Insulin-like Growth Factor-I Receptor Signaling Pathway Induces Resistance to the Apoptotic Activities of SCH66336 (Lonafarnib) through Akt/Mammalian Target of Rapamycin – Mediated Increases in Survivin Expression

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Abstract Although preclinical studies have suggested that farnesyltransferase inhibitors (FTI) have promising antitumor activity, clinical trials have shown that FTI activity in patients is actually limited. The mechanism that induces resistance to FTI treatment is still not fully understood. The FTI SCH66336 has been shown to induce apoptotic and antiangiogenic activities in a subset of head and neck squamous cell carcinoma (HNSCC) and non – small cell lung cancer (NSCLC) cell lines. We therefore investigated the mechanisms mediating resistance to the therapeutic activities of SCH66336 in HNSCC and NSCLC. Our various analyses showed that insulin-like growth factor-I receptor (IGF-IR) activation interferes with the antitumor activity of SCH66336 in HNSCC and NSCLC cells. Treatment with SCH66336 activated the IGF-IR/phosphatidylinositol 3-kinase/Akt pathway, leading to increased mammalian target of rapamycin (mTOR)-mediated protein synthesis of survivin in a subset of HNSCC and NSCLC cell lines that were insensitive to the apoptotic activities of the drug. Inhibition of IGF-IR, Akt, or mTOR or the knockdown of survivin expression abolished resistance to SCH66336 and induced apoptosis in the cells. Overexpression of survivin by the use of adenoviral vector protected SCH66336-sensitive HNSCC cells from the apoptotic activities of the drug. Our results suggest that expression of phosphorylated IGF-IR, phosphorylated Akt, phosphorylated mTOR, and survivin serves as biological markers of SCH66336 responsiveness in HNSCC and NSCLC cells and that SCH66336 induces survivin expression through an IGF-IR/Akt/mTOR – dependent pathway. Thus, combining inhibitors of IGF-IR, phosphatidylinositol 3-kinase/Akt, mTOR, or survivin with SCH66336 may be an effective anticancer therapeutic strategy for patients with HNSCC or NSCLC.

Head and neck squamous cell carcinoma (HNSCC) is diagnosed annually in an estimated 600,000 patients worldwide (1). In the United States, the annual incidence is estimated to be 31,000 with ~7,400 associated deaths (2). First-line therapy for locally advanced HNSCC is typically surgery and/or radiotherapy (3, 4). However, radical surgery for HNSCC is difficult and disfiguring (3). In addition, tumors recur after first-line therapy in approximately one third of all patients with HNSCC, underscoring the need for more effective treatment strategies.

The Ras signaling pathway is invariably activated (5) in several types of human cancers, including head and neck cancer, through the overexpression of the epidermal growth factor receptor, mutation(s) in *Ras*, and/or the downstream activation of Ras-mediated pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt (5–8), resulting in deregulated cell proliferation, angiogenesis, cell transformation, cytoskeletal reorganization, and suppression of apoptosis (9). The idea of targeting *Ras* is therefore appealing. Because Ras requires posttranslational modification, including farnesylation, to ensure its cell membrane localization and further engagement of the raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase kinase, MAPK, ral/Gef, and PI3K, farnesyltransferase inhibitors (FTI) have been developed to block Ras activation (10). However, previous findings have suggested that FTIs have several targets other than Ras, including RhoB, the centromere-binding proteins CENP-E and CENP-F, insulin-like growth factor (IGF)-binding protein-3, heat shock protein 90, Ras homologue enriched in brain, and other proteins not yet identified (11–14).

FTIs have been shown to inhibit tumor cell growth *in vitro* (15) and *in vivo* (16). In human tumor cell lines, FTIs induce the accumulation of cells in the G_2-M phase of the cell cycle, which is often correlated with p53-dependent $p21^{waf/cip1}$ induction in many cell types (15). FTIs have also been shown

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to inhibit the growth of chemically induced lung tumor with K-Ras mutations in immunocompetent mice (17) and the growth of human bladder, colon, and fibrosarcoma tumor xenografts (16).

SCH66336 (lonafarnib) was one of the first FTIs to be evaluated in clinical trials (18, 19). SCH66336 was shown to have antiproliferative and apoptotic activities in a wide variety of cancer cells *in vitro*. We previously showed that SCH66336 could also induce apoptotic and antiangiogenic activities in a subset of HNSCC and non–small cell lung cancer (NSCLC) cell lines (12, 13, 20). In spite of the promising antitumor activities of SCH66336 observed in preclinical studies, however, researchers have found unsuccessful therapeutic activities of SCH66336 in clinical trials. A large-scale phase III clinical trial in patients with advanced NSCLC showed that no additional benefit was gained by adding SCH66336 to standard first-line chemotherapy (21). In addition, no response or even modest activity has been observed in patients with colon cancer or chronic myeloid leukemia (22, 23).

In this study, we investigated the mechanisms involved in the resistance of HNSCC cells to the therapeutic activities of SCH66336. Here, we report that SCH66336 induces survival response that involves the IGF-I receptor (IGF-IR)/PI3K/Akt/mammalian target of rapamycin (mTOR)–mediated synthesis of antiapoptotic survivin protein. Consequently, inactivation of IGF-IR/phosphorylated Akt (pAkt)/mTOR–mediated survivin protein synthesis and knockdown of survivin protein render HNSCC cells sensitive to SCH66336 treatment.

Materials and Methods

Cell lines and reagents. The human HNSCC cell lines UMSCC38, UMSCC22A, UMSCC10B, SqCC/Y1, and SqCC35 were established originally by Drs. Michael Reiss (Yale University, New Haven, CT) and Thomas Carey (University of Michigan, Ann Arbor, MI). TR146 cells were provided by Dr. A. Balm (The Netherlands Cancer Institute, Amsterdam, the Netherlands). Normal human oral keratinocytes were obtained from Dr. Reuben Lotan (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Human NSCLC cell lines 226B, 226Br, and H460 were obtained from the American Type Culture Collection. The cell lines used in this study were derived from primary cancers or metastases from patients with HNSCC or NSCLC (24, 25). The HNSCC cell lines were cultured in DMEM, and NSCLC cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified environment with 5% CO_2. Normal human oral keratinocytes were cultured in keratinocyte serum-free medium (Invitrogen) supplemented with recombinant human epidermal growth factor and bovine pituitary extract.

We obtained 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide from Sigma-Aldrich. The class I PI3K inhibitor LY294002, the MAPK/extracellular signal-regulated kinase kinase inhibitor U0126, and the IGF-IR tyrosine kinase inhibitor AG1024 were purchased from Chemicon. Another IGF-IR tyrosine kinase inhibitor, OSI-868, was obtained from OSI. The mTOR inhibitor rapamycin was purchased from Calbiochem-Novabiochem. Antibodies against procaspase-3, active caspase-3, XIAP, survivin, phosphorylated IGF-IR, pAkt, Akt, phosphorylated $p70^{s6k}$, $p70^{s6k}$, phosphorylated 4E-binding protein 1 (4E-BP1), 4E-BP1, phosphorylated c-Raf, and phosphorylated MAPK were purchased from Cell Signaling Technology; anti-actin, H-Ras, K-Ras, IGF-IRβ, and MAPK were obtained from Santa Cruz Biotechnology; and α-tubulin was purchased from Sigma. SCH66336 {i.e., [+]4-(2-[4-(8-chloro-3,0-dibromo-6,11-dihydro-5-benzocyclohepa (1,2-β)pyridin-11-yl)-1-piperidinyl]-2-oxoethyl)-1-piperidinecarboxamide} was provided by Schering-Plough Research Institute. Synthetic small interfering RNA (siRNA) that targets survivin was purchased from Ambion. Adenoviral vector expressing constitutively active survivin, referred to as Ad-survivin, was provided by Dr. Dario C. Altieri (University of Massachusetts Medical School, Worcester, MA; ref. 26). Empty vector (Ad-EV) was used as a control.

Proliferation assay. HNSCC cells were seeded at 3,000 per well in 96-well plates (Becton Dickinson). After 1 day, the medium was changed to medium with or without 5 μmol/L SCH66336. DMSO (0.1%) was used as a control. After 3 days of incubation, cell proliferation was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described elsewhere (27).

siRNA transfection and virus infection. To assess the effects of survivin in SCH66336-induced apoptosis in HNSCC cells, TR146 and UMSCC38 cells (2×10^5) in 35-mm dishes were transfected with 60 nmol of scrambled siRNA or siRNA specifically targeting survivin by the use of Oligofectamine (Invitrogen) for 6 h. siRNA-transfected cells were incubated with or without 5 μmol/L SCH66336 for 48 h for fluorescence-activated cell sorting analysis or Western blotting. UMSCC10B, SqCC/Y1, and SqCC35 cells (8×10^5) in 60-mm dishes were infected with Ad-EV or Ad-survivin with 10 plaque-forming units/cell for 2 h in medium without FBS. After 36 h, cells were incubated with or without 5 μmol/L SCH66336 for 48 h for fluorescence-activated cell sorting analysis or Western blotting. TR146 cells were infected with Ad-EV or adenovirus expressing dominant-negative IGF-IR (Ad-dnIGF-IR) with the indicated plaque-forming unit/cell for 2 h in medium without FBS. After 24 h, cells were incubated with or without 5 μmol/L SCH66336 for 72 h for the cell proliferation assay.

Western blotting. Western blotting was done as described elsewhere (13). Briefly, equivalent amounts of protein (25 μg) were resolved by SDS-PAGE in 8% to 15% gels (80 V for 10 min and 100 V for 1-1.5 h) and transferred overnight at 20 V to a nitrocellulose membrane. Blocking, washing, incubation with primary or secondary antibody, and development were done as described elsewhere (13).

Reverse transcription-PCR. Total RNA was isolated from the cells with the use of Trizol reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA as templates in a 50 μL reaction by using the Taqman reverse transcriptase reagents according to the manufacturer's protocol (Applied Biosystems). The reaction was incubated at 25°C for 10 min, at 48°C for 30 min, and inactivation at 95°C for 5 min. After inactivation, the cDNA was stored at -20°C until use. Reverse transcription-PCR was done by amplification of the genes using cDNA template generated as described above and corresponding gene-specific primer sets. The primer sequences are as follows: survivin, 5′-GCATGGGTGCCCCGACGTTG-3′ (sense) and 5′-GCTCCGGCCAGAGGCCTCAA-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5′-GGTGAAGGTCGGTGTGAACGGATTT-3′ (sense) and 5′-AATGCCAAAGTTGTCATGGATGACC-3′ (antisense).

To avoid amplification of genomic DNA, the primers of each genes were chosen from different exons. PCR was carried out in a total volume of 25 μL containing 2 μL of cDNA solution and 0.2 μmol/L of sense and antisense primers. The reverse transcription-PCR exponential phase was determined on 28 to 33 cycles to allow quantitative comparisons among the cDNAs developed from identical reactions. Amplification products (8 μL) were resolved in 2% agarose gel, stained with ethidium bromide, and visualized in a transilluminator and photographed.

Cell cycle and apoptosis analysis. All cells (nonadherent and adherent) were harvested, washed, fixed with 1% paraformaldehyde and 70% ethanol, and stained using an APO-BRDU kit (Phoenix Flow Systems) according to the manufacturer's protocol. Flow cytometric analysis was done by using a Coulter EPICS Profile II flow cytometer (Coulter Corp.) equipped with a 488-nm argon laser. Approximately 10,000 events (cells) were evaluated for each sample.

Results

Effect of SCH66336 on the proliferation of HNSCC cells. In the experiments described in previous report (20), some of the same HNSCC cell lines used in the current study (TR146, UMSCC38, and SqCC/Y1 cells) showed apoptotic response after treatment with SCH66336 (1 μmol/L) for >3 days in the presence of 5% FBS. However, we recently observed a rather large differential response in HNSCC cell lines. We recently found that a 2-day treatment with 5 μmol/L SCH66336, a dose achievable *in vivo* (~ 8 μmol/L; ref. 28), was sufficient to induce apoptosis in the FTI SCH66336-sensitive HNSCC cell lines (12) but not in the resistant cell lines (12). To study the mechanism of SCH66336 resistance in HNSCC cells, we chose a 5 μmol/L SCH66336 in this experiment. We increased the dose of SCH66336 to 5 μmol/L, which is not optimal for observing the apoptotic activities of the drug in the HNSCC cell lines used in our study.

Flow cytometric analysis on a subset of HNSCC cell lines stained with propidium iodide showed a mild increase in the cell population in the G_0-G_1 phase (TR146, UMSCC22A, and UMSCC38), G_2-M phase (UMSCC10B), or sub-G_0-G_1 phase (SqCC/Y1, UMSCC10B, and SqCC35) of the cell cycle after the treatment with SCH66336 (5 μmol/L) for 48 h in medium containing 10% FBS (Fig. 1*A*). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based fluorescence-activated cell sorting analysis revealed no apoptotic response in the TR146, UMSCC22A, or UMSCC38 cell lines after the same treatment (Fig. 1*B*). In contrast, SqCC/Y1, UMSCC10B, and SqCC35 cells remained sensitive to the apoptotic activities of SCH66336 (Fig. 1*B*). These findings indicated that cell survival pathways activated in a high serum condition protected the TR146, UMSCC22A, and UMSCC38 cells from apoptotic activities of SCH66336.

SCH66336 induces survivin expression, which mediates resistance to apoptotic activities of the drug in HNSCC cells. We attempted to find the molecules that are involved in HNSCC cell resistance to SCH66336 treatment. We first assessed the K-Ras pathway status of the cell lines used in our study. Western blot analysis showed that the basal expression levels of K-Ras, H-Ras, and phosphorylated c-Raf (Ser^{338}), a downstream of Ras, did not show obvious difference between the HNSCC cell lines and normal human oral keratinocytes (Fig. 2*A*). Further, consistent with the previous report (20), the response of the HNSCC cell lines to the SCH66336 treatment (Fig. 1*A*) did not seem to be correlated with the status of the K-Ras pathway.

Because inhibitors of apoptosis proteins are involved in the sensitivity of tumor cells to chemotherapeutic drugs (26, 29), we next correlated the apoptotic response (Fig. 2*B*, *top*) with the expression of inhibitors of apoptosis proteins, including survivin and XIAP, in TR146, UMSCC38, and SqCC/Y1 cells after the treatment with SCH66336. TR146 cells with marginal apoptotic response (Fig. 2*B*, *bottom*) showed no detectable changes in the expression levels of active caspase-3, XIAP, and survivin after the drug treatment (5 μmol/L for 48 h) in the medium containing 0.1% FBS (Fig. 2*B*, *bottom*). Under the same conditions, UMSCC38 and SqCC/Y1 cells showed increases in apoptotic cell population and expression of active caspase-3 in parallel with decreased XIAP and survivin expression (Fig. 2*B*, *bottom*). When the drug was treated in the presence of 10% FBS, TR146, UMSCC22A, and UMSCC38 cells all showed a pronounced increase in survivin expression with no change in procaspase-3 and active caspase-3 levels (Fig. 2*C*). In contrast, SqCC/Y1, UMSCC10B, and SqCC35 cells

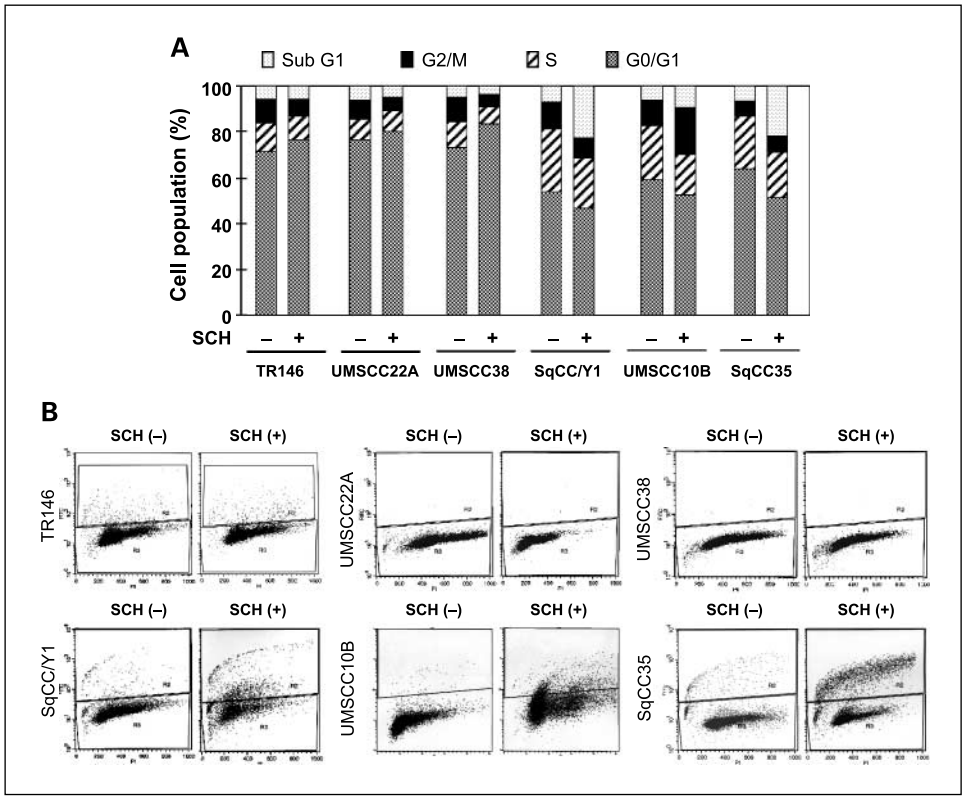

Fig. 1. The effect of SCH66336 on HNSCC cells. A subset of HNSCC cells was treated with 0.1% DMSO or SCH66336 (*SCH*; 5 μmol/L) for 48 h and analyzed. Percentage of cells in specific phases of the cell cycle (sub-G_1, G_0-G_1, S, and G_2-M) by flow cytometric analysis (*A*) and a flow cytometry–based terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (*B*).

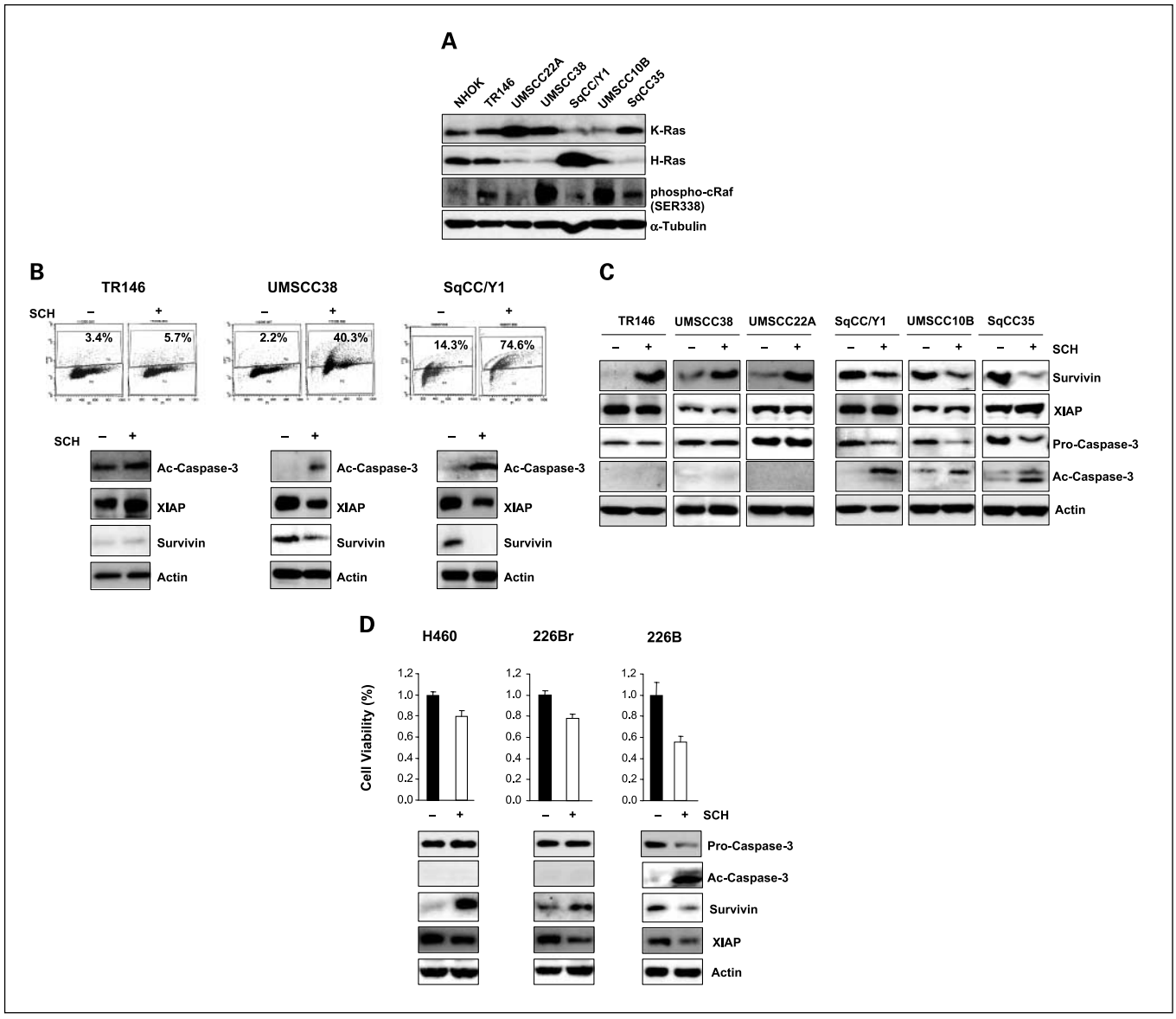

Fig. 2. The effect of SCH66336 on the expression of survivin. *A,* Western blot analysis in a normal human oral keratinocytes (*NHOK*) and in a subset of HNSCC cells. *B,* flow cytometry – based terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay (*top*) and Western blot analysis (*bottom*) in TR146, UMSCC38, and SqCC/Y1 cells incubated for 48 h in medium with 0.1% FBS and in the presence or absence of 5 μmol/L SCH66336. *C,* Western blot analysis in a subset of HNSCC cells, which were incubated for 48 h in 10% FBS medium with or without 5 μmol/L SCH66336. *D,* cell proliferation (72 h) and Western blot analysis (48 h) of H460, 226Br, and 226B NSCLC cell lines, which were treated with 5 μmol/L SCH66336.

showed a notable decrease in the survivin and procaspase-3 levels in an association with an increase in the active caspase-3 level after the treatment with SCH66336 (Fig. 2*C*). XIAP expression remained unchanged in these cells after the drug treatment. We further investigated the response of NSCLC cells to SCH66336 treatment. H460 and 226Br cell lines treated with SCH66336 showed no major differences in cell viability or procaspase-3 and active caspase-3 expression but a marked increase in survivin expression (Fig. 2*D*). The H226B cell line showed a substantial decrease in cell number, survivin protein level, and caspase-3 cleavage after drug treatment (Fig. 2D). Altogether, these data suggest that the expression of survivin, but not of XIAP, is involved in the apoptotic activities of SCH66336 in HNSCC and NSCLC cells.

To determine the role of survivin in SCH66336-mediated apoptosis in HNSCC cells, we transfected TR146 and UMSCC38 cells with siRNA specifically targeting survivin and treated them with SCH66336 for 48 h. We found it interesting that apoptosis was induced by the transfection of survivin (18.5% in TR146 cells and 7.8% in UMSCC38 cells) and was notably increased by treatment with SCH66336 (33.1% in TR146 cells and 20.0% in UMSCC38 cells; Fig. 3*A*). The induction of apoptosis was further confirmed by the cleavage of caspase-3, detected by Western blot analysis, in the cells transfected with survivin siRNA and then treated with SCH66336 (Fig. 3*B*). We then examined the apoptotic response of SqCC/Y1 and UMSCC10B cells to SCH66336 treatment when overexpression of survivin was induced by infection with

adenoviral vector containing survivin. The caspase-3 cleavage induced by SCH66336 was substantially prohibited in the cells overexpressing survivin (Fig. 3*C*). Thus, survivin seemed to play an important role in the resistance of HNSCC cells to SCH66336.

Activation of the IGF-IR/Akt pathway in HNSCC cells induces resistance to the apoptotic activities of SCH66336. We investigated the mechanism by which SCH66336 regulates survivin expression. The transcriptional activity of nuclear factor-κB (30), hypoxia-inducible factor-1α (31), and p53 on survivin expression has been reported in cancer cells (32). Given the effects of SCH66336 on these transcription factors (33), we tested the effects of SCH66336 on survivin mRNA expression in HNSCC cell lines. Reverse transcription-PCR analysis revealed that mRNA levels of survivin were mildly decreased in sensitive cells (SqCC/Y1) and increased in the resistant cells (TR146 and UMSCC38) in response to the SCH66336 cells (Fig. 4*A*).

Activation of IGF-IR and the consequent activation of IRS-I and Akt have been known to mediate resistance to antitumor agents in various cancers (34, 35). Hence, we examined the status of the IGF-IR/Akt signaling pathway in HNSCC cells after treatment with SCH66336. SCH66336 induced increases in the unfarnesylated H-Ras in all of the HNSCC cell lines used in this experiment (Fig. 4*B*), indicating the effectiveness of the drug in inhibiting the activation of Ras by farnesyltransferase. The expression of pIGF-IR and pAkt was increased in SCH66336-resistant TR146, UMSCC22A, and UMSCC38 but decreased in the sensitive SqCC/Y1, UMSCC10B, and SqCC35 cells after drug treatment (Fig. 4*B*). Meanwhile, SCH66336 induced increased levels of p44/42MAPK, an indicative activated 44/42MAPK, in all of the HNSCC cell lines used in this experiment. These findings indicate that Akt was activated in the HNSCC cell lines that were insensitive to the apoptotic activities of SCH66336 through an IGF-IR–dependent but Ras- and MAPK-independent mechanism.

Inhibition of IGF-IR activation sensitizes HNSCC cells to SCH66336. To investigate the role of IGF-IR in the resistance to apoptosis induced by SCH66336, we determined whether blocking the IGF-IR pathway can sensitize the resistant cells to SCH66336. To this end, we infected TR146 and UMSCC38 cells with Ad-dnIGF-IR (36) before treating them with SCH66336. The number of TR146 and UMSCC38 cells was substantially decreased by the SCH66336 treatment when the IGF-IR pathway was inhibited (Fig. 4*C*). The effects of Ad-dnIGF-IR transfection on UMSCC38 cells were more obvious than those on TR146 cells, probably due to the sensitivity of the UMSCC38 cells to the adenoviral vector as determined by the response of the cells to the control adenoviral vector (Ad-EV). We found an approximate 20% decrease in the viable number of UMSCC38 cells after the infection with 10 plaque-forming units/cell of Ad-dnIGF-IR. In contrast, less than a 10% decrease in viability was observed in the Ad-EV–infected UMSCC38 cells. SCH66336 treatment induced a mild (6%) but statistically significant decrease in the viable cells infected with 50 plaque-forming units/cell of Ad-dnIGF-IR ($P = 0.005$, Student's t test). We further examined the effects of IGF-IR tyrosine kinase inhibitors, AG1024, on survivin expression and the apoptotic response of HNSCC cells to SCH66336. We observed substantially enhanced inhibition of cell proliferation in TR146 and UMSCC38 cells treated with SCH66336 and AG1024 (Fig. 4*D*). Moreover, Western blot analysis revealed that combined treatment with AG1024 and SCH66336 increased the level of active caspase-3 in TR146 and UMSCC38 cells (Fig. 4*D*, *bottom*), suggesting that survivin expression induced by the IGF-IR pathway protected the cells from apoptosis.

SCH66336 induces protein synthesis of survivin in HNSCC cells through the IGF-IR/Akt/mTOR pathway. We investigated whether activation of the IGF-IR/Akt pathway is involved in the expression of SCH66336-induced survivin in HNSCC cells. SCH66336-induced increases in pAkt, p4E-BP1, and survivin expression were all suppressed by the combined treatment of SCH66336 plus LY294002 but not with SCH66336 plus U0126 (Fig. 5*A*). Moreover, when the PI3K pathway was blocked by the LY294002 treatment, caspase-3 was activated (Fig. 5*A*) and cell proliferation was further inhibited by SCH66336 (Fig. 5*B*). In contrast, combined treatment with U0126 did not affect cell proliferation or the expression of survivin or active caspase-3 (Fig. 5*A* and *B*).

Because mTOR, which is activated by the IGF-IR/Akt pathway, plays a key role in mRNA translation (37), we investigated whether survivin expression was enhanced by the activation of mTOR. Indeed, phosphorylation of the eukaryotic translation initiation factor 4E-BP1, a substrate of mTOR

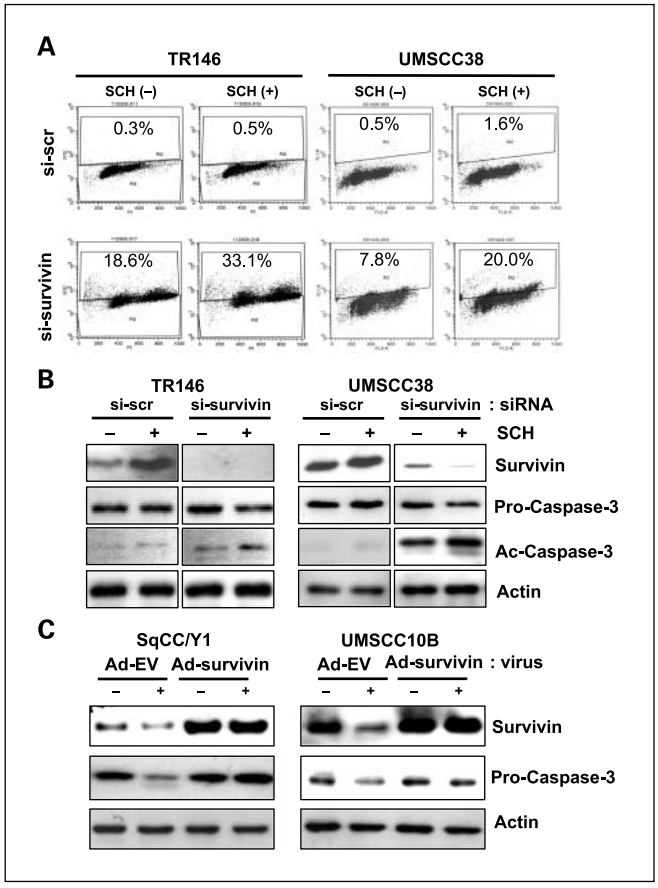

Fig. 3. Survivin mediates resistance to SCH66336 in cancer cells. Flow cytometry–based terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (*A*) and Western blotting (*B*) in TR146 and UMSCC38 cells after transfection with control siRNA (*si-scr*) or survivin siRNA (*si-survivin*) followed by treatment with 5 μmol/L SCH66336 for 48 h. *C,* Western blotting in SqCC/Y1, UMSCC10B, and SqCC35 cells infected with adenoviral vector expressing survivin (*Ad-survivin*) or empty vector (*Ad-EV*) followed by 5 μmol/L SCH66336 for 48 h. Ac-caspase-3, active caspase-3.

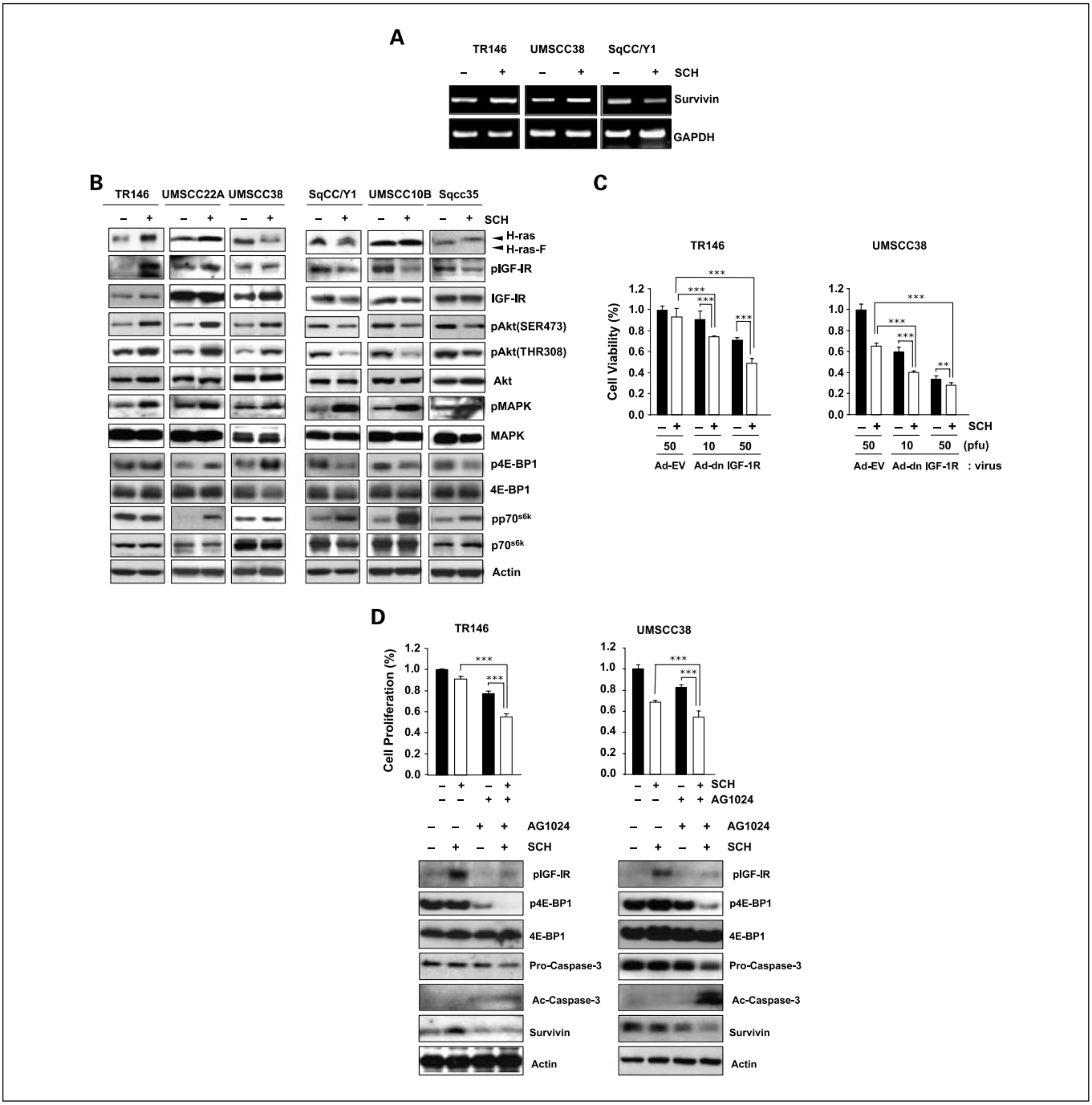

Fig. 4. SCH66336 induced survivin expression through the IGF-IR pathway. *A,* reverse transcription-PCR analysis of survivin in TR146, UMSCC38, and SqCC/Y1 cells after treatment with 5 μmol/L SCH66336 for 48 h. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the control. *B,* Western blotting in a subset of HNSCC cells incubated for 48 h in the medium containing 10% FBS with or without 5 μmol/L SCH66336. *C,* proliferation of TR146 and UMSCC38 cells after adenoviral vector (*Ad-EV*) or adenoviral vector expressed truncated α-subunit of IGF-IR (*Ad-dnIGF-IR*) into the medium after treatment with 5 μmol/L SCH66336 for 48 h. Cell proliferation (72 h; *D*) and Western blotting (48 h; *D, bottom*) in TR146 and UMSCC38 cells after 5 μmol/L SCH66336 and IGF-IR tyrosine kinase inhibitor AG1024 treatment. **, $P < 0.01$; ***, $P < 0.001$, compared with indicated control.

activation (Fig. 4*B*), was well correlated with the induction of survivin expression (Fig. 2*C*). In contrast, the level of pp70^{s6k} expression increased in all of the HNSCC cell lines (Fig. 4*B*). Decreased levels of pAkt, p4E-BP1, pp70^{s6k}, and survivin expression (Fig. 5*C*), as well as notably enhanced antiproliferative effects (Fig. 5*D*), were also shown in TR146 cells treated with rapamycin. Interestingly, combined treatment with AG1024 plus SCH66336 also decreased the protein levels of p4E-BP1 in TR146 and UMSCC38 cells, activated caspase-3 expression, and suppressed survivin expression (Fig. 4*D*). Therefore, it is plausible that the activation of IGF-IR induced by SCH66336 treatment stimulated mTOR-mediated protein synthesis of survivin, which in turn protected HNSCC cells from apoptosis.

Discussion

Numerous studies have shown the critical role of the IGF-IR/Akt pathway in the survival of cancer cells against the cytotoxic effects of chemotherapy, radiotherapy, and several molecular targeting agents (20, 34, 35, 38). In this study, we showed that SCH66336 induced activation of IGF-IR and its downstream mediators Akt and mTOR, resulting in survivin protein synthesis that mediates resistance to the apoptotic activities of SCH66336 in HNSCC cells. Consequently, inhibition of the IGF-IR/Akt/mTOR signaling pathway decreased survivin expression, resulting in restoration of the apoptotic activities of SCH66336 in HNSCC cells.

Much evidence has identified survivin as a prognostic marker for a variety of human cancers (39, 40), including HNSCC (26, 29, 39). Recent findings have shown that survivin mediates resistance to cancer therapy; overexpression of survivin is correlated with resistance to radiotherapy or chemotherapy (39, 41, 42). The inhibition of survivin expression by siRNA or the expression of mutant survivin (T34A) sensitized breast, cervical, prostate, lung, and colorectal cancer cells to apoptotic stimuli (26, 43). In these studies, the suppression of survivin expression and/or activity inhibited tumor development through a dual mechanism of inducing tumor cell apoptosis and suppressing angiogenesis (43). Based on these findings, we hypothesized that survivin expression may be involved in the sensitivity to SCH66336 treatment. In support of our hypothesis, our results showed the important role of survivin in the resistance of HNSCC cells to the apoptotic response of SCH66336. We found that (*a*) SCH66336 induced IGF-IR activation and survivin expression in a subset of HNSCC and NSCLC cell lines, which inversely correlated with the sensitivity of the cells to the apoptotic activities of the drug; (*b*) the blockade of IGF-IR by the use of AG1024 or Ad-dnIGF-IR or the inactivation of Akt by the use of LY294002 was sufficient to suppress SCH66336-induced phosphorylation of Akt and survivin expression and to restore apoptotic activities of the drug; (*c*) decreased survivin expression by specific siRNA led to increased susceptibility to apoptosis induced by SCH66336; and (*d*) the overexpression of survivin rendered SCH66336-sensitive HNSCC cells resistant to the apoptotic activities of the drug. These findings indicate that induced expression of survivin through the IGF-IR/Akt/mTOR pathway is responsible for the sensitivity of the HNSCC cells to SCH66336. Because the IGF-IR signaling pathway plays a major role in cell proliferation and survival, inhibition of the pathway was expected to decrease cell viability. Indeed, the treatment with Ad-dnIGF-IR or AG1024 effectively decreased the expression of phosphorylated IGF-IR and viability of TR146 and UMSCC38 cells in a dose-dependent manner. Blockade of IGF-IR signaling by these treatments also blocked the SCH66336-induced activation of IGF-IR and significantly enhanced the apoptotic

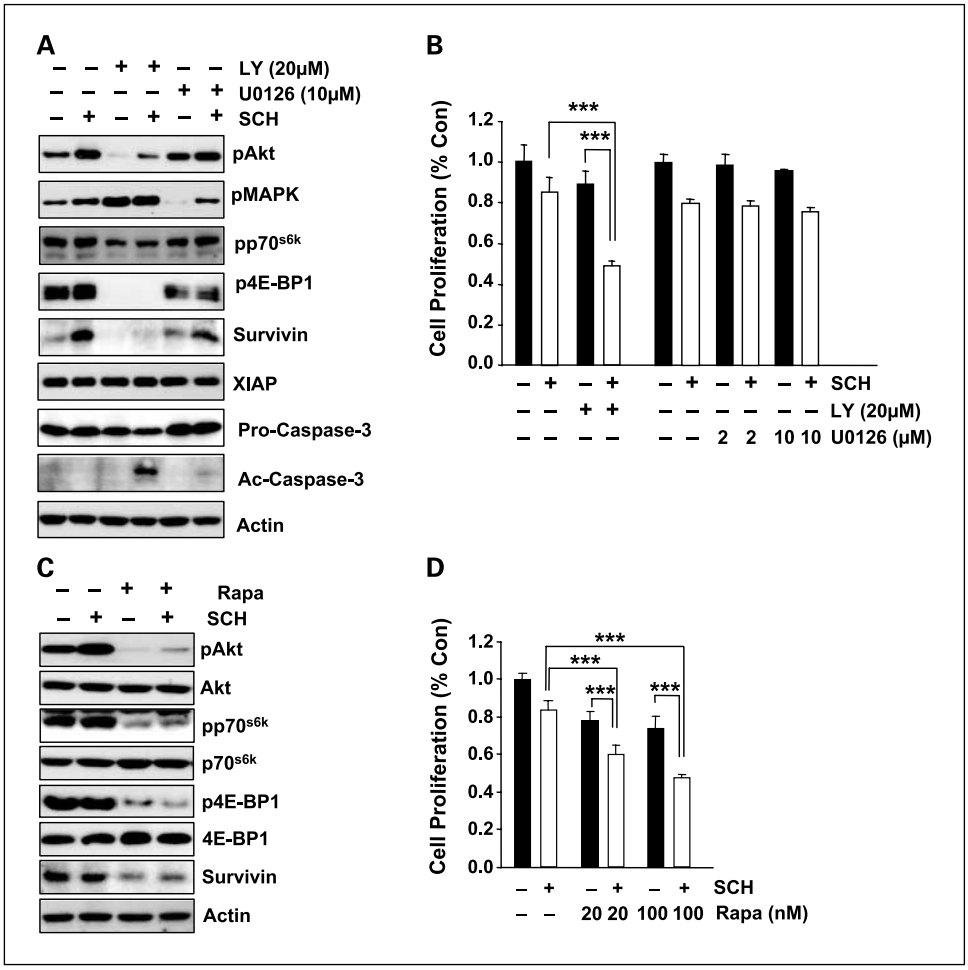

Fig. 5. Inhibition of Akt/mTOR pathway enhances the apoptotic effect of SCH66336 in HNSCC cells. Western blot analysis (48 h; *A*) and cell proliferation (72 h; *B*) in TR146 cells after treatment with 5 μmol/L SCH66336 plus the PI3K inhibitor LY194002 (*LY*) or 5 μmol/L SCH66336 plus the MAPK/extracellular signal-regulated kinase kinase inhibitor U0126. Ac-caspase-3, cleaved caspase-3. Western blot analysis (48 h; *C*) and cell proliferation (72 h; *D*) of TR146 cells after treatment with 5 μmol/L SCH66336 plus 100 nmol/L mTOR inhibitor rapamycin (*Rapa*). ***, $P < 0.001$, compared with indicated control.

activities of the drug. Although blockade of IGF-IR signaling with AG1024 effectively reduced phosphorylated IGF-IR expression, the effects of the drug treatment on phosphorylated 4E-BP1 and survivin expression were mild, probably due to the other signaling pathways that influenced the activation/expression of 4E-BP1 and survivin. In contrast, direct blockade of survivin by the use of siRNA almost completely abolished survivin expression, induced apoptosis on its own, and sensitized the cells to apoptotic activities of SCH66336, indicating an important role of survivin in preventing apoptosis in HNSCC cells. We, along with other researchers, have shown that the induction of survivin is involved in the survival of cancer cell lines against several therapeutic agents; overexpression of survivin protects a variety of cancer cells from the apoptotic activities of gefitinib (44), erlotinib (45), deguelin (46), Taxol (47), and radiotherapy (48). Therefore, the mechanism of survivin overexpression does not seem to depend specifically to FTI.

We addressed the role of mTOR in protein translational regulation of survivin. We were surprised to find that treatment with SCH66336 resulted in the phosphorylation of 4E-BP1, indicative of mTOR activation, in the HNSCC cell lines resistant to the apoptotic activities of the drug. Moreover, combined treatment with SCH66336 plus rapamycin, a mTOR inhibitor, decreased survivin expression and provoked apoptosis in HNSCC cells with weak SCH66336 sensitivity. Therefore, resistance and sensitivity to SCH66336 in HNSCC may be determined at least in part by the ability of the cancer cells to stimulate IGF-IR/Akt/mTOR–mediated synthesis of survivin expression, which protects HNSCC cells from apoptosis. Of interest, rapamycin treatment suppressed pAkt levels in TR146 cells. The effects of rapamycin on the decreased level of pAkt expression in these HNSCC cell lines contradict the previous results showing an increase in pAkt level in leukemia, prostate, and breast cancer cells after the rapamycin treatment (49, 50). However, recent reports have also shown that prolonged rapamycin treatment suppresses mTOR complex 2 assembly and consequently inhibits Akt phosphorylation in cancer cells, endothelial cells, or mice when treated for >24 h (51). We also observed an unexpected activation of p44/42MAPK in the SCH66336-treated HNSCC cell lines, which is not consistent with the previous finding showing inactivation of p44/42MAPK in glioma cells and Ras-transformed Rat 2 fibroblast after the SCH66336 treatment (52, 53). This unexpected MAPK activation was also observed in NSCLC cells treated with SCH66336 (54), and the molecular mechanism of this activation was not elucidated. These findings indicate that the response of cancer cells to molecularly targeted therapy varies depending on the cellular context.

In conclusion, we have described, to the best of our knowledge for the first time, that the activation of the IGF-IR/Akt/mTOR pathway and consequent induction of survivin expression play an important role in inducing HNSCC cell survival against SCH66336 treatment. Further, pharmacologic inhibition or genetic manipulation of IGF-IR, PI3K, or mTOR pathways sensitized HNSCC cells to SCH66336 through the inhibition of survivin expression. Multiple mechanisms, including activity/expression of Akt and Bcl-xL (20), IGF-binding protein-3 (12, 55), nuclear factor-κB (33), and death receptor 5 (56), have been suggested to be involved in the apoptotic activities of SCH66336. For patients with SCH66336-resistant HNSCC, this newly identified resistance mechanism against SCH66336 could provide the basis for new therapeutic strategies, such as combinations of SCH66336 and inhibitors of IGF-IR, Akt, mTOR, and survivin. Further studies are warranted to investigate the mechanism of differential regulation of IGF-IR activation in HNSCC cells. In addition, clinical trials are needed to validate whether such combined treatments could enhance the objective response and survival rates in patients with HNSCC.

References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 1999;80:827–41.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
3. Martinez JC, Otley CC, Okuno SH, Foote RL, Kasperbauer JL. Chemotherapy in the management of advanced cutaneous squamous cell carcinoma in organ transplant recipients: theoretical and practical considerations. Dermatol Surg 2004;30:679–86.
4. Browman GP, Hodson DI, Mackenzie RJ, Bestic N, Zuraw L. Choosing a concomitant chemotherapy and radiotherapy regimen for squamous cell head and neck cancer: a systematic review of the published literature with subgroup analysis. Head Neck 2001;23:579–89.
5. Katz ME, McCormick F. Signal transduction from multiple Ras effectors. Curr Opin Genet Dev 1997;7:75–9.
6. Mendelsohn J. The epidermal growth factor receptor as a target for cancer therapy. Endocr Relat Cancer 2001;8:3–9.
7. Rodriguez-Viciana P, Warne PH, Dhand R, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 1994;370:527–32.
8. Massarelli E, Liu DD, Lee JJ, et al. Akt activation correlates with adverse outcome in tongue cancer. Cancer 2005;104:2430–6.
9. Campbell PM, Der CJ. Oncogenic Ras and its role in tumor cell invasion and metastasis. Semin Cancer Biol 2004;14:105–14.
10. Crul M, de Klerk GJ, Beijnen JH, Schellens JH. Ras biochemistry and farnesyl transferase inhibitors: a literature survey. Anticancer Drugs 2001;12:163–84.
11. O'Regan RM, Khuri FR. Farnesyl transferase inhibitors: the next targeted therapies for breast cancer? Endocr Relat Cancer 2004;11:191–205.
12. Oh SH, Kim WY, Kim JH, et al. Identification of insulin-like growth factor binding protein-3 as a farnesyl transferase inhibitor SCH66336-induced negative regulator of angiogenesis in head and neck squamous cell carcinoma. Clin Cancer Res 2006;12:653–61.
13. Han JY, Oh SH, Morgillo F, et al. Hypoxia-inducible factor 1α and antiangiogenic activity of farnesyltransferase inhibitor SCH66336 in human aerodigestive tract cancer. J Natl Cancer Inst 2005;97:1272–86.
14. Basso AD, Mirza A, Liu G, Long BJ, Bishop WR, Kirschmeier P. The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity. J Biol Chem 2005;280:31101–8.
15. Ashar HR, James L, Gray K, et al. The farnesyl transferase inhibitor SCH 66336 induces a G(2)→M or G(1) pause in sensitive human tumor cell lines. Exp Cell Res 2001;262:17–27.
16. Nagasu T, Yoshimatsu K, Rowell C, Lewis MD, Garcia AM. Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res 1995;55:5310–4.
17. Lantry LE, Zhang Z, Yao R, et al. Effect of farnesyltransferase inhibitor FTI-276 on established lung adenomas from A/J mice induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 2000;21:113–6.
18. Adjei AA, Erlichman C, Davis JN, et al. A phase I trial of the farnesyl transferase inhibitor SCH66336: evidence for biological and clinical activity. Cancer Res 2000;60:1871–7.
19. Eskens FA, Awada A, Cutler DL, et al. Phase I and pharmacokinetic study of the oral farnesyl transferase inhibitor SCH 66336 given twice daily to patients with advanced solid tumors. J Clin Oncol 2001;19:1167–75.
20. Chun KH, Lee HY, Hassan K, Khuri F, Hong WK, Lotan R. Implication of protein kinase B/Akt and Bcl-2/Bcl-XL suppression by the farnesyl transferase inhibitor SCH66336 in apoptosis induction in squamous carcinoma cells. Cancer Res 2003;63:4796–800.
21. Blumenschein G, Ludwig C, Thomas G. A randomized phase III trial comparing lonafarnib/carboplatin/paclitaxel versus carboplatin/paclitaxel (CP) in chemotherapy-naive patients with advanced or metastatic non-small cell lung cancer (NSCLC) [abstract O-082]. In: Proceedings from the 11th World Conference on Lung Cancer, Barcelona, Spain, 2005.
22. Sharma S, Kemeny N, Kelsen DP, et al. A phase II trial of farnesyl protein transferase inhibitor SCH 66336, given by twice-daily oral administration, in

patients with metastatic colorectal cancer refractory to 5-fluorouracil and irinotecan. Ann Oncol 2002;13:1067–71.
23. Borthakur G, Kantarjian H, Daley G, et al. Pilot study of lonafarnib, a farnesyl transferase inhibitor, in patients with chronic myeloid leukemia in the chronic or accelerated phase that is resistant or refractory to imatinib therapy. Cancer 2006;106:346–52.
24. Krause CJ, Carey TE, Ott RW, Hurbis C, McClatchey KD, Regezi JA. Human squamous cell carcinoma. Establishment and characterization of new permanent cell lines. Arch Otolaryngol 1981;107:703–10.
25. Carney DN, Gazdar AF, Bepler G, et al. Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Res 1985;45:2913–23.
26. Mesri M, Wall NR, Li J, Kim RW, Altieri DC. Cancer gene therapy using a survivin mutant adenovirus. J Clin Invest 2001;108:981–90.
27. Alley MC, Scudiero DA, Monks A, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988;48:589–601.
28. Liu M, Bryant MS, Chen J, et al. Antitumor activity of SCH 66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and wap-ras transgenic mice. Cancer Res 1998;58:4947–56.
29. Altieri DC. Validating survivin as a cancer therapeutic target. Nat Rev Cancer 2003;3:46–54.
30. Mitsiades CS, Mitsiades N, Poulaki V, et al. Activation of NF-κB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 2002;21:5673–83.
31. Peng X-H, Karna P, Cao Z, Jiang B-H, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1α signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 2006;281:25903–14.
32. Mirza A, McGuirk M, Hockenberry TN, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21:2613–22.
33. Takada Y, Khuri FR, Aggarwal BB. Protein farnesyltransferase inhibitor (SCH 66336) abolishes NF-κB activation induced by various carcinogens and inflammatory stimuli leading to suppression of NF-κB-regulated gene expression and up-regulation of apoptosis. J Biol Chem 2004;279:26287–99.
34. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res 2002;62:200–7.
35. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J Natl Cancer Inst 2001;93:1852–7.
36. Lee CT, Park KH, Adachi Y, et al. Recombinant adenoviruses expressing dominant negative insulin-like growth factor-I receptor demonstrate antitumor effects on lung cancer. Cancer Gene Ther 2003;10:57–63.
37. Vaira V, Lee CW, Goel HL, Bosari S, Languino LR, Altieri DC. Regulation of survivin expression by IGF-1/mTOR signaling. Oncogene 2007;26:2678–84.
38. Jiang K, Coppola D, Crespo NC, et al. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. Mol Cell Biol 2000;20:139–48.
39. Grabowski P, Kuhnel T, Muhr-Wilkenshoff F, et al. Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma. Br J Cancer 2003;88:115–9.
40. Miller M, Smith D, Windsor A, Kessling A. Survivin gene expression and prognosis in recurrent colorectal cancer. Gut 2001;48:137–8.
41. Monzo M, Rosell R, Felip E, et al. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. J Clin Oncol 1999;17:2100–4.
42. Kennedy SM, O'Driscoll L, Purcell R, et al. Prognostic importance of survivin in breast cancer. Br J Cancer 2003;88:1077–83.
43. Blanc-Brude OP, Mesri M, Wall NR, Plescia J, Dohi T, Altieri DC. Therapeutic targeting of the survivin pathway in cancer: initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis. Clin Cancer Res 2003;9:2683–92.
44. Morgillo F, Kim WY, Kim ES, Ciardiello F, Hong WK, Lee HY. Implication of the insulin-like growth factor-IR pathway in the resistance of non-small cell lung cancer cells to treatment with gefitinib. Clin Cancer Res 2007;13:2795–803.
45. Morgillo F, Woo JK, Kim ES, Hong WK, Lee HY. Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib. Cancer Res 2006;66:10100–11.
46. Jin Q, Menter DG, Mao L, Hong WK, Lee H-Y. Survivin expression in normal human bronchial epithelial cells: an early and critical step in tumorigenesis induced by tobacco exposure. Carcinogenesis 2007. In press.
47. Pratt MA, Niu MY, Renart LI. Regulation of survivin by retinoic acid and its role in paclitaxel-mediated cytotoxicity in MCF-7 breast cancer cells. Apoptosis 2006;11:589–605.
48. Lu B, Mu Y, Cao C, et al. Survivin as a therapeutic target for radiation sensitization in lung cancer. Cancer Res 2004;64:2840–5.
49. Ikezoe T, Nishioka C, Bandobashi K, et al. Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells. Leuk Res 2007;31:673–82.
50. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006;66:1500–8.
51. Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 2006;22:159–68.
52. Glass TL, Liu TJ, Yung WK. Inhibition of cell growth in human glioblastoma cell lines by farnesyltransferase inhibitor SCH66336. Neuro-oncol 2000;2:151–8.
53. Brassard DL, English JM, Malkowski M, Kirschmeier P, Nagabhushan TL, Bishop WR. Inhibitors of farnesyl protein transferase and MEK1,2 induce apoptosis in fibroblasts transformed with farnesylated but not geranylgeranylated H-Ras. Exp Cell Res 2002;273:138–46.
54. Janmaat ML, Rodriguez JA, Gallegos-Ruiz M, Kruyt FA, Giaccone G. Enhanced cytotoxicity induced by gefitinib and specific inhibitors of the Ras or phosphatidyl inositol-3 kinase pathways in non-small cell lung cancer cells. Int J Cancer 2006;118:209–14.
55. Lee HY, Chun KH, Liu B, et al. Insulin-like growth factor binding protein-3 inhibits the growth of non-small cell lung cancer. Cancer Res 2002;62:3530–7.
56. Sun SY, Liu X, Zou W, Yue P, Marcus AI, Khuri FR. The farnesyltransferase inhibitor lonafarnib induces CCAAT/enhancer-binding protein homologous protein-dependent expression of death receptor 5, leading to induction of apoptosis in human cancer cells. J Biol Chem 2007;282:18800–9.