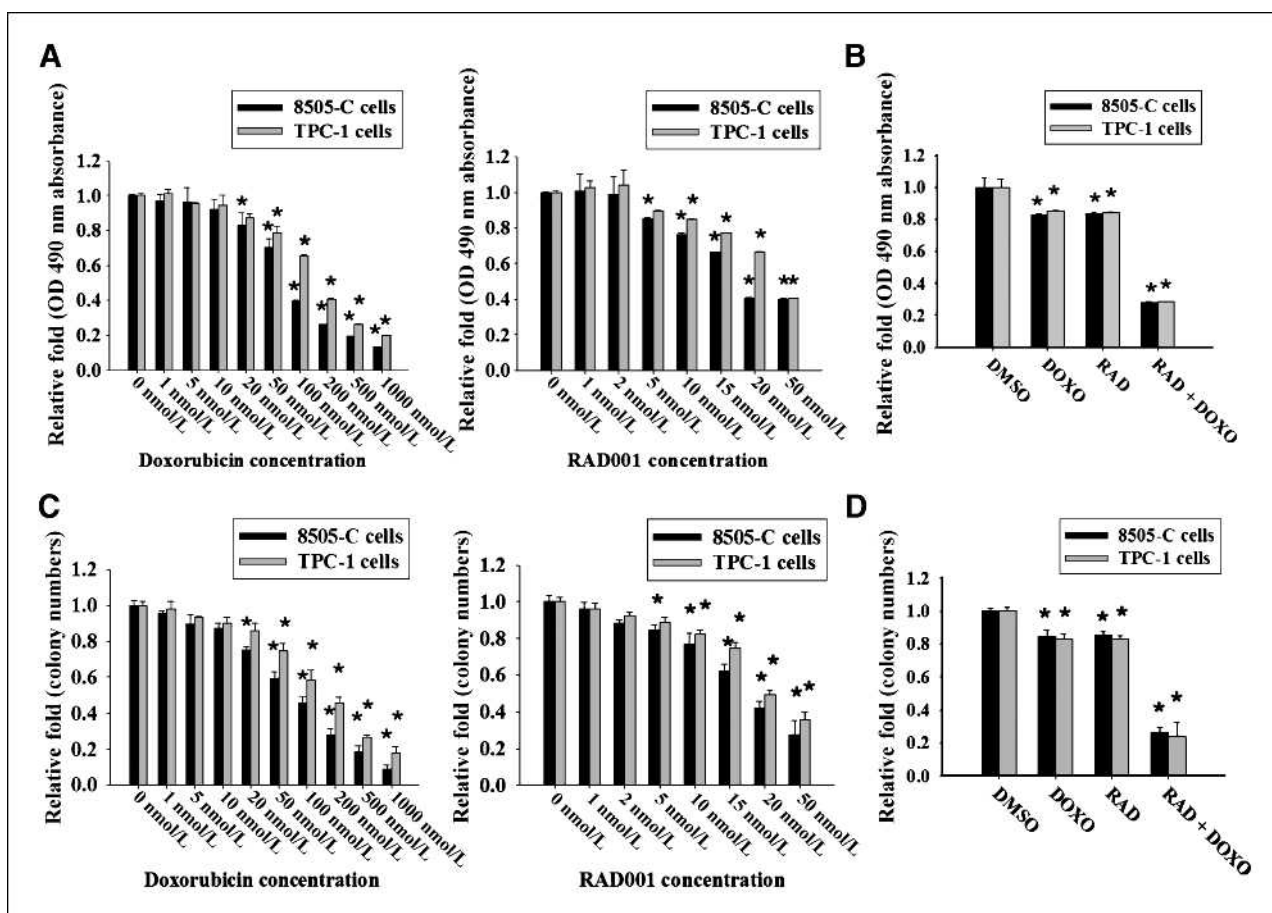
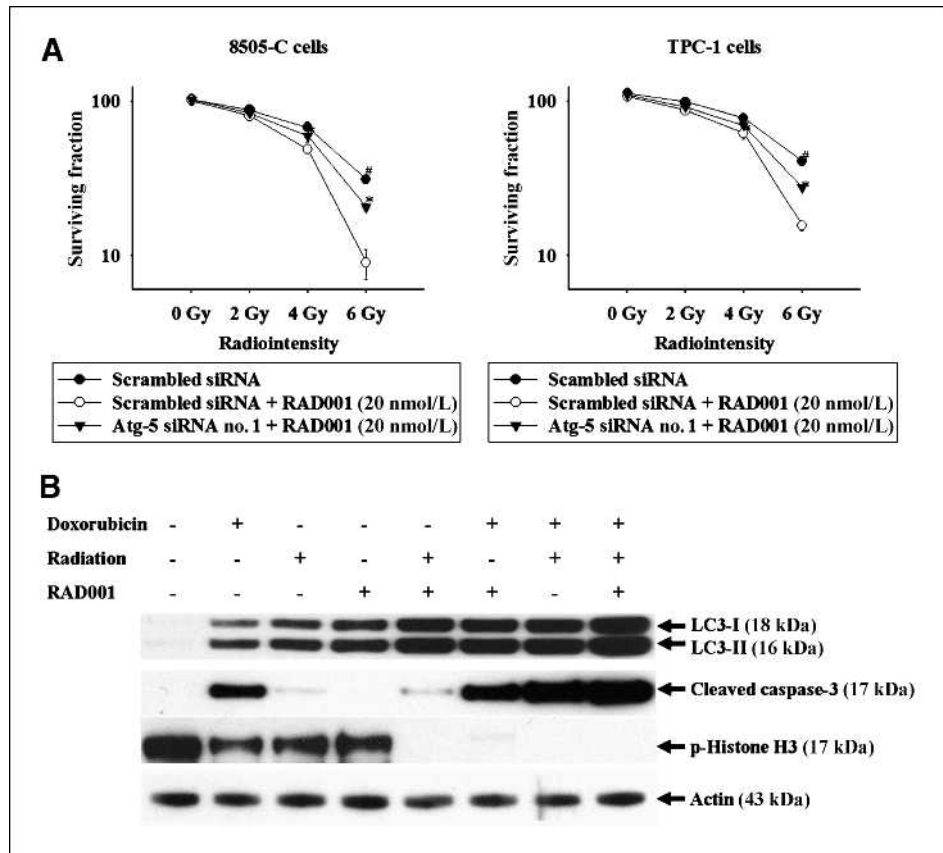


FIGURE 4. RAD001 plus doxorubicin exhibits synergistic effects on suppressing cell survival in 8505-C and TPC-1 cells. 8505-C and TPC-1 cells were treated with various concentration of doxorubicin and RAD001 as indicated for 48 h. Treated cells were subjected to MTS (A) or clonogenic survival assay (B). 8505-C and TPC-1 cells were also treated with DMSO, doxorubicin (20 nmol/L for 8505-C cells and 50 nmol/L for TPC-1), RAD001 (5 nmol/L for 8505-C cells and 10 nmol/L for TPC-1), or doxorubicin + RAD001 (20 nmol/L of doxorubicin + 5 nmol/L of RAD001 for 8505-C cells and 50 nmol/L of doxorubicin + 10 nmol/L of RAD001 for TPC-1 cells) for 48 h. Treated cells were subjected to MTS (C) or clonogenic survival assay (D). Similar experiments were repeated three times. Histograms represent relative fold of OD490 nmol/L absorbance or colony numbers (*, $P < 0.05$). All data are relative multiples of expression compared with untreated cells. Columns, mean of three experiments; bars, SE.

FIGURE 5. RAD001 enhances radiosensitivity through activating autophagy in 8505-C and TPC-1 cells. **A**, 8505-C or TPC-1 cells were treated DMSO or 20 nmol/L of RAD001 for 24 h as indicated. Treated cells were exposed to 0, 2, 4, or 6 Gy of radiation as indicated. After 10 d, colonies were stained and counted colony numbers were graphed. *, $P < 0.05$, indicating a significant statistical difference between radiation exposure–decreased colony formation numbers with or without RAD001 treatment. Atg-5 siRNA no. 1 + DMSO experiments were done (data not shown). **B**, 8505-C or TPC-1 cells were treated with or without doxorubicin (1,000 nmol/L), radiation (6 Gy), and RAD001 (20 nmol/L) as indicated. Cell lysates were prepared and LC3-I and LC3-II and cleaved caspase-3 protein levels as well as histone H3 phosphorylation levels were monitored by Western blotting. Reprobing against actin was done to ensure equal protein loading.



pathways. We used a kinome array technique, based on the multiplexed coupling of tyrosine kinase–specific antibodies to polystyrene microspheres beads, to simultaneously determine the phosphorylation status of 71 unique tyrosine kinases. In this analysis, Src and Met were the most differentially phosphorylated in scrambled versus Atg-5 siRNA–transfected cells, and in control versus RAD001–treated cells (Fig. 6A). We validated the kinome array results by immunoblotting; this confirmed that Atg-5 siRNA decreased Src but increased Met activity, whereas RAD001 increased Src but decreased Met activity (Fig. 6B).

Because autophagic activation modulated Src and Met, we determined if either or both were essential for the augmentation in chemosensitivity and radiosensitivity observed during autophagy. We treated PTC cell lines with dasatinib, a Src inhibitor, to determine if it reversed the effects of RAD001. RAD001 decreased the IC_{50} by 36% and 31%, whereas RAD001 plus dasatinib decreased the IC_{50} by 62% and 52% in 8505-C and TPC-1 cells, respectively. Furthermore, the addition of dasatinib to RAD001 also significantly improved radiosensitivity (Supplementary Fig. S3). Therefore, these results show that dasatinib enhanced, rather than attenuated, the anticancer effects of RAD001.

In contrast, Atg-5 siRNA increased the IC_{50} by 81% and 71%, whereas Atg-5 siRNA in combination with the Met inhibitor PHA665752 increased the IC_{50} by only 3% and 6% in 8505-C and TPC-1 cells, respectively. There-

fore, PHA665752 abrogated the effects of Atg-5 knockdown on chemosensitivity. Furthermore, RAD001 and PHA665752 induced similar effects on chemosensitivity and radiosensitivity with no significant additive effects in combination (Supplementary Figs. S3A and S4A). Together, these results suggest that the effects of autophagic activation on chemosensitivity and radiosensitivity are mediated largely through Met.

Discussion

RAD001 potently activates autophagy in PTC cell lines and human PTC specimens maintained *ex vivo*. RAD001 synergistically enhances both chemosensitivity to doxorubicin and radiosensitivity, and seems to do so through autophagy. That RAD001 elicits these effects is of considerable interest as it inhibits mTOR, a signaling protein that is a convergence point for many signaling pathways. Furthermore, mTOR elicits the cellular response to extracellular stimuli by mediating changes of cellular protein synthesis. That RAD001 acts through autophagy is supported by the observation that Atg-5 knockdown completely abrogates the effect of RAD001 on chemosensitivity. Because Atg-5 is an essential component of the autophagosome with no other known function, our results suggest that RAD001 enhancement of chemosensitivity is mediated through autophagy.

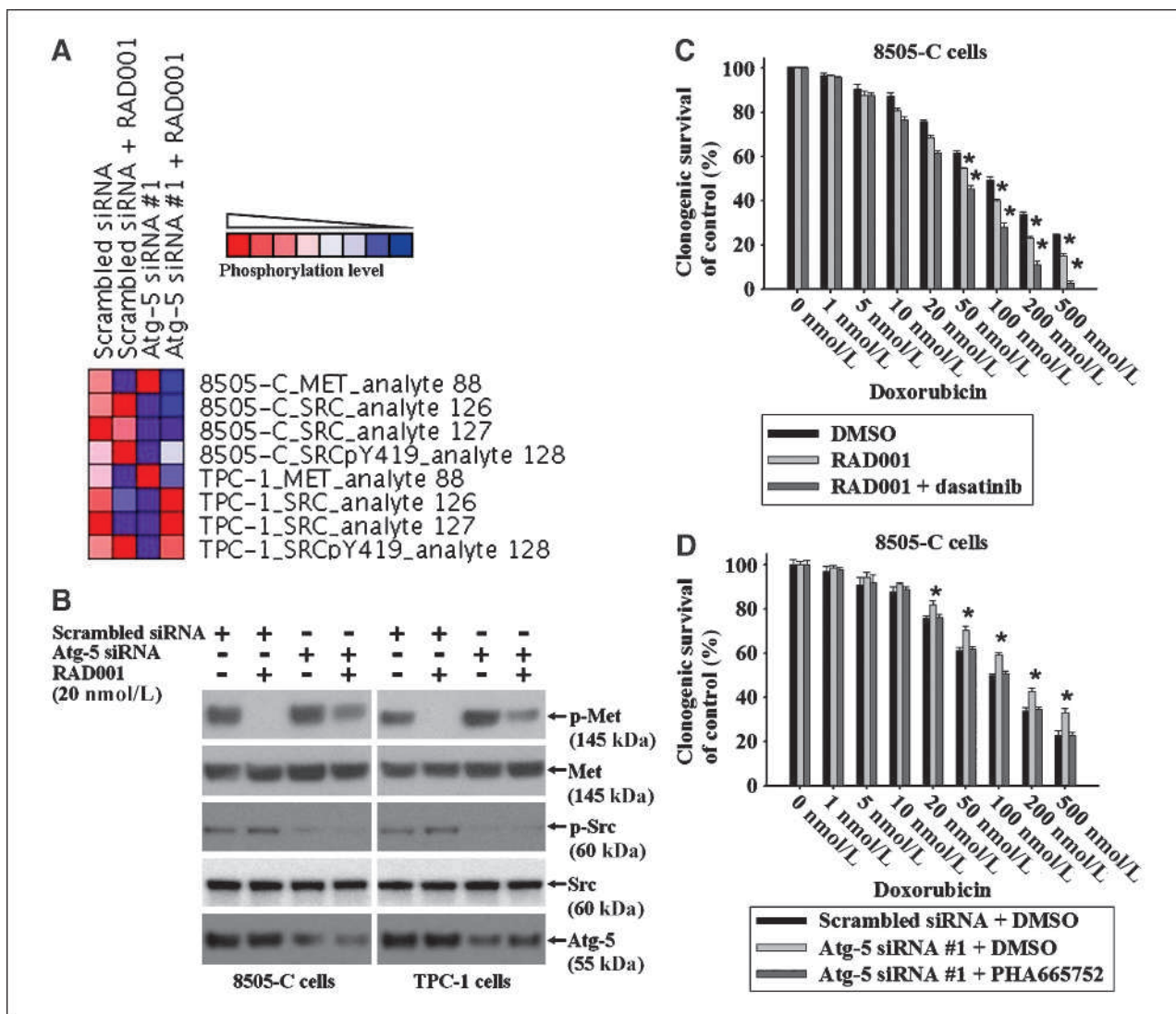


FIGURE 6. Atg-5 RNAi and RAD001 treatment modulate Met and Src phosphorylation in 8505-C and TPC-1 cells. 8505-C and TPC-1 cells were transfected with scrambled or Atg-5 siRNA no. 1 and then exposed to DMSO or 20 nmol/L of RAD001 for 24 h. Cell lysates were collected and then subjected to kinome array analysis. Tyrosine kinase phosphorylation levels were tested by single or multiple antibodies. A, the raw data were acquired processed and positive signals were normalized to fold-over backgrounds. The normalized readings were log transformed and converted into a .gct file and the HeatMap diagram was generated using the GenePattern 3.0 software. B, cell lysates were also collected and then subjected to Western blotting analysis of phosphorylated Met and SRCpY419. Reprobing against total MET and Src were done to ensure equal loading. Atg-5 expression levels were also assayed to monitor RNAi transfection efficiency. C, 8505-C cells were treated with DMSO, RAD001 (20 nmol/L), or RAD001 (20 nmol/L) plus dasatinib (100 nmol/L) for 24 h. D, 8505-C cells were transfected with scrambled or Atg-5 siRNA no. 1 and then coincubated with or without 0.25 μ mol/L of PHA665752 for 24 h. Treated cells were coincubated in the absence or presence of different concentrations of doxorubicin. The IC₅₀ of doxorubicin was determined by the clonogenic survival assay. The absorbance of each well is proportional to cell survival, and the absorbance of 0 nmol/L of doxorubicin-treated cells was set to 100% survival. The Y-axis indicates the relative percentage of survival. The bars represent the average of three independent experiments. *, $P < 0.05$, indicating a significant statistical difference.

Because autophagy is often considered a prosurvival phenomenon, our findings might be somewhat unexpected. One explanation for our observations is that RAD001 might promote autophagy-mediated programmed cell death, a nonapoptotic escape pathway important in cells that are defective or deficient in apoptosis (30). PTC is intrinsically resistant to cytotoxic chemotherapies, partly due to defects in apoptosis (16). This is relevant to a variety of malignan-

cies, many with known deficiencies in apoptosis that are resistant to conventional cytotoxic chemotherapy, such as gemcitabine-refractory metastatic pancreatic cancer (31–37).

Because of the potent synergistic anticancer effects of RAD001 with doxorubicin, we characterized the effects of RAD001 on cell signaling. The most prominent alterations detected from autophagy blockade with Atg-5 knockdown were Met activation and Src inactivation. Consistent with this

observation, the most prominent alterations during autophagy activation by RAD001 were Met inactivation and Src activation. Our results suggest that the chemosensitizing and radiosensitizing effects of autophagic activation are mediated largely through Met. If the chemosensitizing effects of RAD001 were mediated through Src activation, Src inhibition would at least partially abrogate these effects. However, a Src inhibitor potentiated, rather than reversed, the chemosensitization from RAD001. Of note, a recent report showed that Src inhibition activated autophagy in glioma cells (38). This report, and the results of our current study, suggest that combination treatment with a Src inhibitor might increase RAD001 efficacy.

In contrast, Atg-5 knockdown increased doxorubicin chemoresistance and Met phosphorylation. The addition of a Met inhibitor to Atg-5 knockdown cells abrogated this effect. This suggests that the effects of autophagy on chemosensitivity are mediated mostly through Met. This is the first study to directly associate autophagic activation with Met activation. A recent study showed that a cyclooxygenase-2 inhibitor downregulated both mTOR expression and Met activation in hepatocellular carcinoma cells (39). Although this was associated with autophagic activation, the association between autophagy and Met phosphorylation was not identified. Interestingly, RAD001 has excellent activity in papillary renal carcinoma, a malignancy driven by a constitutively active Met mutation (6, 7).

Ionizing radiation induces autophagy in various cell lines (40–43). However, it is unclear if autophagy promotes or prevents cell death in cancer cells exposed to ionizing radiation. In mouse embryonic fibroblasts, RAD001 promotes autophagy and reduces radioresistance (41). Furthermore, autophagy inhibition by Atg-5 siRNA or 3-MA increases the radiosensitivity of human lung cancer cells (42). In contrast, treatment with 3-MA or bafilomycin A1, another autophagy inhibitor, radiosensitizes malignant glioma cells, suggesting that autophagy may provide protection from ionizing radiation (40). Our data show that RAD001 by itself increases the radiosensitivity of PTC (Fig. 5). These observations imply that the autophagic response to radiation exposure might be a pro-survival or pro-death mechanism, depending on cellular context.

There are several issues that remain to be addressed. First, the concept of autophagy-driven programmed cell death is controversial. Some cancer cell lines that lack the machinery to execute apoptosis could still die in a nonnecrotic manner in a process associated with autophagy (44), which can be abrogated by knocking down Atg-5 (45). In the end, it is

difficult to determine if autophagy is the mechanism of cell death, or if it is a survival response triggered by stresses that occur near death (46).

Here, we used RAD001 to induce autophagy. Because mTOR is an important regulator of several effector pathways, it is unclear how much of its anticancer activity could be attributed to autophagy. However, our experiments using Atg-5 knockdown suggest an autophagy-specific effect for RAD001. Lastly, our work did not include *in vivo* studies, and as such, we did not investigate issues related to efficacy and toxicity in animals. However, RAD001 trials are already under way for other malignancies, and the toxicity profiles are acceptable (47). We have already initiated a RAD001 trial for patients with advanced PTC resistant to radioactive iodine treatment (Dana-Farber Cancer Institute protocol no. 09-049).

In summary, the induction of autophagy in combination with cytotoxic chemotherapy is a promising therapeutic strategy for patients with refractory PTC. The synergistic effects of RAD001 and doxorubicin suggest that improvements in therapeutic efficacy could be achieved with lower overall toxicity. RAD001 improves sensitivity to doxorubicin and ionizing radiation through the attenuation of Met. Future studies should determine the efficacy and safety of combining RAD001 with conventional cytotoxic chemotherapy and radiation therapy for malignancies that are resistant to conventional therapy due to intrinsic resistance to apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Orlo H. Clark (University of California at San Francisco, San Francisco, CA) for kindly providing TPC-1 cells; Dr. Sareh Parangi (Massachusetts General Hospital, Boston, MA) for providing 8505-C cells; and Adelaide Carothers for her assistance with this work.

Grant Support

Osteen Junior Faculty Research Grant from the Department of Surgery at Brigham and Women's Hospital.

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Received 04/14/2010; revised 06/26/2010; accepted 07/22/2010; published OnlineFirst 08/24/2010.

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