Id-1 activation of PI3K/Akt/NFκB signaling pathway and its significance in promoting survival of esophageal cancer cells

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Abbreviations: Id-1, inhibitor of differentiation or DNA binding; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; NFκB, nuclear factor kappa B; GSK3, glycogen synthase kinase 3β; IκB, inhibitor of kappa B; ESCC, esophageal squamous cell carcinoma; MDM2, mouse double minute 2; mTOR, mammalian target of rapamycin; PARP, poly (ADP-ribose) polymerase; TUNEL, TdT-mediated dUTP nick-end labeling; TNF-α, tumor necrosis factor-α.

Introduction

Inhibitor of differentiation or DNA binding (Id-1) is a member of the helix-loop-helix proteins. It lacks the basic domain for DNA binding and functions as a dominant inhibitor of the basic helix-loop-helix transcription factors by forming heterodimers, thus inhibiting gene expression (1). Increased expression of Id-1 has been found in many types of human cancer including esophageal squamous cell carcinoma (ESCC) (2). Recently, Id-1 has been shown to play an important role in the regulation of cell proliferation and survival in human cancers (3,4). In addition, Id-1 promotes invasion and metastasis of human cancers (5), and the level of Id-1 protein expression is associated with poor prognosis in several types of cancers (6,7). Id-1, therefore, may play a critical role in tumorigenesis and cancer progression. The poor prognosis in several types of cancers (5), and the level of Id-1 protein expression is associated with poor prognosis in several types of cancers (6,7). Id-1, therefore, may play a critical role in tumorigenesis and cancer progression. The poor prognosis in several types of cancers (5), and the level of Id-1 protein expression is associated with poor prognosis in several types of cancers (6,7). Id-1, therefore, may play a critical role in tumorigenesis and cancer progression. Id-1 induces serum-independent cell proliferation that is associated with inactivation of the p16/IpR pathway (3,8,9). The growth promoting function of Id-1 also involves activation of the mitogen-activated protein kinase (MAPK) signaling pathway in prostate cancer cells (3,10). In our recent paper, we reported that Id-1-induced cell proliferation of esophageal cancer cells was associated with up-regulation of mouse double minute 2 (MDM2), but not the key members of the p16/Rb pathway (11). Our findings suggest that the oncogenic function of Id-1 in esophageal cancer may preferentially involve signaling pathways different from that of other cancers.

Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase that generates second messengers involved in regulation of a wide spectrum of cellular functions including proliferation, survival and invasion (12). One of its major effectors is Akt (protein kinase B). The PI3K/Akt pathway is frequently activated in many types of human cancers including ESCC (13–15), and has been linked to cancer development for some time. The pathway controls several growth-regulatory transcription factors. One of the prominent examples is nuclear factor kappa B (NFκB) (16,17), a heterodimeric transcription factor that is sequestered in the cytoplasm as an inactive form by inhibitor of kappa B (IκB) (18). Phosphorylation of IκB frees NFκB, and allows its nuclear translocation, binding and subsequent activation of target genes (19). In addition to being involved in immune and inflammatory responses, NFκB also regulates cell proliferation, apoptosis and migration, and is constitutively activated in a number of human cancers including ESCC (20–22). Therefore, these evidences suggest that the PI3K/Akt/NFκB signaling pathway may be associated with tumorigenesis in ESCC. However, the mechanism responsible for PI3K/Akt/NFκB activation in ESCC is largely unknown.

Esophageal cancer ranks as the eighth most common cancer in the world (23), and ESCC is the most common form throughout the Asia-Pacific region. Since both Id-1 over-expression and PI3K/Akt/NFκB activation are frequent events in ESCC, and are associated with tumor progression of ESCC (14,15,21,24), we investigated whether there is any association between Id-1 and PI3K/Akt/NFκB signaling. We examined the effects of ectopic and down-regulated Id-1 expression on the PI3K/Akt/NFκB pathway and the anti-apoptotic role of Id-1 in ESCC cells. In addition, we studied the effects of pharmaceutical inhibitors of PI3K/Akt and NFκB on the anti-apoptotic function of Id-1 in ESCC cells. Our results suggest that Id-1 induces the activation of the PI3K/Akt/NFκB signaling pathway, leading to increased resistance to tumor necrosis factor-α (TNF-α)-induced apoptosis.

Materials and methods

Cell culture and drugs

Human ESCC cell line, HKESC-3, was established from a well-differentiated ESCC (25). The cell line has serum-dependent Id-1 expression. The establishment of stable Id-1-expressing clones and empty vector control clones from this cell line was described previously (11). The pooled stable transfectants, designated HKESC-3-Id-1 and HKESC-3-pBabe, respectively, and another ESCC cell line KYSE510 with high endogenous Id-1 expression even under serum-starved condition (26) were maintained in RPMI 1640 (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37°C in 5% CO2. The HEK293 cell line, obtained from American Type Culture Collection (Rockville, MD), was maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum. TNF-α (PeproTech EC, London, UK) was diluted in culture medium to obtain the desired concentration. Unless specified otherwise, the HKESC-3-Id-1 and HKESC-3-pBabe cells were cultured in serum-free medium for 24 h before treatment or collection.

Expression vector and transient transfection

The vector containing full-length Id-1 cDNA (pDNA3-Id-1) or empty vector control (pDNA3) (27) was transiently transfected into HEK293 cells using Fugene 6 transfection reagent (Roche Diagnostics, GmbH, Mannheim, Germany).
according to the manufacturer’s protocol. Cells were collected 48 h after transfection for Western blot.

**Knock down of Id-1 expression using siRNA**

A siRNA duplex targeting Id-1 (si-Id-1) was purchased from Dharmacon (Chicago, IL) and dissolved in RNase-free distilled water. The siRNA target site is 5'-UAACAGGUGCUCUCUAAGC-3', and the si-genome duplex RNA sequence was validated previously to successfully inhibit Id-1 in a nasopharyngeal carcinoma cell line (28). The Dharmacon siCONTROL non-targeting siRNA (catalog number D-001210-02) was used as irrelevant siRNA control (si-CON). Cells were seeded into six-well plates and left for 24 h until ~50% confluent. A 5 μl aliquot of siRNA solution (20 μM) and 4 μl of Lipofectamine 2000 (Invitrogen) were each mixed with 250 μl of RPMI 1640 culture medium. The two mixtures were combined and incubated for 20 min at room temperature, then added to the cells to give a final concentration of 100 nM siRNA. The siRNA-transfected cells were used 48 h post-transfection.

**Preparation of nuclear extracts**

Cells (5 × 10⁶) collected after trypsinization were washed with ice-cold phosphate-buffered saline and the pellet resuspended in 200 μl of ice-cold Buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 2.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol, 1 μg/ml NaF and 0.5 mM phenylmethylsulfonlfyl fluoride). After incubation on ice for 15 min, 10 μl of 1% Nonidet P-40 was added. The mixture was left at room temperature for 4 min, and then vortexed vigorously for 20 s. The cell nuclear pellet was collected by centrifugation at 3000 rpm for 3 min, washed with 100 μl of Buffer B, and collected again after centrifugation. The pellet was then resuspended in 50 μl of Buffer C (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.9, 0.4 M NaCl, 1 mM ethylenediaminetetraacetic acid, 2.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol, 1 μg/ml NaF and 1 mM phenylmethylsulfonlfyl fluoride), shaken vigorously at 4°C for 15 min and then centrifuged at 14,000 r.p.m. for 15 min at room temperature. The supernatant was recovered as nuclear extract and then transferred to a fresh Eppendorf tube containing 10% v/v of glycerol. The protein concentration was assayed using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) before use for Western blotting or electrophoretic mobility shift assay.

**Western blot analyses**

Preparation of whole-cell lysates and immunoblotting were described previously (11,29). The primary antibodies used include p-Akt (Ser-473), Akt, p-glycogen synthase kinase 3β (GSK3β) (Ser-9), GSK3β, p-IκB (Ser-32/36), caspase-3, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell signaling Technology, Beverly, MA), and Id-1, Id-2, Id-3, Id-4, NFκB/p65, histone H1 and actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Electrophoretic mobility shift assay**

NFκB DNA-binding activity was detected using an EMSA kit (Panomics, Redwood, CA) according to manufacturer’s instruction. Briefly, the nuclear extract was incubated in 1 × binding reaction mixture including biotin-labeled double-stranded NFκB consensus oligonucleotide for 30 min at 20°C. The mixture was separated on a non-denaturing polyacrylamide gel and then transferred to a nylon membrane (Amersham). The membrane was baked for 1 h at 80°C in a dry oven, then transferred to a UV crosslinker oven for 3 min. The shifted bands corresponding to the protein–DNA complexes were visualized relative to the unbound dsDNA. The bands were visualized after exposure to BioMax Light Film (Kodak, Rochester, NY).

**TdT-mediated dUTP nick-end labeling assay**

End labeling of exposed 3'-OH ends of DNA fragments was undertaken with the TdT-mediated dUTP nick-end labeling (TUNEL) in situ cell death detection kit Fluorosecin (Roche Diagnostics) as described by the manufacturer. Briefly, the cells were fixed with 4% paraformaldehyde for 1 h, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min before incubating in TUNEL reaction mixture. The cells were subsequently stained with 4',6-diamidino-2-phenylindole and visualized under a fluorescence microscope with a ×40 objective. Eight representative areas were randomly selected. At least 500 4',6-diamidino-2-phenylindole-positive cells were scored. The percentage of apoptotic cells was determined by dividing the number of TUNEL-positive cells by the total number of cells (4',6-diamidino-2-phenylindole-positive cells) in the corresponding area.

**Results**

**Effect of Id-1 over-expression and silencing on Akt signaling pathway**

Esophageal cancer cells with stable ectopic Id-1 expression (HKESC-3-Id-1) and the vector control cells (HKESC-3-pBabe) were used to investigate whether ectopic Id-1 expression had any effect on the Akt signaling pathway. These pooled populations of stably transfected clones were derived from parental cells that showed nearly undetectable Id-1 expression under serum-free condition (Figure 1A). Since Akt is activated through phosphorylation (30), we detected the expression of the Ser 473 phosphorylated form of Akt and its downstream target, GSK3β, in the cell lysates. As shown in Figure 1B, there was increased phosphorylation of Akt and GSK3β in HKESC-3-Id-1, although the total levels of each protein remained similar to
those of the vector control (HKESC-3-pBabe). To eliminate the possibility that the activation of Akt pathway was due to the drug selection during the establishment of the stable cell lines, we repeated the experiments by transiently transfecting HEK293 cells with different amounts of Id-1 expression vector. As shown in Figure 1C, phosphorylated Akt was up-regulated in these cells, and the increase corresponded to the level of Id-1 expression. We next determined the effect of Id-1 gene silencing on Akt signaling pathway. Inhibition of Id-1 expression was achieved through transient transfection with siRNA against Id-1 (si-Id-1) in KYSE510, an ESCC cell line with high inherent Id-1 expression (Figure 1A). The siRNA used was specific for Id-1, and did not affect the expression of other Id proteins. Compared with control cells transfected with an irrelevant siRNA (si-CON), the pooled si-Id-1 transiently transfected cells showed decreased expression of the phosphorylated form of Akt and GSK3β (Figure 1D). Taken together, these results indicate that Id-1 activates Akt signaling pathway, and that this function is not confined to ESCC cells.

**PI3K is required for Id-1 activation of Akt signaling pathway**

Since activated Akt is the predominant and essential mediator of PI3K function (31), we hypothesized that Id-1 activates Akt through the PI3K signaling pathway. To examine whether PI3K was involved in the induction of Akt in the Id-1-over-expressing cells, esophageal cancer cells with stable ectopic Id-1 expression (i.e. HKESC-3-Id-1) were treated with a PI3K-specific inhibitor LY294002. As shown in Figure 2A, treatment with LY294002 attenuated Id-1-induced phosphorylation of Akt and GSK3β in HKESC-3-Id-1, indicating the significance of PI3K in the Id-1 activation of Akt pathway. To confirm this observation, we transiently transfected HEK293 cells with different amounts of Id-1 expression vector and studied the effects on the expression levels of Akt, GSK3β and their phosphorylated forms in the presence of LY294002. Figure 2B shows that inhibition of PI3K with LY294002 abolished the dose-dependent up-regulation of p-Akt and p-GSK3β by Id-1 seen in a parallel experiment without the addition of LY294002. These data show that PI3K is involved in the Id-1 induction of Akt activity.

**Effect of Id-1 expression on NFκB signaling pathway**

We next investigated whether Id-1 expression had any effect on NFκB activity. Since NFκB translocates to the nucleus upon activation, we studied the expression of the most abundant subunit of NFκB, p65, in the nuclear extracts and whole lysates of Id-1-over-expressing cells and the vector control cells by Western blot. As shown in Figure 3A, there was increased nuclear translocation of p65 in HKESC-3-Id-1, as indicated by increased p65 expression in the nuclear extract, compared with that of the HKESC-3-pBabe vector control cells. Since phosphorylation of IκB is required for NFκB activation (19), we also compared the expression level of the phosphorylated form of IκB in the whole-cell lysates of the two cell lines, and found increased phosphorylated IκB in HKESC-3-Id-1 (Figure 3A). Moreover, transient transfection of HEK293 cells with increasing amounts of Id-1 expression plasmids resulted in a dose-dependent increase in nuclear p65 (Figure 3B), thus demonstrating that Id-1 was directly involved in the activation of NFκB signaling pathway. Furthermore, electrophoretic mobility shift assay showed that the HKESC-3-Id-1 cells had higher NFκB DNA-binding activity than the empty vector control cells (Figure 3C, lane 3 compared with lane 2). We also tested the effect of Id-1 gene silencing on NFκB in the KYSE510 ESCC cells transiently transfected with si-Id-1 and found decreased phosphorylation of IκB and reduced nuclear translocation of p65 (Figure 3D). Taken together, these observations support that Id-1 functions as an upstream regulator of the NFκB signaling pathway.

**Id-1-induced NFκB activation is mediated through PI3K/Akt**

Since NFκB is a downstream target of PI3K/Akt signaling pathway (16,17), and our results above indicated that Id-1-activated both PI3K/Akt and NFκB, we hypothesized that PI3K/Akt signaling pathway is required for the Id-1 activation of NFκB. As shown in Figure 3E, inhibition of PI3K activity in HKESC-3-Id-1 cells using the PI3K inhibitor LY294002 resulted in reduced phosphorylation of Akt and IκB, as well as decreased nuclear translocation of p65 (lane 3). On the other hand, treatment with the NFκB inhibitor Bay11-7082 attenuated phosphorylation of IκB and nuclear translocation of NFκB subunit p65, but did not affect Akt activity (lane 4). This suggests that Id-1-induced activation of NFκB is mediated through the PI3K/Akt signaling pathway.

**Id-1 protects ESCC cells from TNF-α-induced apoptosis through activation of PI3K/Akt/NFκB signaling pathway**

It has been documented that the PI3K/Akt/NFκB signaling pathway plays a role in preventing cells from undergoing apoptosis (32). Since our results indicated that Id-1-induced up-regulation of the PI3K/Akt/NFκB signaling pathway in ESCC cells, we reasoned that activation of PI3K/Akt/NFκB by Id-1 may protect ESCC cells against apoptosis. The parental cell line HKESC-3 was tested for sensitivity to TNF-α (up to 200 ng/ml) in serum-free medium (Figure 4A). We then treated HKESC-3-Id-1 (consisting of pooled stable clones of Id-1 over-expressing ESCC
cells) and HKESC-3-pBabe (vector control cells) with TNF-α at a dose of 50 ng/ml for up to 24 h. As shown in Figure 4B and C, the treatment elicited a significantly weaker apoptotic response in HKESC-3-Id-1, compared with the vector control (4.8 versus 24.5% apoptotic cells at 24 h). Western blot analysis of apoptosis-related proteins, caspase 3, PARP and their cleaved fragments in the cell lysates at different time points showed increasing levels of cleaved fragments of caspase 3 and PARP in the vector control cells, which was not apparent in the Id-1-over-expressing cells (Figure 4D). These results demonstrated that the Id-1-over-expressing cells were more resistant to TNF-α-induced apoptosis than the control cells. The time course experiment also showed a rapid and marked elevation of phosphorylated Akt and nuclear p65 in the HKESC-3-Id-1 cells, and the increase was sustained throughout the remaining duration of the treatment. In contrast, the vector control cells showed a relatively delayed and weaker stimulation of Akt phosphorylation, and only a transient increase in p65 nuclear translocation. Notably, the p65 expression level in the nuclear extract of the control cells returned to basal level at 4 h, prior to increased apoptosis indicated by elevated expression levels of cleaved fragments of caspase 3 and PARP.

Fig. 3. Effects of Id-1 modulation on NFκB signaling pathway. (A) Id-1-over-expressing ESCC cells (HKESC-3-Id-1) and vector control cells (HKESC-3-pBabe) were compared for the expression of phosphorylated IκB (p-IκB) and nuclear p65 by Western blot. Nuclear histone and actin were included as loading controls. The Id-1-over-expressing cells showed up-regulated p-IκB and increased nuclear translocation of p65, indicating increased NFκB activity, compared with vector control cells. (B) HEK293 cells transiently transfected with increasing doses of Id-1 expression vector were also compared for nuclear p65 expression, and the increase was found to be proportional to the level of Id-1 expressed. (C) Electrophoretic mobility shift assay was carried out to determine the effect of Id-1 on NFκB DNA-binding activity. Binding activity was indicated by a labeled shifted band at the top of the gel. Higher binding activity was evident in the nuclear extract/DNA mixture of HKESC-1-Id-1 (lane 3), compared with that of HKESC-3-pBabe (lane 2). In lane 4, addition of excess unlabeled dsDNA probe eliminated the shifted band, thus confirming that the intensity of the shifted bands was a measure of NFκB DNA binding. (D) The effect of Id-1 gene silencing on NFκB activity was assessed in the KYSE510 ESCC cells transiently transfected with siRNA against Id-1 by Western blot. Knock down of Id-1 expression through transfection with siRNA resulted in reduced p-IκB and nuclear p65, indicating suppression of NFκB activity. (E) HKESC-3-Id-1 cells were treated with 50 μM PI3K inhibitor LY294002, 20 μM NFκB inhibitor Bay11-7082 or vehicle (dimethyl sulfoxide) for 12 h. Western blot showed decreased expression of p-IκB and reduced nuclear translocation of p65 in LY294002-treated cells (lane 3), compared with untreated (lane 1) and vehicle-treated cells (lane 2). Treatment with NFκB inhibitor Bay11-7082 did not affect Akt activity, but decreased phosphorylation of IκB and nuclear translocation of p65.
seen in Figure 4. Compared with HKESC-3 (Figure 4A), the KYSE510 cell line was more resistant to TNF-α treatment (Figure 5A), which made it a good model for studying the effect of Id-1 gene silencing on apoptotic response. As shown in Figure 5B and C, knock down of Id-1 expression in KYSE510 cells transiently transfected with si-Id-1 significantly increased the sensitivity to TNF-α-induced apoptosis.

Fig. 4. Up-regulation of Id-1 induced PI3K/Akt/NFκB signaling pathway and protected ESCC cells from TNF-α-induced apoptosis. (A) HKESC-3 parental cells showed dose-dependent increase in percentage of apoptotic cells after treatment with 50–200 ng/ml TNF-α for 24 and 48 h. (B–D) Pooled Id-1 stable transfectant (HKESC-3-Id-1) and vector control cells (HKESC-3-pBabe) were treated with TNF-α (50 ng/ml) for up to 24 h. (B) The Id-1-over-expressing cells had significantly lower percentage of apoptotic cells than the vector control at 12 and 24 h after TNF-α treatment (*P < 0.001, Student’s t-test). Data represent mean ± SD of three independent experiments. (C) Apoptotic cells were detected by TUNEL staining (green) and nuclei of all cells counterstained with 4',6-diamidino-2-phenylindole (blue). (D) Phosphorylation of Akt, nuclear p65 as well as apoptotic markers were detected using Western blot, with the inclusion of actin and nuclear histone as loading controls. Note rapid and pronounced induction of Akt and NFκB activities in HKESC-3-Id-1, compared with the vector control. The control cells showed elevated levels of cleaved caspase-3 and cleaved PARP at later time points, indicating increased apoptosis.
apoptosis. Moreover, whereas induction of Akt and NFκB activities was sustained after 12 h of TNF-α treatment in the control cells, the effect was more transient in the si-Id-1 transfected KYSE510 cells and was followed by increased expressions of cleaved caspase 3 and PARP (Figure 5D).

To provide further proof that activation of PI3K/Akt/NFκB mediates the anti-apoptotic effect of Id-1, we investigated whether suppression of PI3K/Akt/NFκB could increase the sensitivity of Id-1-over-expressing cells to TNF-α-induced apoptosis in HKESC-3-Id-1 cells (Figure 6). In the presence of TNF-α, LY294002 treatment
one of the mechanisms responsible for protecting ESCC cells from activation of the PI3K/Akt/NFκB signaling pathway. Treatment with Bay11-7082 did not affect Akt but suppressed NFκB activity, indicated by down-regulation of phosphorylated IκB and nuclear p65. The results also demonstrated that, in the presence of TNF-α, regardless of whether the PI3/Akt or the NFκB part of the PI3/Akt/NFκB pathway was inhibited, there was increased apoptosis, accompanied by increase in cleaved caspase 3 and PARP. Taken together, these results illustrate that PI3/Akt acts upstream of NFκB, and that this pathway plays a role in mediating the anti-apoptotic effect of Id-1.

**Discussion**

In this study, our results indicate for the first time that Id-1 induces activation of the PI3/Akt/NFκB signaling pathway, which may be one of the mechanisms responsible for protecting ESCC cells from TNF-α-induced apoptosis.

Several novel points arise from this study. First, we provide the first evidence that Id-1 is an upstream regulator of the PI3/Akt pathway (Figures 1 and 2). Our finding that Id-1 activates PI3/Akt is significant since activation of PI3/Akt is a frequent event in many types of human cancers (13,20), but the mechanisms underlying its activation are not fully understood. Recently, it was reported that the Env1 oncogene increases the sensitivity of colon cancer cells to taxol-mediated apoptosis through activation of PI3/Akt (33). Whether a similar association exists between the PI3/Akt pathway and other known oncoproteins warrants further investigation.

Second, in discovering an association between Id-1 and PI3/Akt pathway, we might have identified an important missing link in the downstream signaling of Id-1 that is responsible for some of its oncogenic functions. One of downstream target implicated in the anti-apoptotic function of Id-1 in cancer cells is NFκB, but the association has only been demonstrated in prostate cancer cells (29). Our results showed that Id-1 over-expression induced phosphorylation of IκB, nuclear translocation of p65 and NFκB DNA-binding activity in ESCC and HEK293 cells confirmed this (Figure 3), but a critical question remains as to how Id-1 activates NFκB. Since our data showed that ectopic Id-1 expression also activated the Akt pathway and protected ESCC cells from TNF-α-induced apoptosis (Figures 4 and 5), and treatment with specific PI3K and NFκB inhibitors indicated that PI3/Akt and NFκB pathways acted upstream of NFκB (Figure 6), we believe that the PI3/Akt pathway serves as the link between Id-1 and NFκB in promoting cancer cell survival. Previously, we reported that Id-1 up-regulates MDM2 and promotes cell proliferation in ESCC (11). Since MDM2 is a direct target of Akt (12) and is known to induce cell proliferation by directly stimulating E2F1 (34), it is possible that Id-1 acts through PI3/Akt and MDM2 to drive G1/S progression. In addition to cell cycle stimulation and anti-apoptotic function, Id-1 is known to promote invasion and metastasis of breast cancer cells (35,36), and PI3/Akt/NFκB is documented to be involved in the regulation of cellular invasion and metastasis (12,37), it may be one of the mechanisms by which Id-1 exerts its effects on cancer progression.

Third, our findings that endogenous Id-1 expression could be inhibited by siRNA to suppress the PI3/Akt/NFκB signaling pathway and hence increase the sensitivity of ESCC cells to TNF-α-induced apoptosis suggest a potentially novel therapeutic strategy for esophageal cancer. Although PI3/Akt/NFκB is unlikely to be the sole mediator of Id-1-dependent cell survival, its functions in regulating cell cycle, cell growth, driving tumorigenesis and imparting chemoresistance to anticancer treatment makes it an attractive target for cancer therapy (12). A number of candidate drugs targeting this pathway, such as inhibitors of PI3K, epidermal growth factor receptor, platelet-derived growth factor receptor and mammalian target of rapamycin (mTOR), as well as monoclonal HER2 antibody, have been studied. Although the PI3K inhibitors wortmannin and LY294002 have been extensively evaluated in cultured cells as research tools, the non-selectivity of these compounds within the PI3K family and the short half-life of wortmannin and poor water solubility of LY294002 have limited their clinical use. Rapamycin, an inhibitor of the Akt downstream mTOR, also has poor aqueous solubility and chemical stability, although it has significant anti-proliferative activity in several murine tumor systems (38). A rapamycin analog, CCI-779, with improved pharmaceutical properties and comparable efficacy was approved in phase I and II of clinical studies; phase III trials are in progress (39). However, inhibitors of mTOR may not block all the functions of PI3/Akt pathway because they only affect one of the many downstream pathways of PI3/Akt signaling. While new reagents targeting this pathway are being developed and tested, perhaps, consideration should be given to targeting Id-1 as an alternative strategy in cancer therapy since evidence to date indicates that Id-1 has multiple effects on tumor progression including tumor growth, invasion, angiogenesis, metastasis and drug resistance. This notion is supported by a study showing that systemically targeting Id-1 expression using antisense Id-1 cDNA reduced the metastatic spread of 4T1 breast cancer cells in syngeneic BALB/c mice (5). From a therapeutic standpoint, since Id-1 is over-expressed in many types of human cancer but present at very low levels in normal tissues, targeting Id-1 may provide a ‘safe’ alternative strategy in cancer therapy.
low levels in normal adult tissues (2), inhibition of Id-1 should have very little side effects on normal tissues. Furthermore, with the development of improved delivery systems in RNA interference technology and the recent success in application of therapeutic siRNA in non-human primates (40), RNAi-based therapeutic reagents targeting Id-1 may be a promising alternative or adjunct to cytotoxic chemotherapy for esophageal cancer.

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