Inhibitor of Differentiation 1 Contributes to Head and Neck Squamous Cell Carcinoma Survival via the NF-κB/Survivin and Phosphoinositide 3-Kinase/Akt Signaling Pathways

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Abstract

Purpose: A key issue in cancer is apoptosis resistance. However, little is known about the transcription factors that contribute to cellular survival of head and neck squamous cell carcinoma (HNSCC).

Experimental Design: Three batches (54, 64, and 38) of HNSCC specimens were used for cellular and molecular analyses to determine the major molecular signaling pathways for cellular survival in HNSCC. Animal models (cell culture and xenografts) were used to verify the importance of apoptosis resistance in HNSCC.

Results: Inhibitor of differentiation (Id) family member, Id1, was significantly upregulated in clinical HNSCC specimens and acted to protect keratinocytes from apoptosis. Transfection of HNSCC cells with Id1 in vitro induced the phosphorylation of Akt (p-Akt) via phosphoinositide 3-kinase and increased the expression of survivin via NF-κB. Blockage of both pathways by specific inhibitors (LY294002 and IκBαM, respectively) abrogated Id1-induced cell survival of keratinocytes. In vivo studies showed that increased expression of Id1 allowed nontumorigenic keratinocytes (Rhek-1A) to become tumorigenic in nude mice by increased expression of survival genes such as p-Akt and survivin. More importantly, short interfering RNA for Id1 significantly reduced HNSCC tumor volume of HNSCC in xenograft studies. Analysis of clinical data verified the importance of the Id1 downstream molecule, survivin, in the prognosis of HNSCC patients.

Conclusions: The above data, taken together, suggest that Id1 and its downstream effectors are potential targets for treatment of HNSCC because of their contribution to apoptosis resistance. Clin Cancer Res; 16(1); 77–87. ©2010 AACR.

Cancer cells are by nature apoptosis resistant. Currently, the reasons for head and neck squamous cell carcinoma (HNSCC) apoptosis resistance are poorly understood. Under normal conditions, there are two major pathways for cellular apoptosis in cancer. The first is the mitochondrial pathway characterized by intracellular caspase activation with cytochrome c participation. This is under the control of inhibitor of apoptosis proteins (IAP), which inhibit caspases (1), and is known as the intrinsic pathway. The second is the death receptor (DR) signaling pathway triggered by extracellular factors [exemplified by tumor necrosis factor-α (TNF-α)] and is known as the extrinsic pathway.

The expression level of TNF-α is elevated in the milieu of HNSCC (2). However, cancer cells survive in such a high TNF-α–enriched environment perhaps with limited cellular apoptosis possibly due to the NF-κB activation in HNSCC (3), which regulates IAPs (4–6), including survivin and Bcl-2 (7–9). This suggests that cellular survival plays a critical role in HNSCC through cellular apoptosis blockade.

Inhibitor of differentiation 1 (Id1) is a transcription factor that has been identified in esophageal SCC and is related to distant metastasis within 1 year of esophagectomy (10). However, little is known about this transcription factor involved in HNSCC cellular survival. Id1 is a transcription factor that reduces cellular differentiation and increases cellular proliferation. Additionally, it is potentially upregulated in squamous cancer compared with normal skin in a very preliminary case series of squamous cancer (11). However, Id1 may also be a candidate gene for cellular survival of HNSCC, as Id1 is served as an oncogene in many tumors (12–14) and is involved in prostate cancer survival (15) and esophageal SCC (10). We have recently reported that Id1 increases the proliferation of keratinocytes within 24 hours in vitro (16) and esophageal SCC (10) but arrests cell growth thereafter, suggesting its involvement in cellular survival.
Translational Relevance

Further investigations into head and neck cancer causation will allow for the development of novel therapies designed to intervene at newly identified targets. Apoptosis resistance is very important in head and neck cancer, as no therapies currently abrogate apoptosis resistance as a second-line treatment for failed primary disease. The current study focuses on inhibitor of differentiation 1 (Id1) and effector molecules that are likely associated with apoptosis resistance in this disease. The findings of the current study suggest that Id1 and its effectors are important in the apoptosis resistance and are potential targets for therapy of head and neck cancer. The relevance is increased because the findings are reverse translated from a cohort of genically identified poor outcome patients. Next, the findings were confirmed in preclinical molecular studies, and then the concepts were applied to other patient cohorts. This study suggests that Id1-mediated signaling is an important pathway for apoptosis resistance in head and neck cancer.

Recent studies indicate that Id1 is possibly linked to NF-κB in keratinocytes (16, 17) and prostate epithelial cells (7). NF-κB has been shown to increase the resistance of HNSCC cell lines to radiation (8) and improve survival of lymphoma cells in vitro (9). Therefore, it is possible that Id1, via NF-κB, increases HNSCC apoptosis resistance. In addition, a recent study indicated that the expression of survivin (baculoviral IAP repeat-containing 5) is under the control of NF-κB (18). Survivin, a member of the IAP family, is not detectable in normal tissues but highly upregulated in certain cancers such as adenocarcinomas of the lung, pancreas, colon, breast, and prostate (19–23). We know that survivin is also highly expressed in HNSCC (24–26), but we do not know whether survivin is under the control of Id1 via NF-κB in HNSCC.

Finally, increases in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway are also reported to aid in apoptosis resistance (27). Akt phosphorylates proapoptotic factors such as BAD and procaspase-9 as well as forkhead transcription factor family that induces the expression of proapoptotic factors such as Fas ligands (27), making them inactive or downregulated. Whether this pathway is important for tumorigenesis of HNSCC is poorly understood at the present time.

In this study, we hypothesized that Id1 is linked to the survival of HNSCC via regulation of the NF-κB/survivin and PI3K/Akt pathways following the initial effects on cellular proliferation. Our in vivo and in vitro data showed that Id1 upregulated survivin via a NF-κB–dependent mechanism and simultaneously activated the Akt pathway via PI3K, contributing to keratinocyte survival. In addition, Rhek-1A, a nontumorigenic keratinocyte cell line (28), became tumorigenic in mice after stable transfection with Id1. Additionally, inhibition of Id1 expression in HNSCC with short interfering RNA (siRNA) significantly reduced xenografted tumor growth in nude mice. These findings, taken together, show the existence of an Id1/NF-κB/survivin/Akt signaling axis in head and neck cancer apoptosis resistance.

Materials and Methods

Clinical specimens. Surgical specimens (first batch: 41 HNSCC and 13 normal tissues) were collected from the clinical patients who underwent surgery at the Department of Otolaryngology, University of Minnesota Hospital and Clinics, and used for microarray analysis, as previously published (29). This database was interrogated with respect to genes of interest in the present study. Control specimens were biopsies of normal tissues close to the cancer site. Total RNA from 54 tissue specimens was isolated using Trizol (Invitrogen) for microarrays. The second batch of specimens (64 HNSCC and 12 vocal cord polyps) was collected from clinical patients who underwent surgery at the Department of Otolaryngology, Sun Yat-sen University, and used for evaluation of survivin by immunohistochemistry and clinical prognosis analysis. The third batch of 50 surgical specimens (38 HNSCC and 12 normal tissues) was collected from the Department of Otolaryngology, University of Minnesota, and used for dual-labeling immunohistochemistry for Id1, NF-κB p65 subunit, phosphorylated Akt (p-Akt), and survivin. All specimens and clinical data in this study were procured, handled, and maintained according to the protocols approved by each Institutional Review Board.

Cell culture. Three cancer or squamous cell lines, CA9-22, Rhek-1A, and HOK16B, were used in this study. CA9-22 is a cell line established from oral cancer tissue (30, 31), HOK16B is a cell line derived from human papillomavirus transfection of keratinocytes in the oral cavity (32), and Rhek-1A is a cell line established from the human foreskin and immortalized with SV40 large T antigen and Kirsten sarcoma viruses (33). CA9-22 was maintained in RPMI 1640 (Life Technologies, Invitrogen), HOK-16B was maintained in keratinocyte basal medium (Lonza), and Rhek-1A was maintained in Eagle’s MEM (Invitrogen; ref. 34). During transient transfection of cells, Opti-MEM (Invitrogen) was used (hereafter referred to as transfection medium).

Molecular reagents. The Id1 cDNA from a HNSCC specimen was cloned into a plasmid with enhanced green fluorescent protein (Clontech; ref. 35), and Id1 siRNA was constructed in a similar way, as previously described (36). The p65 cDNA was cloned as previously described (37, 38). IκBα mutant (IκBαM; a kind gift of Dr. Inder Verma, Salk Institute, La Jolla, CA), a dominant-negative NF-κB (39, 40), is a superrepressor of NF-κB activity. Pyrrolidine dithiocarbamate (PDTC) was used as an alternative inhibitor of the NF-κB activity (41). Camptothecin is a universal inducer of apoptosis. A specific PI3K inhibitor, LY294002, was used as a specific PI3K inhibitor.
Immunohistochemistry. HNSCC and control tissues were fixed in 10% formalin, cut in a thickness of 4 μm, deparaffinized, and incubated for 90 min with the following primary antibodies: Id1 from Santa Cruz Biotechnology (1:400 dilution), NF-κB p65 subunit from Abcam (ab7970, 1:100 dilution), an "activated" form of p65 from Chemicon (MAB 3026, 1:10 dilution), and survivin from R&D Systems (1:400 dilution). Sections were then washed and incubated with FITC- and TRITC-conjugated secondary antibodies (IgG, Zymed) using the protocols as previously described (36, 42). Dual-labeled immunohistochemistry was done, as previously described (43), to determine coexpression of Id1 and NF-κB p65 subunit, Id1 and survivin, as well as p-Akt and survivin on clinical HNSCC specimens (batch 3) and/or mouse xenografted tumors.

Affymetrix microarrays. Affymetrix microarrays were done as previously described (29). Briefly, cDNA, prepared from 10 μg of total RNA using the double-strand DNA synthesis kit (Invitrogen), was reverse transcribed into cRNA and labeled with biotin-streptavidins using the BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics). Biotin-streptavidin–labeled cRNA was hybridized to Affymetrix U133 Plus 2.0 arrays according to the manufacturer's protocol. Arrays were scanned using the Affymetrix GeneChip Scanner 3000, and data were collected using the Affymetrix GeneChip Operating Software (GCOS) version 1.4. Biotin-streptavidin–labeled cRNA was hybridized to Affymetrix U133 Plus 2.0 arrays according to the manufacturer's protocol. Arrays were scanned using the Affymetrix GeneChip Scanner 3000, and data were collected using the Affymetrix GeneChip Operating Software (GCOS) version 1.4. Biotin-streptavidin–labeled cRNA was hybridized to Affymetrix U133 Plus 2.0 arrays according to the manufacturer's protocol. Arrays were scanned using the Affymetrix GeneChip Scanner 3000, and data were collected using the Affymetrix GeneChip Operating Software (GCOS) version 1.4. Biotin-streptavidin–labeled cRNA was hybridized to Affymetrix U133 Plus 2.0 arrays according to the manufacturer's protocol. Arrays were scanned using the Affymetrix GeneChip Scanner 3000, and data were collected using the Affymetrix GeneChip Operating Software (GCOS) version 1.4.
was then hybridized to the human U133A arrays. Expression of the Id1, Id2, Id3, Id4, PI3K, Akt1, Akt2, Akt3, NF-κB subunits, Bcl-2, and survivin genes in the HNSCC tissue specimens was identified as previously described (36). A pathway analysis tool module (PEGG) was used for evaluation of cell cycle progression and apoptosis. The results were presented as "z-scores," which weigh the entire pathway gene activity and give scores for upregulated and downregulated genes separately. Important genes involved in cellular survival were individually analyzed using t test embedded in GeneSifter software and data are presented as individual heat maps or merged heat maps with P values. In addition, Affymetrix microarray analysis was done on xenografted mouse tumors and HNSCC cell lines (CA9-22, SCC9, and NA) against their respective controls, in a similar manner.

**Apoptotic assays.** Nucleic acid stain was done using Yopro-1 (Molecular Probes), which selectively passes through the plasma membranes of apoptotic cells and labels apoptotic cells green in color (44). Cells at 40% confluence were incubated with Id1 and empty vector, and apoptosis was induced by the addition of 50 μmol/L camptothecin and stained with Yopro-1. Results are presented as percentage of apoptotic cells over total cells.

**Trypan blue exclusion.** Briefly, cells were transfected in transfection medium with Id1 and empty vector at 1.4 μg/mL for 16 h, recovered in cell culture medium for 24 h, treated with and without PDTC at 50 μmol/L or IκBαM at 1.4 μg/mL, and then harvested for evaluation of cell numbers after staining with trypan blue dye. Briefly, cells were washed in PBS and incubated in 0.3 mL of 0.05% trypsin-EDTA solution for 10 min. Five microliters of the trypsin solution were mixed with 5 μL of trypan blue and transferred to a hemacytometer for counting. Results are presented as viable cells (by 1 × 10⁴).

**Fluorescence-activated cell sorting.** Cell cultures (60% confluence) were transfected in transfection medium with Id1 and empty vector at 1.4 μg/mL for 16 h, recovered in cell culture medium for 24 h, and then harvested for evaluation of antibody-stained positive cells as previously described (45). Apoptosis analysis was done with Annexin V/7-aminoactinomycin D (7-AAD) and Annexin V-allophycocyanin as instructed by the

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**Fig. 2.** Cell survival–related genes are significantly upregulated in HNSCC specimens. A compared with control tissues, HNSCC and xenografted tumor specimens showed a significant increase in mRNA transcripts for Id1, Id2, Akt3, RelB, and survivin (heat maps). Empty vector, Immunoistochemistry showed that Id1 (dark purple) was coexpressed with NF-κB (intense red, green arrows; black arrows in B indicate faint red); Id1 (red, in the nuclei) coexpressed with survivin (green, in the cytosol; C); p-Akt (red, in the cytosol) with survivin (green, in the cytosol; D); p-Akt with p65 (E, white arrowheads); and Id1 and p65 in xenografts (F, white arrowheads). F, yellow, NF-κB p65 subunit (activated form) was active in the edge of the xenografted mouse tumor and coexpressed with Id1 in the cellular nuclei. Id1 is expressed in the entire xenograft tumor, whereas active p65 is expressed only in the cells that are located in the edge of xenograft tumors. B and F, regular microscopic graphs. C, D, and E, confocal microscopic graphs. Scale bars, 10 μm.
manufacturer's manual (BD Biosciences). Results are presented as percentage of viable cells (in a total of 10,000 cells counted per sample) against total cells. Effects of empty vector and Id1 on cellular survival were determined by Yopro-1, trypan blue exclusion, and caspase-3 or Annexin V/7-AAD in this study. To study whether Id1-induced cellular survival occurs via PI3K, LY294002 at 50 μmol/L was incubated with cells transfected with Id1.

Fig. 3. Id1 is highly related to survival of Rhek-1A cells. Yopro-1 stain showed that Id1-transfected Rhek-1A cells obviously reduced the apoptotic cell numbers (B) compared with empty vector control (A). C, statistical analysis showed a significant difference of apoptotic cell numbers between empty vector–transfected and Id1-transfected cells. Id1 significantly reduced the expression of caspase-3 by FACS (D) but significantly increased the activation of Akt (p-Akt) by FACS (E). Compared with those of empty vector (F), Id1 activated NF-κB p65 subunit by immunohistochemistry using activated p65 antibody (G). H, left, IκBαM, a specific inhibitor of NF-κB, abrogated the effects of Id1 on the NF-κB activation by FACS. LY294002, a specific inhibitor of the PI3K, abrogated the Id1-expressing viable keratinocytes (7-AAD–negative and Annexin V–negative cells) by FACS (H, right), whereas PDTC and IκBαM canceled out the effect of Id1 on cellular viability by trypan blue exclusion (TBE; I). It is noted that transfection with empty vector and Id1 causes cellular apoptosis to some degree. Scale bars, 50 μm.
for 24 h and then harvested for evaluation of cellular survival by Annexin V/7-AAD.

**Luciferase assays.** Cells cultured in a 12-well plate with 60% confluence were transfected with the Id1 cDNA at 1.4 μg/mL and cotransfected with NF-κB luciferase/β-galactosidase reporters, respectively, at 1.4 μg/mL for 16 h in the transfection medium and recovered in culture medium for 24 h. Cells were harvested for luciferase assays, as previously described (46). The activity of NF-κB luciferase over β-galactosidase (internal control) is presented as a relative luciferase activity. The Tropix dual reporter kit (Applied Biosystems) was used with a Berthold TriStar flash injection luminometer.

**Tumor xenograft in nude mice.** Cells stably transfected with Id1 and empty vector for up to 6 mo were sorted via FACSMaria cell sorter (BD Biosciences) and expanded in culture. Nude mice (six per group) were inoculated with 1 × 10^6 cells via s.c. flank injection. After injection, tumor volume was measured weekly up to 10 wk. At 10 wk, tumors were harvested for evaluation of immunohistochemistry and global gene expression profiles by Affymetrix microarrays. To study the importance of Id1, a specific siRNA for Id1 in pSilencer 1.0-U6 (siRNA-A, 5′-aaccgcaagggcaagggTTCAGAGAaccttgctcaattgggtt-3′) was used for knocking down the Id1 gene in HNSCC cells. The siRNA control, made in our early study (36), was used. Briefly, CA9-22 cells, positive for Id1 expression, were stably transfected with siRNA construct, as above, and CA9-22 cells were then injected into six nude mice for tumor growth. Tumor volume was measured again, as above, from 1 to 10 wk with control cells stably transfected with nonspecific siRNA and empty vectors. For confirmation of Id1 protein inhibition in specific and nonspecific siRNA-transfected xenografts, immunohistochemistry was done using Id1 antibody and nonspecific IgG as an immunohistochemistry control.

**Statistical analysis.** The Student’s t test was used for evaluation of differences of global gene expression between controls and experiments in vitro, whereas the Kaplan-Meier survival test was used for calculation of mean and median survival time of both survivin+ and survivin− patients as well as making survival curves. Log rank (Mantel-Cox) was then used for verification of the above data. Cox regression univariate and multivariate analyses were used for evaluation of correlates between survivin expression and clinical data (HNSCC stage, differentiation, lymph node metastasis, and distal metastasis). Fisher’s exact test was also used for evaluation of clinical data when the data distribution pattern is extremely uneven (such as age and distal metastasis). P values of <0.05 were considered significant.

**Results**

**Cell cycle progression and apoptosis are imbalanced in the HNSCC specimens.** To evaluate the overall activities of cell cycle and apoptosis pathways, we used a pathway analysis parameter (z-score, a normal z-score usually between +2 and −2 as defined by GeneSifter). Normal z-scores for both cell cycle progression and apoptosis were close to 0 when 13 controls were randomly split into two groups and compared with each other. Similarly, the z-scores for both cell cycle and apoptosis within the HNSCC samples (intragroups) were −1.59 to +0.41 and −0.63 to +0.15 when the 41 HNSCC specimens were randomly split into...
two groups and compared with each other. These data indicate that z-scores for the cell cycle progression and apoptosis vary little within intragroups, either control or cancer groups. However, the z-scores varied dramatically between intergroups (HNSCC versus control specimens). Among 100 genes studied on the U133A chips in the cell cycle, the expression of 34 genes was altered (>1.5-fold, with z-score from 4.70 to −2.07) compared with Id1-transfected cells (vector controls). These data suggest that many HNSCC cells go through the cell cycle and proliferate but few cells undergo apoptosis. To exclude the possibility of DR downregulation, we also analyzed the extrinsic pathway on these HNSCC microarrays. It was found that genes involved in the extrinsic pathway were upregulated in HNSCC versus control specimens (e.g., TNF-α/Fas/TNF-related apoptosis-inducing ligand/DR5/Fas-associated via death domain/Mort1; Fig. 1). This suggests that apoptosis resistance in HNSCC cannot be attributed to the downregulation of the extrinsic pathway.

**Genes specific to cellular survival pathways are significantly upregulated in the HNSCC specimens.**

The results from this initial interrogation of the microarray data led us to postulate that cell survival–related genes might be upregulated. Consequently, we then interrogated the Affymetrix microarray data for cell survival pathway–related genes. The following individual cell survival genes were significantly upregulated in the clinical HNSCC versus control specimens: Id1, Id2, PI3K, Akt3, NF-κB2, Ret B, survivin, and Bcl-2A1 (Fig. 2A). Next, to determine a preliminary link between the above genes (id1, p-Akt, NF-κB, and survivin), dual-labeled immunohistochemistry was done on 50 HNSCC specimens (from batch 3). As expected, Id1 and activated NF-κB, Id1 and survivin, p-Akt and survivin, as well as p-Akt and activated NF-κB were coexpressed in the HNSCC specimens and/or xenografted tumor sections. Representative data are shown in Fig. 2B to F. The normal mucosal tissues occasionally showed staining for Id1 and NF-κB but not survivin (Supplementary Data 1). To examine whether Id family members (Id1, Id2, and Id3) are extensively expressed in HNSCC specimens, the expression of Id1, Id2, and Id3 mRNA transcripts was examined on 41 HNSCC specimens individually. The results indicated that genes for cell survival, including Id1, Id2, Id3, survivin, Akt3, and NF-κB, were upregulated in 58% to 78% of HNSCC specimens (Supplementary Data 2). Id1 was found in both the cytosol and the nuclei of HNSCC specimens. In batch 3 specimens, survivin expression in the cytosol was counted because of its relevance to cellular survival, whereas survivin expression in the nucleus was not counted because of its nonrelevance to cellular survival.

**Id1 increases keratinocyte survival in vitro.**

To study whether Id1 could be linked to cell survival in keratinocytes, we transfected Id1 into Rhek-1A cells and evaluated the keratinocytes for several cell survival markers [apoptosis as judged by caspase-3 fluorescence-activated cell sorting (FACS) and Yopro-1 stain]. The efficacy of this transient transfection in Rhek-1A cells was approximately 57% to 70% by FACS, as previously reported (35), with an increase of Id1 mRNA by reverse transcription-PCR (data not shown). Id1 significantly reduced the apoptotic cell number of Rhek-1A as judged by Yopro-1 stain.
Id1 expression might promote cellular survival in vivo, which in turn increases apoptosis resistance, likely through an inhibition of caspase-3 activation.

Id1 induces tumor growth in nude mice. To test whether Id1 expression might promote cellular survival in vivo, Id1 was stably transfected into Rhek-1A cells and Id1-expressing clones were sorted with FACSMaria and expanded in cell culture and then injected into five nude mice at million cells per injection. Empty vector stably expanded in cell culture and then injected into five nude mice. Id1 expression was stably transfected into Rhek-1A cells and Id1-transfected Rhek-1A cells grew small tumors in 3 of 15 nude mice. The tumor volume in the Id1 groups was significantly larger than that of the empty vector groups (Fig. 4A). To study whether Id1 triggers tumor growth via increased cellular survival or cell cycle progression upregulation, the global gene expression of xenograft tumors was evaluated. Id1-expressing xenografted tumors, unlike empty vector or p65 tumors, were inactive in cell cycle progression (Fig. 4B). The genes for cellular survival, similar to several HNSCC cell lines, were significantly upregulated in the xenograft Id1 tumors compared with empty vector tumors (Fig. 4C), suggesting that Id1-induced tumor growth in nude mice is primarily via increases in cellular survival.

Id1 siRNA inhibits tumor growth in nude mice. To test the importance of Id1 in tumor growth, one of the HNSCC cell lines, CA9-22, which expresses a high level of Id1 and has a z-score similar to overexpressing Id1 tumor, was used for the Id1 gene knockdown with siRNA. Cancer cells with and without siRNA were injected into five nude mice (1 million cell flank injections). Animal experiments were done in triplicate. CA9-22 cells transfected with Id1 siRNA grew smaller tumors than those transfected with control siRNA (Fig. 4D). Simultaneously, xenografted tumors were stained with Id1 to confirm the inhibition of Id1 protein by immunohistochemistry. Nonspecific siRNA-transfected xenografts showed active Id1 protein in the nuclei, whereas specific siRNA showed an inhibitory effect on Id1 protein expression in the nuclei. Sections stained with nonspecific IgG showed no Id1 protein signals (Supplementary Data 4).

Id1 upregulates survivin via NF-κB in keratinocytes. Because survivin and Id1 were coexpressed in clinical tumors (Fig. 2), we postulated that Id1 might control the expression of survivin in HNSCC via NF-κB. To test this hypothesis, Rhek-1A cells were transfected with Id1, cotransfected with NF-κB and β-galactosidase reporters, and harvested for evaluation of NF-κB promoter activity by luciferase assays and survivin by FACS. Id1 significantly increased NF-κB promoter activity, whereas PDTC and λ-BαM abrogated the Id1-induced NF-κB promoter activity (Fig. 5A). Simultaneously, Id1 significantly increased the expression of survivin and Id1-induced survivin expression was abrogated by PDTC and λ-BαM as judged by FACS (Fig. 5B). This suggests that Id1 increases the expression of survivin via NF-κB. In addition, Id1 increased the translocation of NF-κB into the nuclei of Rhek-1A cells (Fig. 5D, activated p65) but PDTC abrogated the translocation of NF-κB into the nuclei of Rhek-1A cells by Id1 (Fig. 5E).

Expression of survivin in HNSCC is highly related to poor prognosis of clinical patients. To verify the importance of survivin expression as a potential factor of poor prognosis, we used an independent set of 64 HNSCC and 12 control specimens and stained for survivin expression (batch 3). Of the 64 clinical HNSCC specimens, 43 (67.2%) were positive for survivin in the cytosol and all the vocal cord polyps were negative for survivin by immunohistochemistry. There was a significant difference in survival time of patients between cytosol survivin+ and survivin− fractions.
(Fig. 6). Log rank (Mantel-Cox) was used for verification of the above survival time data. A significant difference has been seen between survivin+ and survivin− patients in terms of survival time (χ² = 6.498, P = 0.011). Overall mean survival time clearly indicated that patients with survivin− staining in the cytosol had a poor prognosis (mean survival time, 53.3 months; 95% confidence interval, 44.5-62.1 months), whereas patients with survivin+ had mean survival time of 74.809 months (95% confidence interval, 65.4-84.2 months). Median survival time was analyzed, but the data were not included in this study as we could not calculate 95% confidence interval for survivin− patients and median survival has not yet been achieved in this group. The correlations between survivin and clinical data (gender, age, clinical HNSCC classification, differentiation, and metastasis) are summarized in Supplementary Data 5. Association of survivin expression and clinical prognosis (Cox regression multivariate analysis) is presented in Supplementary Data 6.

Discussion

Apoptosis resistance is an important mechanism for tumorigenesis. In the present study, we determined certain antiapoptotic properties of HNSCC using a global gene expression approach using clinical HNSCC specimens in combination with relevant in vitro and growth characteristics in nude mice. The antiapoptotic properties of HNSCC are attributable to the dysregulation of Id1, p-Akt, NF-κB, and survivin. This dysregulation tilts the balance of cell survival and apoptosis. Id1 contributes to this dysregulation via the PI3K/Akt and NF-κB/survivin signaling pathways. The degree of change is sufficient to transform nontumorigenic cells into tumorigenic cells, allowing growth of tumors in nude mice, a characteristic feature of survival gene expression.

Id1-induced cell survival is in part blocked by NF-κB inhibitors (IκBαM and PDTC) or Pi3K inhibitor (LY294002). Interestingly, p65 alone mainly caused cell proliferation that is obviously different from Id1 in terms of tumorigenesis. If true, interesting future work in head and neck carcinogenesis examining the balance of cell survival versus proliferation may be designed around interactions between Id1 and NF-κB. In our clinical HNSCC specimens, approximately 60% to 75% of patients fit into this cellular survival pattern. The principal difference between xenografts and clinical specimens is that cell cycle progression was not active in Id1 xenografts compared with the clinical specimens. This difference may be attributed to genetic mutations, such as tumor suppressor p53, and epigenetic changes of clinical HNSCC.

We have noted that a constitutive increase of NF-κB by p65 stable transfection in keratinocytes is critical for cell cycle progression as judged by a pathway analysis parameter (z-score). As seen in this study, p65-induced tumor (xenografted) scored 2.82. However, a study by Hinata et al. (47) showed that NF-κB is involved in the maturation of human skin epithelial cells. Clearly, further work needs to be done on NF-κB function in normal skin epithelium and why it seems contradictory to NF-κB function in aerodigestive squamous cancer. Unlike p65, Id1-induced xenografts scored very low (~4.75) in cell cycle progression, representing a different mechanism in inducing tumor growth of nude mice characterized by cellular survival. In the current study, apoptosis is marginally increased with a z-score of 2.24 in the HNSCC specimens, perhaps attributable to the expression of a few proapoptotic genes such as BIK, BAX, and BAG2 against a background of a greater number of antiapoptotic genes.

Activation of NF-κB by survival factors in normal cells usually increases the expression of Bcl-2 but not survivin. Interestingly, activation of NF-κB by Id1 leads to the expression of survivin, instead of Bcl-2, in the xenograft tumors in this study. This may explain why Id1 transfectants are tumorigenic. As seen in this study, TNF-α is expressed in clinical HNSCC specimens but has not been shown to be apoptosis promoting preclinically in head and neck cancer (48). The reason this does not lead to increased apoptosis may be attributable to IAPs. The IAP members include X-linked IAP and cellular IAP (c-IAP). c-IAP binds to TNF receptor–associated factors (TRAF1 and TRAF2), thus interfering with activation of proteases and canceling out subsequent apoptosis. NF-κB also induces TRAF1, TRAF2, c-IAP1, and c-IAP2 to suppress caspase-8 activation (5). In addition, NF-κB prevents cellular apoptosis by suppressing PTEN expression (49) through the PI3K/Akt pathway. Inhibition of NF-κB by IκBαM attenuates resistance of human HNSCC to TNF-α caspase-mediated cell death (3). This may in part explain why increased TNF-α expression in HNSCC has a limited effect on cellular apoptosis of HNSCC.

Due to the blockage of the extrinsic pathway for cellular apoptosis, HNSCC is highly dependent on the intrinsic pathway for cell death. Unfortunately, this pathway is also inhibited. Both Id1 and NF-κB are overexpressed in HNSCC, which contributes to cellular survival. NF-κB regulates survivin (50), whereas Id1 strengthens this regulation via an increase of the NF-κB promoter activity, which contributes to an increase of NF-κB constitutively. Therefore, apoptosis for HNSCC is largely blocked in the intrinsic pathway. If this is true, one should be able to construct an in vivo animal model using Id1 as a trigger. Indeed, injection of Id1-transfected keratinocytes into nude mice induced the tumor growth likely primarily due to cellular survival. Conversely, interference of Id1 expression by siRNA in HNSCC cells reduced the tumor growth in vivo probably secondary to cell survival downregulation. However, we could not exclude the possibility that Id1 reduces the tumor volume by inhibition of angiogenesis. Our recent unpublished data indicate that Id1 contributes to angiogenesis by regulating the expression of angiogenic factors such as vascular endothelial growth factor, interleukin-8, and cyclooxygenase-2.

Id1 has recently been recognized as a clinical outcome predictor in esophageal squamous carcinoma (10). In this study, we correlated survivin with clinical outcomes...
of patients. As expected, survivin is highly related to the clinical prognosis of HNSCC. The reason why we exclusively focus on survivin instead of Id1 is in part because of a relatively small number of HNSCC specimens collected recently for Id1 protein analysis (batch 3) and in part because of the above study (10). We believe that focusing on the entire Id1/NF-κB/survivin signaling pathway or downstream key molecules specific for cellular survival is more relevant to clinical prognosis than an upstream molecule that has extensive effects on multiple signaling pathways. In addition, survivin is uniquely expressed in cancer cells but not in normal cells, whereas Id1 is mainly expressed in cancer cells but is occasionally seen in the skin basal cells and proliferating fibroblasts surrounding the tumor cells. The function of Id1 may also be offset by other helix-loop-helix transcription factors, such as E-box proteins, which are involved in cellular differentiation acting against Id1. In HNSCC, we have observed that some Id1-positive specimens are associated with well-differentiated cancer cells. This suggests that Id1 alone does not determine the cellular fate (proliferation or differentiation). It is the interaction between Id1 and its antagonists (helix-loop-helix transcription factors) that determines the cell fate. If this is true, Id1 predominant HNSCC may not necessarily be poorly differentiated but surely committed to cellular survival. High survivin levels in Id1+ patients may predict apoptosis resistance in HNSCC Id1+ but survivin- cells would also be expected to be apoptotically resistant; however, in this scenario, the action of Id1 may be attenuated by other helix-loop-helix genes that do not promote apoptosis resistance. This requires further investigation.

In summary, these data support the rationale of pharmacologic inhibition of the Id1/NF-κB/survivin or Id1/PI3K/Akt pathways for HNSCC cancer therapy and suggest that inhibition of Id1 or its downstream molecule survivin removes the protection of HNSCC cells from apoptosis. Therefore, these HNSCC properties may be of significant clinical utility for head and neck cancer radiochemosensitization to improve long-term patient outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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