Inhibitor of Differentiation/DNA Binding 1 (ID1) Inhibits Etoposide Induced Apoptosis in a c-Jun/c-Fos-dependent Manner

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ABSTRACT

ID1, inhibitor of differentiation/DNA binding 1, acts an important role in metastasis, tumorigenesis and maintenance of cell viability. It has been shown that the upregulation of ID1 is correlated with poor prognosis and the resistance to chemotherapy of human cancers. However, the underlying molecular mechanism remains elusive. Here, we determined for the first time that up-regulating ID1 upon etoposide activation was mediated through AP-1 binding sites within the ID1 promoter, and confirmed that ID1 enhanced cell resistance to DNA damage-induced apoptosis in ESCC cells. Ablation of c-Jun/c-Fos or ID1 expression enhanced etoposide-mediated apoptosis through inhibiting activity of caspase 3 and PARP cleavage. Moreover, c-Jun/c-Fos and ID1 were positively correlated in human cancers. More importantly, simultaneous high expression of ID1 and c-Jun or c-Fos was correlated with poor survival in cancer patients. Collectively, we demonstrate the importance of c-Jun/c-Fos-ID1 signaling pathway in chemoresistance of esophageal cancer cells, and provide considerable insight into understanding the underlying molecular mechanisms in ESCC cell biology.

INTRODUCTION

Esophageal cancer remains one of the most virulent malignancies with ranking eighth in incidence and sixth in cancer-related mortality worldwide (1). These malignancies are particularly prevalent in China and other countries in Asia, where esophageal squamous cell carcinoma (ESCC) is most common (1). A 5-year overall survival (OS) rate has not been improved
evidently in spite of the progressed surgical techniques and incorporation of new therapeutic approaches in the past decades (2). Adjuvant chemotherapy for ESCC could reduce postoperative recurrence and improve survival (3,4). Nevertheless, accumulating evidence shows that cancer often acquires resistance to chemotherapy after nonlethal exposure (2,5). Thus, an integrated view of chemoresistance will provide a more useful approach for designing novel therapies for this devastating disease.

ID1 is a member of the helix-loop-helix (HLH) protein family, and contribute to tumorigenesis by inhibiting cell differentiation, stimulating proliferation and facilitating tumour neoangiogenesis (6-9). ID1 was found to be overexpressed in diverse human tumour types including prostate, breast, colon and esophagus. The overexpression of ID1 is frequent (93%) in human primary ESCC (10). ID1 expression correlates directly with tumor invasion, metastasis and poor prognosis in ESCC (6,11,12). Notably, ID1 was involved in chemotherapy and radiotherapy resistance in human cancer including pancreatic adenocarcinoma, breast cancer, lung cancer, colorectal cancer and esophageal cancer, and becomes a new potential therapeutic target (13-17). ID1 is transactivated in the context of 5-FU therapy, which provides a resource for future study addressing the molecular mechanisms of chemotherapy in breast cancer (18). In the study of p53 protecting cells from arsenic caused cell cycle arrest, ID1 is more extensive induced by arsenite in p53+/+ cells rather than p53-deficient cells, which display greater resistance to arsenite-induced mitotic arrest and apoptosis (19). Recent study reported that competitive binding between ID1 and E2F1 to Cdc20 regulates E2F1 degradation and thymidylate synthase expression to promote esophageal cancer chemoresistance. The ID1-E2F1-IGF2 regulatory axis has important implications for cancer prognosis and treatment (12). These data indicate that ID1 is up-regulated by chemotherapeutic drugs and may be involved in chemoresistance. However, the mechanisms of ID1 affecting chemoresistance have yet to be elucidated.

The transcription factor AP-1 is a menagerie of dimeric basic region-leucine zipper (bZIP) proteins that recognize either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGACGTCA-3′) (20). AP-1 is a mammalian transcription factor and collectively describes a group of structurally and functionally related members of the Jun protein family (c-Jun, JunB and JunD) and Fos protein family (c-Fos, FosB, Fra-1 and Fra-2) (20,21). It has been reported that AP-1 is required for cell survival and involved in multi-drug resistance (22,23). Recent researches demonstrated that the aberrantly high levels of ID1 expression in cancer are often a consequence of transcriptional induction by many proteins which are activated in a constitutive manner in cancer cells and affect chemoresistance (24-26). Bearing in mind the key roles of AP-1 and ID1 in chemoresistance, the transcriptional regulation between ID proteins and AP-1 is of particular interest.

In this study, we report that ID1 conferred etoposide chemoresistance through inhibiting caspase 3 activity and PARP cleavage. Ablation of ID1 promoted etoposide induced apoptosis. Mechanistically, c-Jun/c-Fos bound directly to the
ID1 promoter region, and activated its transcription in vivo. Ectopic expression of c-Jun/c-Fos enhanced ID1 transactivation. Conversely, knockdown of c-Jun/c-Fos inhibited ID1 transactivation. Overexpression of ID1 rescued cells from apoptosis in c-Jun/c-Fos knockdown cells. The expression level of ID1 was positively correlated with c-Jun/c-Fos in human cancers. More importantly, analysis of gene expression profiles of multiple cancer types indicated that high expression of ID1 and c-Jun or c-Fos is correlated with poor survival in cancer patients. These findings suggested that c-Jun/c-Fos was involved in chemosensitivity pathways and contributed to the regulation of ID1 in response to chemotherapeutic drugs induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions—Human ESCC cell lines (KYSE series) were generous gifts from Dr. Shimada Y of Kyoto University. KYSE30, 140, 180 and 450 cell lines used in this study were originally established from primary esophageal squamous cell carcinoma tissue samples after surgery in ESCC patients, who had not received prior cytotoxic therapy, whereas KYSE150 were from patients who had received cytotoxic therapy previously (27). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL streptomycin and 100 U/mL penicillin.

Patient Tissue Samples—Tissue samples from 34 patients with ESCCs were used for ID1, c-Jun and c-Fos mRNA expression analysis, and patients were consecutively recruited at the Chinese Academy of Medical Sciences Cancer Hospital (Beijing). At recruitment, informed consent was obtained from each subject. This study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute and Hospital. Tissue microarray from 110 patients with ESCC was previously made in our lab (28).

Plasmids Construction and Site-Directed Mutagenesis—Full-length cDNA of human ID1 was cloned into the mammalian expression vector pLVX. The promoter region of ID1 (-2209--163) was cloned into the pGL3-basic vector, designed as ID1-pro-2000. One point mutation was introduced into target site by mutagenesis PCR. The resulting construct was verified by direct sequencing. c-Jun and TAM67 expression plasmids were generated in our laboratory. c-Fos expression plasmids were provided by Dr. Marta Barbara Wisniewska of University of Warsaw (Warsaw, Poland).

Western Blot Analysis—Western blot was performed as described previously (29). Antibodies were used as follows: ID1, cleaved caspase 3, PARP, p53, c-Jun and c-Fos (Santa Cruz, Delaware, CA, USA), and β-actin (Sigma-Aldrich, St. Louis, Mo, USA).

Immunohistochemistry—Immunohistochemistry were performed as described previously (30). The human ESCC tissue microarray was subjected to immunohistochemistry using antibodies against ID1 (Santa Cruz, Delaware, CA, USA).

siRNA Transfection, RNA Isolation and PCR Analysis—Cells were transfected with siRNAs (10nM) by HiperFect (Qiagen) following the manufacturers’ protocol. ID1 siRNA (GS3397; Qiagen), c-Jun siRNA (GS3725; Qiagen), c-Fos siRNA (GS8061; Qiagen) and negative control siRNA (1027310; Qiagen) were purchased from Qiagen. RNA purification and
qRT-PCR were performed as described previously (31). The primers used are listed in Table 1.

Chromatin Immunoprecipitation Assay and Luciferase Assay—The luciferase assay and ChIP was performed as described previously (29). The antibodies against c-Jun and c-Fos were used from Beijing Golden Bridge Biotechnology Company.

Cell Proliferation Assay—Cell proliferation assay was performed as described previously (31).

Cell Apoptosis Assay—Apoptosis assay was measured using the BD FITC Annexin V Apoptosis Detection Kit (Becton, Dickinson and Company) according to the manufacturer’s protocol. Briefly, cells were digested with trypsin-EDTA into single-cell suspensions and then collected. The resuspended cells (1x10^5) were centrifuged at 1,000 rpm for 5 min to remove the supernatant, and the cells were resuspended in 100 μl Annexin V binding solution and transferred into a 5-ml culture tube. Annexin V-FITC (3 μl) was added to the solution, and incubated at room temperature for 15 minutes in the dark, followed by addition of 400 μl Annexin V binding solution and propidium iodide (PI) (3 μl) was added for flow cytometry.

Statistical Analysis—We statistically evaluated experimental results using two-independent sample t tests, one-way analysis of variance test, and Pearson correlation analysis. Survival analysis was performed by PROGgeneV2, a web-based resource combining genomic/clinical database and analysis tools that enable single/multiple gene-based prognostic assessment (32). All tests of significance were set at P<0.05.

RESULTS

ID1 Expression was Induced by Etoposide in Esophageal Cancer Cells—Previous studies indicated that ID1 was commonly up-regulated by chemotherapeutic drug treatment (18,19). To evaluate the possible role of ID1 in ESCC, we first analyzed ID1 expression in ESCC tumor tissues and ESCC cell lines KYSE150, KYSE30, KYSE140, KYSE450, KYSE180, KYSE410. qRT-PCR and immunohistochemistry results indicated that the expression of ID1 was high in primary ESCC tumors rather than tumor-adjacent normal tissues (Fig. 1A and B). qRT-PCR and Western blot results showed that ID1 was low in KYSE150 and KYSE30, moderate in KYSE140 and KYSE450, but high in KYSE180 and KYSE410 cells (Fig. 1C). To examine whether therapeutic drug influences ID1 expression, we measured the expression of ID1 in ESCC cells treated with etoposide, and a time-dependent stimulation of endogenous ID1 in different ESCC cell lines was observed (Fig. 1D). These results indicated that ID1 might play an important role in ESCC cell resistance to etoposide.

Overexpression or Knockdown of ID1 Moderately Influences Cell Resistance to Etoposide—To evaluate the role of ID1 in response to DNA damage, we first measured IC50 of etoposide in KYSE150, KYSE140, KYSE450 and KYSE180 ESCC cells. As shown in Fig. 2A, the IC50 of KYSE180 and KYSE450 cells was higher than that of KYSE150 and KYSE140 cells which might due to increased expression of endogenous ID1. Next, we examined the effect of etoposide on KYSE150 and KYSE450 cells with ectopic expression of ID1. As shown in Fig. 2B, cell viability was significantly increased by overexpression of ID1 in response to etoposide, indicating that overexpression of ID1 enhanced cell resistance to etoposide in esophageal cancer cells. To further study whether ID1 may influence

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cellular chemoresistance, we measured the cell apoptosis in pLVX and pLVX-ID1 cells treated with etoposide. Overexpression of ID1 significantly reduced etoposide-induced cell apoptosis in KYSE450 cells (Fig. 2C). Previous study showed that ID1 indirectly repressed p53, which promotes chemoresistance in HCT116 cells (19). Consistent with the previous report, we also found that p53 was markedly decreased following etoposide treatment in ID1 stable transfectants, which further inhibited caspase 3 activity and PARP cleavage (Fig. 2D). Conversely, silencing of ID1 enhanced etoposide-induced apoptosis in KYSE450 cells (Fig. 2E and F). Taken together, overexpression of ID1 could enhance cell resistance to etoposide while knockdown of ID1 reduced the chemoresistence in ESCC cells.

Up-regulating ID1 upon Etoposide Activation Is Mediated through AP-1 Binding Sites—To explore whether increase of ID1 induced by etoposide was in transcriptional or post-transcriptional regulation, we constructed the promoter of ID1 (2kb), and transfected KYSE450 cells treated with or without etoposide to examine ID1 transcriptional activity. As shown in Fig. 3A, etoposide treatment dramatically activated the promoter of ID1 in KYSE450 cells. These data indicated that the increased expression of ID1 was due to the transcriptional activity in response to etoposide, and may involve in other transcriptional factors. Previous study revealed that AP-1 regulates responsive promoter via binding to its canonical TGAG/CTCA motif or TGACGTCA boxes located in the promoter regions of the target genes (33). To further determine the direct regulation of ID1 by AP-1, we first searched for putative AP-1 binding sites on the human ID1 promoter. Remarkably, we identified one potential AP-1 binding site at -919 to -911 bp upstream of the ID1 ATG codon. Moreover, the putative TGACGTCA site is highly conserved among different species (Fig. 3B), suggesting that ID1 might be a direct target gene of c-Jun/c-Fos. To confirm whether the putative AP-1 binding site was involved in transactivation, we next used ID1 promoter reporter constructs containing either wild-type or mutant putative AP-1 binding site. These constructs were cotransfected with or without c-Jun/c-Fos, and then luciferase activity was determined. As shown in Fig. 3C, a 5-fold increase of wild-type putative AP-1 binding site co-transfected with c-Jun/c-Fos was observed, but no significant activation of the mutant was found, indicating that c-Jun/c-Fos activates ID1 through the conserved AP-1 binding site. We also observed a 2.5-fold increase of wild-type putative AP-1 binding site treated with etoposide, but no significant activation of the mutant, indicating that etoposide-induced ID1 promoter activation requires this conserved site (Fig. 3D). To further examine whether AP-1 regulates ID1 gene transcription in vivo, we performed a quantitative ChIP assay using samples with or without etoposide treatment and antibodies against c-Jun and c-Fos. Results showed that AP-1 specifically binds the promoter region encompassing the putative conserved AP-1 binding site of the ID1 gene especially after etoposide treatment. In contrast, IgG did not precipitate detectable DNA (Fig. 3E), providing additional evidence to support the active role of c-Jun/c-Fos in ID1 gene transcription in vivo.

The Activation of ID1 Required c-Jun and c-Fos in Response to Etoposide—Recent studies demonstrated that the aberrantly high expression of ID1 in cancers is often a consequence of
transcriptional induction by many proteins which are activated in a constitutive manner (25,26). Our previous study indicated that among the AP-1 family of transcription factors, c-Jun/AP-1 could bind and activate the expression of a series of genes in esophageal cancer cells (34,35). Therefore, we speculated that transcriptional regulation by AP-1 might contribute to the underlying mechanism of ID1 involved in esophageal cancer cells response to etoposide. To clarify this, we examined c-Jun and c-Fos expression in KYSE450 cells treated with etoposide. qRT-PCR and Western blot results clearly showed that c-Jun/c-Fos and ID1 expression was induced by etoposide (Fig. 4A and B), suggesting that c-Jun and c-Fos may involve in the transcription of ID1 responding to etoposide. To examine the transcriptional mechanism, we performed experiments using c-Jun, c-Fos, TAM67 expression plasmids or c-Jun and c-Fos siRNA in KYSE450 ESCC cells. TAM67 is a dominant-negative form of c-Jun that interacts broadly with all AP-1 transcription factors to inhibit transactivation (36). As shown in Fig. 4C, ectopic expression of c-Jun/c-Fos, rather than TAM67, increased the expression of ID1 in KYSE450 cells. Moreover, compared with control groups, silencing of c-Jun/c-Fos in KYSE450 and KYSE150 cells decreased ID1 expression (Fig. 4D). These results indicated that c-Jun and c-Fos were involved in the transcriptional regulation of ID1 responding to etoposide in ESCC cells.

ID1 Inhibits Etoposide-induced Cell Apoptosis in a c-Jun/c-Fos-dependent Manner in ESCC Cells—To assess whether targeting of c-Jun/c-Fos could contribute to cellular chemoresistance to etoposide, we investigated p53-mediated cell apoptosis by siRNA against c-Jun/c-Fos under the treatment of etoposide. As shown in Fig. 5A, knockdown of c-Jun/c-Fos following etoposide treatment markedly increased the expression of p53, which enhanced activity of caspase 3 and PARP cleavage. Furthermore, we used flow cytometry to investigate cell apoptosis induced by this pathway. As shown in Fig. 5B, the pLVX control group, etoposide treatment induced cell apoptosis by 5.9% in the control cells, as opposed to 19.1% in c-Jun silencing cells and 17.8% in c-Fos silencing cells. The difference is statistically significant (P<0.01). Moreover, we rescued the expression of ID1 after ablation of c-Jun/c-Fos, and then analyzed cell apoptosis under the treatment of etoposide. As shown in Fig. 5B, compared with pLVX control cells, ID1 rescued cells could dramatically reduce cell apoptosis under etoposide treatment. Taken together, these results indicated that etoposide-induced ID1 increase was AP-1-dependent. Elimination of c-Jun/c-Fos or ID1 attenuated the effect of etoposide on the modulation of ID1 and showed the important role of the AP-1-ID1 signaling in mediating the effect of etoposide on ESCC cell apoptosis.

Positive Correlation between c-Jun/c-Fos and ID1 in Human Cancers, and Prognostic Value of High c-Jun/c-Fos and ID1 Expression for Cancer Patients Survival—To evaluate the up-regulation of c-Jun/c-Fos on ID1 expression in human cancers, we examined the mRNA levels of c-Jun/c-Fos and ID1 in diverse human tumors including ESCC, acute myeloid leukemia, ovarian cancer and colorectal cancer. As shown in Fig. 6A, the expression of c-Jun/c-Fos and ID1 are positively correlated in ESCC. The same results were obtained in three GEO database including acute myeloid leukemia (GSE12417), ovarian
cancer (GSE49997) and colorectal cancer (GSE24551). It strikingly supports a positive relationship for c-Jun/c-Fos and ID1 in cancers (Fig. 6B). To investigate the role of c-Jun/c-Fos-ID1 in cancer progression, we first analyzed clinical outcome data using PROGgeneV2 from published studies for correlations between c-Jun/c-Fos-ID1 expression levels and survival of cancer patients. Interestingly, high expression of ID1 and c-Jun or c-Fos is correlated with poor survival in cancer patients (Fig. 6B). These results indicated that concurrent high expression of c-Jun/c-Fos and ID1 may predict poor prognosis of cancer patients.

**DISCUSSION**

Recently, tumor resistance to chemotherapy remains a clinical problem. Functional screens have been directed at finding novel targets affecting sensitivity to chemotherapy, but it remains unclear whether these targets have direct roles in chemoresistance or offer prognostic value to clinicians. ID1 was involved in chemoradioresistant in human cancer, and was exploited as a therapeutic target (13,16-18). Therefore, the biological functions and mechanisms of ID1 under the chemotherapeutic drug treatment need to be intensively studied.

Recent study revealed that competitive binding between ID1 and E2F1 to CDC20 regulates E2F1 degradation and thymidylate synthase expression to promote esophageal cancer chemoresistance (12). Moreover, previous study showed that ID1 was also induced by nicotine, and ectopic expression of ID1 might enhance cell resistance to damage (13). In line with this, we found that ID1 expression was induced by etoposide in esophageal cancer cells (Fig. 1D). These results suggest that ID1 expression was induced by DNA damage response, and maybe correlated with esophageal cancer chemoresistance. Moreover, we found that KYSE180, with a higher endogenous expression of ID1 than KYSE450, KYSE140 and KYSE150 cells, displays greater resistant to etoposide (Fig. 2A). Additionally, our data indicate that overexpression of ID1 can enhance cell resistance to etoposide-induced apoptosis and knockdown of ID1 increased the percentage of the apoptosis (Fig. 2C and E). DNA damage, such as that induced by radiation or chemotherapeutic drugs, is a potent activator of p53 (37). ID1 was interacted with p53 in DNA damage response (16,38). Previous study showed that ID1 is an effector of the p53-dependent DNA damage response pathway and DEC1 represses the transcription of the ID1 gene under the treatment of doxorubicin or camptothecin (38). ID1 upregulates MDM2, a key negative regulator of p53, and promotes p53 protein degradation in human colorectal cancer cells (16). We found that upregulation of ID1 reduced p53 expression, whereas, knockdown of ID1 promoted p53-mediated caspase 3 activity and PARP cleavage under etoposide treatment (Fig. 2D and F). However, the regulatory mechanism of ID1 induced by etoposide still remains elusive.

To explore the molecular mechanism by which the expression of ID1 might be induced with etoposide treatment in ESCC cells, we first detected that etoposide dramatically activated the promoter of ID1 (Fig. 3A), indicating that etoposide induced ID1 expression was at the transcriptional level, which may involve in some transcriptional factors. Next, we explored which
transcription factor was involved in regulating ID1 expression. With computer-aided transcription factor-binding site analysis, we identified one TGACGTCA motif, and it is highly conserved among different species (Fig. 3B). The TGACGTCA motif is one of the most common regulatory elements widely distributed in promoters or enhancers, and was generally regarded as the transcription factor AP-1 family functional binding elements (33). Transcription factor AP-1 is mainly composed of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) (20,21). Previous study showed that AP-1 is required for cell survival and involved in multi-drug resistance (22,23). In this study, using ChIP and dual luciferase reporter assay, we show for the first time that c-Jun/c-Fos can directly bind to the ID1 promoter, thereby activate ID1 transcription and expression in vivo (Fig 3C, and E). Moreover, we found that etoposide increased c-Jun/c-Fos and ID1 expression (Fig. 4A and B) and ectopic expression of c-Jun or c-Fos increased ID1 expression, whereas repressed expression of c-Jun or c-Fos had the opposite effects (Fig. 4C and D). Furthermore, we found that ID1 expression positively correlated with c-Jun/c-Fos in esophageal cancer as well as a variety of other cancer types (Fig. 6A). These results showed that transcription factor AP-1 was involved in regulating ID1 transcription and expression in ESCC.

Apoptosis is a cellular process regulated by the balance of pro- and anti-apoptotic proteins (33,39). Robust and persistent activation of AP-1 in cells containing damaged DNA causes defective replication and may trigger apoptosis through the same mechanisms that induce cell death after constitutive expression of oncogenes (40). Here, we showed that ablation of c-Jun/c-Fos resulted in an increase in the percentage of apoptosis (Fig. 5A and B). Moreover, elimination of c-Jun/c-Fos with ID1 rescued cells could dramatically reduce cell apoptosis under etoposide treatment (Fig. 5B). These results suggest that ID1 inhibits etoposide-induced apoptosis in a c-Jun/c-Fos-dependent manner in human ESCC cells. However, deletion of c-Jun/c-Fos can neither completely abolish the expression of ID1 nor antagonize the effect of etoposide-induced cell apoptosis. This recalls the existence of other transcriptional factors regulating ID1 expression and other downstream targets participating in etoposide-mediated cell apoptosis.

ID1 is known to be associated positively with pathological N stage and could be considered as a prognostic predictor for stage III ESCC patients (11). ID1 and ID3 function together to govern colon cancer-initiating cell (CC-IC) self-renewal by p21 (7). It is also known to be induced by Stat3, and then upregulates MDM2 to promote p53 protein degradation (16). Moreover, ID1 increases thymidylate synthase dependent upon E2F1 to promote cancer chemoresistance (12). These findings indicate that ID1 expression associates with other molecular markers and may not be an independent prognostic factor. Here, we show that simultaneous high expression of ID1 and c-Jun or c-Fos is correlated with poor survival in human cancer patients (Fig. 6B) and further suggests that c-Jun/c-Fos-ID1 regulatory mechanism has clinical significance in human cancer.

Overall, our results are of significance in finding that AP-1 can transcriptionally regulate ID1 in DNA damage response, thus cause chemoresistance to therapeutic drugs in ESCC.
cells. ID1 expression was positively correlated with that of c-Jun and c-Fos in human cancers. More importantly, concurrent high ID1 and c-Jun/c-Fos expression in human tumors is significantly correlated with shorter survival of cancer patients. We also demonstrated the importance of c-Jun/c-Fos-ID1 signaling pathway in chemoresistance of esophageal cancer cells. This will provide an insight to target c-Jun/c-Fos-ID1 for cancer therapeutic strategies. Additionally, our results facilitate the development of innovative anti-cancer strategies and provide considerable insight into understanding the underlying molecular mechanisms in ESCC cell biology.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: Yahui Zhao, Sheng Li and Aiping Luo conducted most of the experiments, analyzed the results, and wrote most of the paper. Zhihua Liu conceived the idea for the project and designed the experiments. Wei Zhang, Hongyan Chen, Yi Li and Fang Ding provided technical assistance and contributed to the preparation of the figures. Furong Huang conducted the luciferase assay. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES
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The abbreviations used are: ESCC, esophageal squamous cell carcinoma; ID1, the inhibitor of differentiation/DNA binding 1; AP-1, activator protein-1; ChIP, chromatin immunoprecipitation; MTS, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium.
FIGURE LEGENDS

FIGURE 1. ID1 expression was induced by etoposide in ESCC cell lines. A, up-regulated ID1 mRNA level was detected in 34 tumors compared with normal adjacent epithelia by qRT-PCR (paired t-test). B, example case showed the expression of ID1 in ESCC tumors and normal counterparts by immunohistochemistry staining on the tissue microarray (upper panel). Quantitative analysis of the ID1 staining between ESCC tissues and the matched normal esophageal epithelia are shown on the lower panel (paired t-test). C, mRNA and protein level of endogenous ID1 was detected in ESCC cell lines by qRT-PCR (left panel) and Western blot (right panel). D, KYSE140, KYSE150 and KYSE450 cells were treated with 10 μM etoposide for the indicated time and harvested. ID1 expression was determined by qRT-PCR (upper panel) and Western blot (lower panel). β-actin was used as a loading control. Data are shown as mean ± SEM from multiple independent experiments, one-way analysis of variance test. *, P<0.05; **, P<0.01; ***, P<0.001.

FIGURE 2. Overexpression of ID1 enhances cellular resistance to etoposide. A, KYSE150, KYSE140, KYSE450 and KYSE180 cells were treated with increasing concentrations of etoposide for 48 hours, and then cell viability was examined by MTS assay. B, KYSE150 and KYSE450 cells stably transfected with empty vector (pLVX) or ID1 (pLVX-ID1) were incubated with DMSO (control) or etoposide (10 μM) for the indicated time, and cell growth was detected using MTS assay. Values were the means ± SD of absorbance at 490 nm for three independent experiments. C and D, ID1 transfectants and empty vector controls were treated with 10 μM etoposide for 48 hours, and then subjected to Annexin V-FITC and PI staining. Values are expressed as a percentage of Annexin V positive versus total cells (C). The expression levels of ID1, p53, cleaved caspase 3 and PARP were examined by Western blot (D). β-actin was used as a loading control. E and F, KYSE450 cells were transiently transfected with negative control (NC) or ID1 siRNA, followed by 10 μM etoposide treatment. Cells were labeled with Annexin V-FITC and PI, and analyzed by flow cytometry. Values are expressed as a percentage of Annexin V positive versus total cells (E). The expression levels of ID1, p53, cleaved caspase 3 and PARP were examined by Western blot (F). β-actin was used as a loading control. Data are expressed as Mean ± SD. *, P<0.05, **, P<0.01, one-way analysis of variance test.

FIGURE 3. Up-regulating ID1 upon etoposide activation is mediated through AP-1 binding sites. A, KYSE450 cells were transiently transfected with the promoter construct of ID1 for 24 hours, and treated with 10 μM or 20 μM etoposide. After 24 hours, the luciferase activity was determined and normalized to an internal cytomegalovirus Renilla luciferase control. B, comparison of nucleotide sequences among seven different species. The AP-1 DNA binding site is represented in shaded box. * is represented for the same nucleotide sequence. C, schematic representation depicts the location of the mutant variant in the 2kb ID1 promoter. TSS stands for transcription start site (upper panel). KYSE450 cells were
cotransfected with ID1 wild-type or mutant luciferase reporters, together with c-Jun/c-Fos or control vector for 24 h. Then luciferase activity was determined and normalized to an internal cytomegalovirus Renilla luciferase control. Data are shown as mean ± SEM from multiple independent experiments (lower panel). D, KYSE450 cells were transfected with ID1 promoter reporter construct containing either wild-type or mutant putative AP-1 binding site and treated with or without 10 μM etoposide, and then the luciferase activity was determined. E, KYSE450 cells were treated with 10 μM etoposide for 4 hours, and then ChIP assays were carried out with antibody against c-Jun, c-Fos or IgG. The percentages of input of coprecipitating DNAs were calculated by qRT-PCR. The data represent the means ± SD of triplicate experiments. *, P<0.05; **, P<0.01; ***, P<0.001, one-way analysis of variance test.

**FIGURE 4.** The activation of ID1 required c-Jun/c-Fos in response to etoposide. A and B, KYSE450 cells were treated with 10 μM etoposide for the indicated time, and the expression of c-Jun/c-Fos and ID1 were determined by qRT-PCR (A) and Western blot (B). C, KYSE450 cells were transiently transfected with c-Jun, c-Fos, c-Jun/c-Fos, TAM67 as described in experimental procedures. After 24 hours, the expression of c-Jun, c-Fos and ID1 was determined by qRT-PCR and Western blot. D, KYSE450 and KYSE150 cells were transiently transfected with c-Jun/c-Fos siRNA as described in experimental procedures. After 24 hours, the expression of c-Jun, c-Fos and ID1 was determined by Western blot. The data represent the means ± SD of triplicate experiments. **, P<0.01; ***, P<0.001, one-way analysis of variance test.

**FIGURE 5.** ID1 inhibits etoposide-induced cell apoptosis in a c-Jun/c-Fos-dependent manner. A, KYSE450 cells were transiently transfected with either negative control (NC) or c-Jun/c-Fos siRNA as indicated. After 24 hours, cells were incubated with DMSO or 10 μM etoposide. The expression of c-Jun, c-Fos, ID1, p53, cleaved caspase 3 and PARP were determined by Western blot. β-actin was used as a loading control. B, KYSE450 cells were transiently transfected with c-Jun/c-Fos siRNA, and rescued ID1 with pLVX-ID1 compared with pLVX for 24 hours. After that, cells were treated with DMSO or 10 μM etoposide, and then subjected to Annexin V-FITC and PI staining. Values are expressed as a percentage of Annexin V positive versus total cells. Data are expressed as Mean ± SD. *, P<0.05, one-way analysis of variance test.

**FIGURE 6.** Positive correlation between c-Jun/c-Fos and ID1 in human cancers, and prognostic value of high c-Jun/c-Fos and ID1 expression for cancer patients survive. A, a statistically significant positive correlation between c-Jun/c-Fos and ID1 mRNA was observed by Pearson’s method in ESCC and patients in three independent published data sets including acute myeloid leukemia (GSE12417), ovarian cancer (GSE49997) and colorectal cancer (GSE24551), Pearson correlation analysis. B, clinical outcome data was analyzed by using PROGgeneV2 from published studies for correlations between c-Jun/c-Fos-ID1 expression levels and survival of cancer patients.
Table 1. qRT-PCR primers

| qRT-PCR primers | Sequences |
|-----------------|-----------|
| ID1 mRNA F      | ACACAAGATCGATCGTTCC |
| ID1 mRNA R      | GGAATCCGAAGTTGGAACC |
| c-Jun mRNA F    | CAACATGCTCAGGGAGACAGG |
| c-Jun mRNA R    | GTTAGCATGAGTTGGCACCC |
| c-Fos mRNA F    | TTACTACCACTCACCACAGG |
| c-Fos mRNA R    | AGTGACCGTGGAATGAAAGT |
| GAPDH F         | GTCGGAGTCAACCGATTTGG |
| GAPDH R         | AAAAGCAGCCCTGATGACC |
Figure 1

A

B

Normal
Cancer

ID1

50 μm

P<0.0001 n=98

ID protein expression

C

ID1 mRNA

Relative mRNA level (RT-qPCR)

KYSE-150
KYSE-30
KYSE-140
KYSE-150
KYSE-140
KYSE-450
KYSE-410

D

ID1 mRNA

Relative mRNA level (RT-qPCR)

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Normal
Cancer

ID1

50 μm

P<0.001 n=34

ID1 mRNA

Relative mRNA level (RT-qPCR)

Normal
Tumor

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Etoposide (10μM)

0 1 2 4 6 hour

ID1

β-actin

Normal
Cancer

ID1

50 μm

P<0.0001 n=98

ID protein expression

KYSE140

KYSE150

KYSE450
Figure 2

A

% of survival

Etoposide (M) 10^-6 10^-5 10^-4 10^-3 10^-2

KYSE450 KYSE150 KYSE180 KYSE140

B

Absorbance at OD 490nm

KYSE150

Days 0 1 2 3 4

KYSE450

Days 0 1 2 3 4

C

Annexin V

Control Etoposide

pLVX pLVX-ID1

pLVX-ID1+Etoposide

D

Apoptosis (%)

Control Etoposide

pLVX pLVX-ID1

pLVX-ID1+Etoposide

ID1 p53 PARP cleaved caspase 3 β-actin

E

Annexin V

Control Etoposide

NC siID1 #1 siID1 #2

F

Apoptosis (%)

NC siID1 #1 siID1 #2

NC siID1 #1 siID1 #2

ID1 p53 PARP cleaved caspase 3 β-actin
Figure 3

A. Graph showing the relative luciferase activity of ID1 promoter mutants with and without 10 or 20 μM etoposide.

B. Table listing the DNA sequences for different species:

- **Human**: TCCGTTTCATTGACGTCA
- **Rhesus**: TCCGTTTCATTGACGTCA
- **Mouse**: TCCGTTTCATTGACGTCA
- **Dog**: TCCGTTTCATTGACGTCA
- **Elephant**: TCCGTTTCATTGACGTCA
- **Chicken**: CGGGCGCGGTCTCAACCGCTG
- **Zebrafish**: TCAAGGCTGAAATGACGTCA

C. Diagram illustrating the ID1 promoter with AP-1 DNA binding site and luciferase assay for CMV-Con and CMV-c-Jun.

D and E. Graphs showing the relative luciferase activity and ID1 promoter binding for different conditions (Control, Etoposide, IgG, c-Jun, c-Fos).
Figure 4

A

B

Etoposide (10 μM)

| Time (hour) | 0  | 0.5 | 1   | 2   | 4   | 6   |
|------------|----|-----|-----|-----|-----|-----|
| c-Jun      |    |     |     |     |     |     |
| c-Fos      |    |     |     |     |     |     |
| ID1        |    |     |     |     |     |     |
| β-actin    |    |     |     |     |     |     |

C

D

| Treatment | KYSE450 | KYSE150 |
|-----------|---------|---------|
| c-Jun     |         |         |
| c-Fos     |         |         |
| ID1       |         |         |
| β-actin   |         |         |

Legend:
- NC: Negative Control
- sic-Jun, c-Fos, c-Jun+c-Fos: Transfection with respective genes
- TAM67: Transfection with TAM67
- +: Transfection present
- -: Transfection absent

Results from RT-qPCR and Western Blot analyses for c-Jun, c-Fos, ID1, and β-actin under different conditions.

Note: Additional details and statistical significance provided in the full text.
Figure 5

A

Control | Etoposide
---------|---------
NC       | NC      | si-c-Jun | si-c-Jun |
---------|---------|----------|----------|
c-Jun    |         |          |          |
ID1      |         |          |          |
p53      |         |          |          |
cleaved caspase 3 | |          |          |
PARP     |         |          |          |
β-actin  |         |          |          |

B

Control | Etoposide
---------|---------
NC       | NC      | si-c-Jun | si-c-Jun |
---------|---------|----------|----------|
PI       |         |          |          |
Annexin V |        |          |          |
---------|---------|----------|----------|

Annexin V

Apoptosis (%)

Control | Etoposide
---------|---------
NC       | NC      | si-c-Jun | si-c-Jun |
---------|---------|----------|----------|
+        |        | -        | -        |
-        | +       | -        | -        |
-        | -       | +        | +        |
-        | -       | -        | +        |
-        | -       | -        | +        |
-        | -       | -        | +        |

NC sic-Jun

NC sic-Fos

Etoposide

NC sic-Jun sic-Fos Etoposide

+        | +       | +       | +       |
-        | +       | -       | +       |
-        | -       | +       | +       |
-        | -       | -       | +       |
-        | -       | -       | +       |
-        | -       | -       | +       |

*
A

ESC Samples (n=34)

ID1

c-Jun

c-Fos

GSE49997 (n=204)

ID1

c-Jun

c-Fos

GSE12417 (n=163)

ID1

c-Jun

c-Fos

GSE24551 (n=173)

ID1

c-Jun

c-Fos

B

GSE12417

Overall Survival

ID1\textsuperscript{low}/c-Jun\textsuperscript{low}

ID1\textsuperscript{high}/c-Jun\textsuperscript{high}

HR=1.39(1.06-1.82)
P=0.0183

Days

GSE49997

Overall Survival

ID1\textsuperscript{low}/c-Jun\textsuperscript{low}

ID1\textsuperscript{high}/c-Jun\textsuperscript{high}

HR=1.33(1.06-1.71)
P=0.0311

Days

GSE24551

Overall Survival

ID1\textsuperscript{low}/c-Jun\textsuperscript{low}

ID1\textsuperscript{high}/c-Jun\textsuperscript{high}

HR=1.64(1.03-2.62)
P=0.0381

Days
Inhibitor of Differentiation/DNA Binding 1 (ID1) Inhibits Etoposide Induced Apoptosis in a c-Jun/c-Fos-dependent Manner
Yahui Zhao, Aiping Luo, Sheng Li, Wei Zhang, Hongyan Chen, Yi Li, Fang Ding, Furong Huang and Zhihua Liu

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