

# Upregulation of Ying Yang 1 (YY1) suppresses esophageal squamous cell carcinoma development through heme oxygenase-1

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(Received April 12, 2013/Revised July 16, 2013/Accepted July 26, 2013/Accepted manuscript online August 6, 2013/Article first published online September 5, 2013)

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest malignancies worldwide. Ying Yang 1 (YY1), a ubiquitously expressed GLI-Krüppel zinc finger transcription factor, plays a regulatory role in a variety of fundamental biological processes, such as embryonic development, growth, apoptosis, differentiation and oncogenic transformation. The purpose of this study was to investigate the expression of YY1 in normal and cancerous esophageal tissues and its function in ESCC development. We found that the expression of YY1 mRNA was significantly increased in the tumor tissues, compared with the para-tissues or normal esophageal tissues. The increased expression of YY1 in tumor samples was further confirmed by immunohistochemistry. Furthermore, the overexpression of YY1 conferred radioresistance to the ESCC TE-1 cells and resulted in markedly reduced cell proliferation. Accordingly, the small interfering RNA-mediated silencing of YY1 expression in TE-1 cells resulted in increased proliferation by enhancing the binding of P21 to Cyclin D1 and CDK4, a protein complex known to mediate cell cycle progression. Moreover, besides P21, heme oxygenase-1 (HO-1) was identified as a YY1 downstream effector, as YY1 stimulated HO-1 expression in esophageal cancer cells. YY1 mediated biological function through transcription of HO-1. Forced expression of HO-1 could moderately suppress proliferation of TE-1 cells. The expression of YY1 significantly correlated with that of HO-1 in ESCC tissues. Taken together, we demonstrated overexpression of YY1 in esophageal carcinoma and identified HO-1 as its target. (*Cancer Sci* 2013; 104: 1544–1551)

Esophageal squamous cell carcinoma (ESCC) remains the sixth most common cause of cancer-related death.<sup>(1)</sup> Despite intensive multimodality therapies, including surgery, radiotherapy and chemotherapy, the prognosis of this disease remains poor. Patients with esophageal carcinomas have a low 5-year survival rate because of late diagnosis and the rapid spread of cancer cells.<sup>(2)</sup> The pathology of ESCC remains complex and largely unknown. Understanding the biological mechanisms involved in esophageal tumor development has emerged as a significant issue in ESCC research. Accumulating evidence indicates that transcription factors have emerged as important players in human cancer development and progression. The aberrant activation of these transcription factors leads to the deregulated expression of multiple gene sets. The activation or inactivation of transcription factors promotes cancer development, cell survival and proliferation and induces tumor angiogenesis.<sup>(3,4)</sup>

Ying Yang 1 (YY1), a ubiquitously expressed GLI-Krüppel zinc finger transcription factor, plays a regulatory role in a variety of fundamental biological processes, such as embryonic

development, growth, apoptosis, differentiation and oncogenic transformation.<sup>(5)</sup> YY1 is capable of influencing gene expression via its ability to directly or indirectly activate or suppress gene transcription.<sup>(6)</sup> Moreover, YY1 has also been shown to regulate its targets through a post-transcriptional mechanism.<sup>(7,8)</sup> The function of YY1 in tumorigenesis and cancer progression has proven to be controversial. YY1 has been shown to activate a variety of oncoproteins by interacting with E1A and C-Myc.<sup>(7,8)</sup> YY1 also attenuates the stability of the tumor suppressor p53, indicating that YY1 acts as an oncogene.<sup>(9)</sup> Some reports also indicate that YY1 mediates the activation of genes with tumor-suppressive functions. A recent report demonstrates that YY1 can suppress the growth of different tumor cell types *in vitro*, including human breast carcinoma cells and glioblastoma cells.<sup>(10)</sup> However, the role of YY1 in cancer development may vary depending on the underlying tissue-specific conditions; further, the biological significance of YY1 in esophageal cancer cells is largely unknown.

One well-characterized YY1 target is P21 (also known as P21<sup>WAF1/Cip1</sup>), which mediates p53-dependent G1 growth arrest.<sup>(11–13)</sup> P21 can be activated in p53-dependent and p53-independent manners and induces G1-phase arrest by inhibition of key Cyclin/Cdk complexes and thus plays a role as a tumor suppressor.<sup>(13,14)</sup> YY1 inhibits P21 transcription in smooth muscle cells by preventing Sp1 occupancy of an upstream element in the P21 promoter, thus perturbing the assembly of the P21-Cdk4-Cyclin D1 complex.<sup>(14)</sup> YY1 can also inhibit P21 complex formation with Cdk4 and Cyclin D1 in cancer cells.<sup>(10)</sup>

Heme oxygenase-1 (HO-1), an inducible isotype of the three HO family isoenzymes, catalyzes the heme ring into carbon monoxide (CO), free iron and biliverdin.<sup>(15)</sup> HO-1 is strongly induced by various stimuli, including heat shock, metals, cytokines and oxidative stress.<sup>(16)</sup> HO-1 and its metabolites have shown an anti-proliferative role in human hepatocellular, breast and prostate cancer cells but an oncogenic role in melanoma and pancreatic cancer.<sup>(17,18)</sup> Beck *et al.*<sup>(15)</sup> revealed a forward feedback interaction between HO-1 and YY1 in both the abdominal aorta of rabbits and the carotid artery of rats. The effects of HO-1 on ESCC development have not yet been analyzed, and there is no current information on the interplay of YY1 and HO-1 in cancer cells.

Here, we investigated the expression of YY1 and its functional consequences in ESCC. We demonstrate that YY1 expression is significantly upregulated in ESCC tissues, compared to normal tissues. In addition, YY1 inhibits cell proliferation in ESCC TE-1 cells through P21 and HO-1.

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## Materials and Methods

**Tissue samples.** For real-time PCR analysis, 24 normal esophageal tissues and 60 human ESCC tissues were used, as previously reported.<sup>(19)</sup> For immunohistochemistry, another 102 ESCC samples and 52 matched, adjacent normal esophageal tissues were collected from the 102 patients. Fifteen normal esophageal tissues were obtained from surgical resections of trauma patients. These tissues were obtained postoperatively between 2010 and 2012 from the Gastrointestinal Center, Jiangyin People's Hospital, Medical School of University of Southeast of China (Jiangyin, China).

All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from the Jiangyin People's Hospital. All diagnoses were based on pathological and/or cytological evidence; the histological features of the specimens were evaluated by two senior pathologists according to the classification criteria from the World Health Organization (WHO).<sup>(20)</sup>

**Real-time PCR.** Total RNA from esophageal tissues was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to cDNA using an oligo(dT)<sub>12</sub> primer and Superscript II (Invitrogen). The mRNA levels of *YY1* and the internal standard *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* were measured by real-time quantitative PCR in triplicate on a Prism 7900 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The specific primers for *YY1* and *GAPDH* were listed in Table 1.

**Immunohistochemistry.** Three-micrometer-thick tissue paraffin sections were deparaffinized and heat-treated with citrate buffer, pH 6.0, for 7 min as an epitope retrieval protocol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min at room temperature, and non-specific-binding sites were blocked with 4% skimmed milk powder for 30 min. Sections were then incubated with the *YY1* or *HO-1* antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h (dilution 1:200) and mixed with 2% skimmed milk powder to again reduce unspecific staining. Biotinylated secondary antibody was then added for 30 min. Avidin-biotin-peroxidase complex (Dako LSAB2 system; DAKO, Carpinteria, CA, USA) was added, and the color was developed using 3,3'-diaminobenzidine. Counterstaining was performed with hematoxylin. All steps were performed at room temperature.

The stained sections were scored using the following criteria: negative (−), <10% of the whole tissue mass stained; weakly positive (+1), 10–25% of the whole tissue mass stained; moderately positive (+2), 25%–75% of the whole tissue mass stained; and strongly positive (+3), >75% of the tissue showed positive.

***YY1* overexpression and RNAi.** The human *YY1* (GenBank accession no. ENST00000262238) coding region was amplified by PCR using a primer pair specific to *YY1*. The amplified fragment was inserted into the pcDNA3.1 vector. The plasmid was then sequenced for confirmation. The shRNA targeting *YY1* and the shRNA control (shRNA-NC) was obtained from Santa Cruz Biotechnology. The siRNA targeting *HO-1* and the siRNA control (siRNA-NC) was obtained from Genepharma (Shanghai, China).

**Cell culture and stable transfection.** The human esophageal cancer cell line, TE-1, was maintained in DMEM supplemented with 10% FBS and antibiotics (Gibco, Grand Island, NY, USA). Cells were grown in a 37°C incubator with 5% CO<sub>2</sub>. To generate a stable control or *YY1*-overexpressing clones, the cells were grown in a 24-well culture plate to 70%–80% confluence and then transfected with either pcDNA3.1 or pcDNA3.1-*YY1* vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was replaced with DMEM containing 600 µg/mL

G418 (Life Technologies, Gaithersburg, MD, USA) 48 h post-transfection. After 3–4 weeks, G418-resistant colonies were selected and screened for *YY1* expression by Western blot.

**Cell viability assay.** Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were plated in 96-well plates. The next day, the cells were transfected with plasmids or siRNAs according to the experimental design. The cells were then incubated with 20 µL MTT (5 mg/mL) for 4 h. After the medium was removed, 100 µL DMSO was added and the optical density (OD) at 490 nm was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

**Clonogenic assay of radiosensitivity.** Stable cell lines were seeded onto six-well plates at 200–2000 cells/well, depending on the dose of radiation. Cells were irradiated using 6-MV X-rays from linear accelerators (Varian Medical Systems, Alto, CA, USA) at a dose rate of 2 Gy/min; a 1.5-cm bolus was used as a compensator. The cells were then grown for 7–10 days to allow for colony formation and were subsequently fixed and stained using crystal violet. Colonies consisting of 50 or more cells were counted as a clone.

**Luciferase assays.** A luciferase reporter containing a fragment of the *HO-1* promoter (−4798 to +16, relative to the transcription start site) was constructed by Shanghai Biobuy (Shanghai, China). For the luciferase assay, the reporter vector with the *HO-1* promoter was co-transfected with pRL-TK (Promega, Madison, WI, USA) to correct transfection efficiency. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). Promoter activities were expressed as the ratio of *Firefly* luciferase to *Renilla* luciferase activities.

**Focus formation.** Cells transfected with the indicated vectors were plated at low density (1000 cells per 6-cm plate), incubated for 10 days and fixed and stained with crystal violet. Foci and colonies containing more than 50 cells were counted using a microscope.

**Western blot.** Cells were lysed in Lysis buffer (Promega) and centrifuged at 4°C for 10 min. The supernatant was collected and subjected to Western-blot. Fifty micrograms of protein from each lysate were fractionated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in PBS-Tween-20 for 1 h at room temperature, the membranes were blotted with the appropriate *YY1*, *HO-1* or *GAPDH* primary antibody (All from Santa Cruz Biotechnology) at a 1:1000 dilution. Membranes were then incubated with the appropriate secondary antibody linked to horseradish peroxidase at a 1:2000 dilution for 1 h at room temperature. After TBST washes, the blot was incubated in the ECL detection kit (Amersham Bioscience, Freiburg, Germany).

**Co-immunoprecipitation.** Co-immunoprecipitation was performed as previously described.<sup>(21)</sup> In short, TE-1 cells were transfected with vectors. After 48 h, the cells were then washed with cold phosphate-buffered saline and harvested by adding 150 µL of IP buffer. Cells were sonicated and centrifuged to remove insoluble material. Following pre-clearing for 1 h at 4°C, total cell extract (200 µg protein) was incubated

**Table 1. Primer sequences for real-time polymerase chain reaction (PCR) analysis**

Gene	Forward primer	Reverse primer
<i>GAPDH</i>	5'-GAAGGTGAA GGTCGGAGTC-3'	5'-GAAGATGGTGATGGGATTTC-3'
<i>YY1</i>	5'-GGATAACTC GGCCATGAGAA-3'	5'-ATAGGGCCTGTCTCCGGTAT-3'

with an anti-P21 antibody (Epitomics, Burlingame, CA, USA) at 4°C with gentle rotation overnight. The antibody–protein complexes were precipitated by addition of 50 µL of protein G agarose and rotation for 2 h at 4°C. The supernatants were then removed by centrifuging, and the pellets were washed with IP buffer and subjected to Western blotting with antibodies against Cyclin D1 (Santa Cruz Biotechnology) and CDK4 (Epitomics).

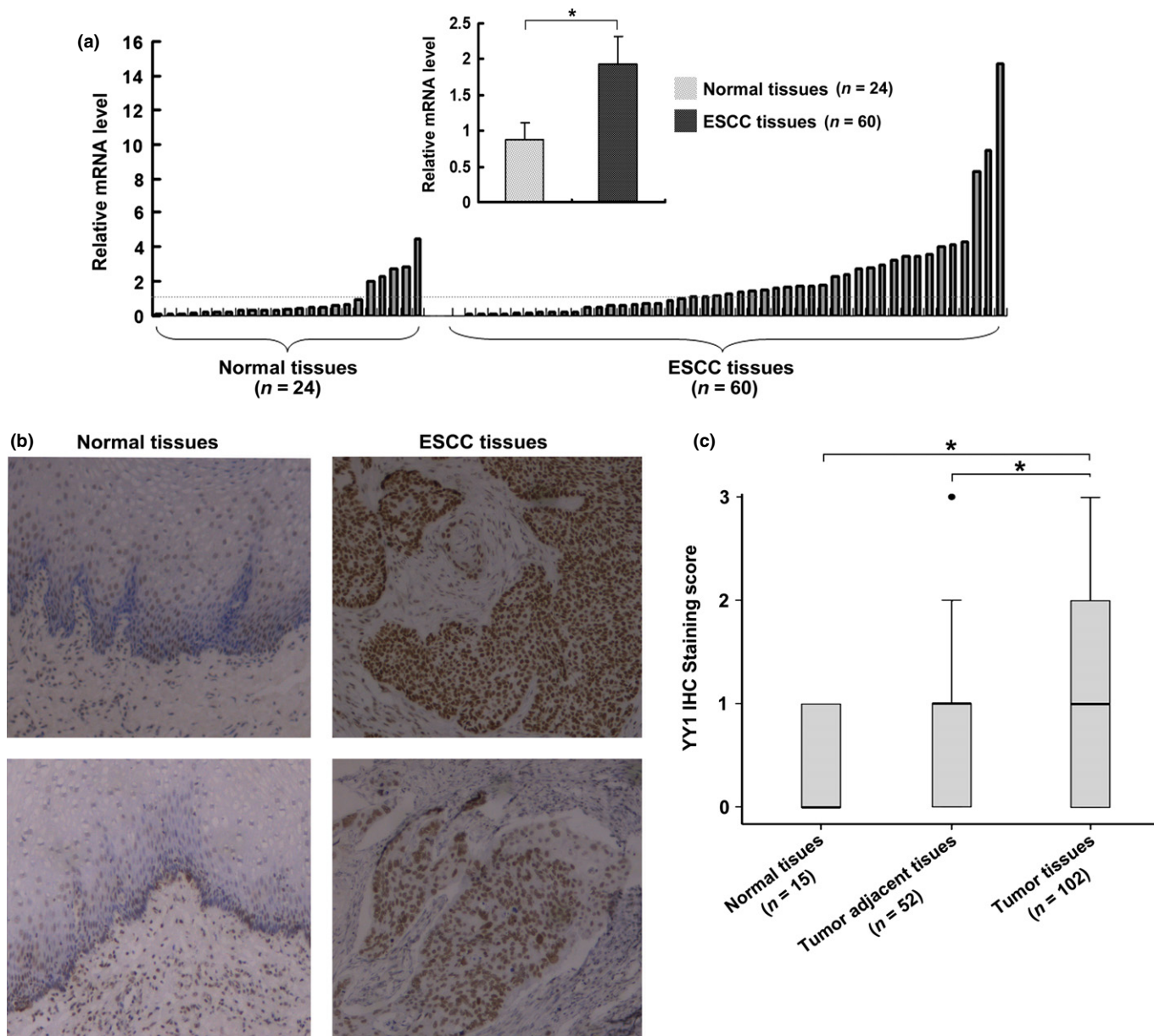
**Adenovirus preparation.** Adenoviruses containing *HO-1* cDNA (Ad-HO-1) and *EGFP* (Ad-EGFP) were used as previously reported.<sup>(22)</sup> Both vectors were propagated in 293A cells. Cells were transfected with a multiplicity of infection (MOI) of 20 virus particles/cell 24 h before being used in experimentation.

**Statistical analysis.** Data were expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. Standard error bars were included for all data

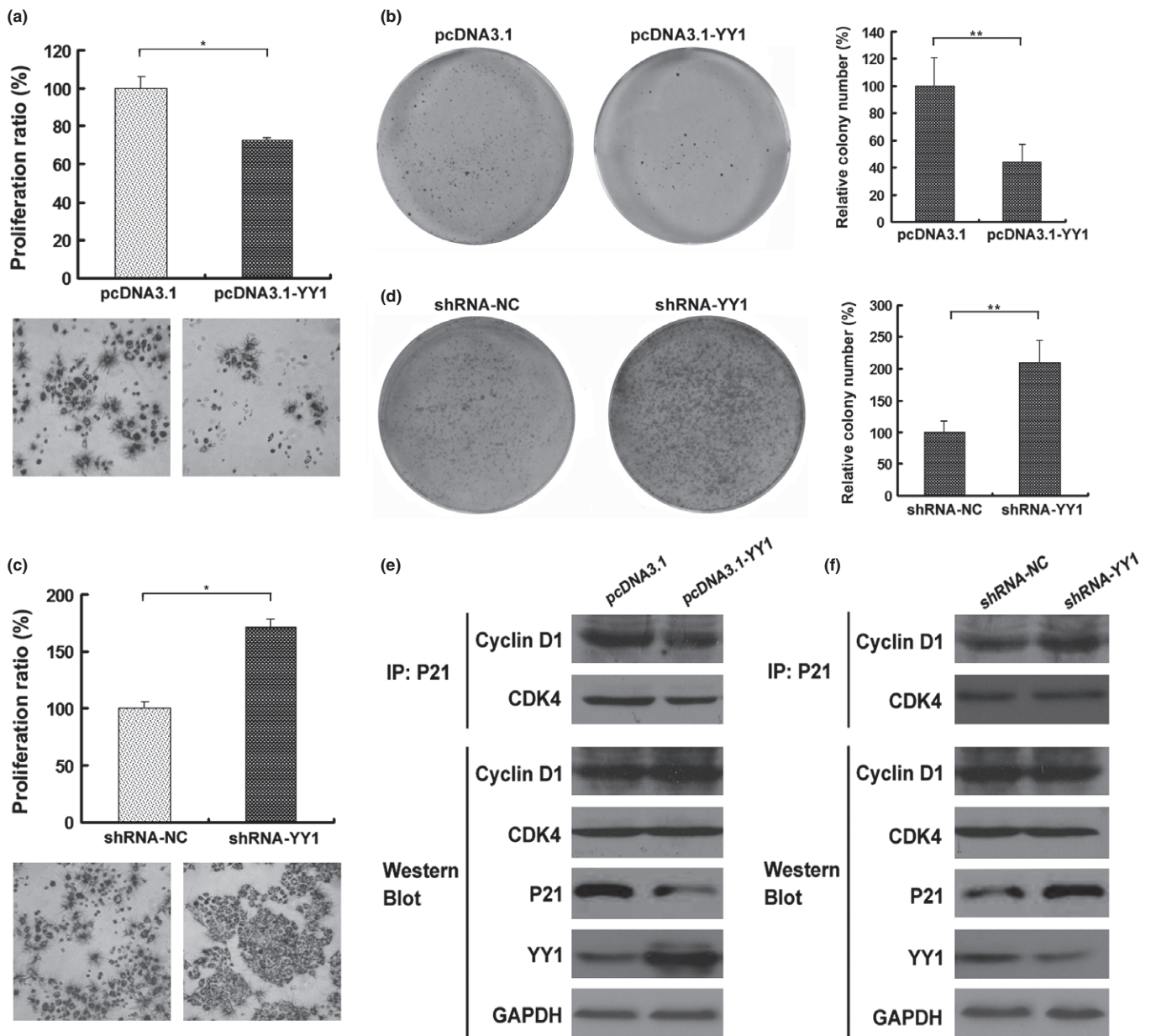
points. The data were then analyzed using Student's *t*-test when only two groups were present or assessed by one-way analysis of variance (ANOVA) when more than two groups were compared. Correlation analysis between IHC staining score of YY1 and HO-1 was performed using Spearman's rho test. Statistical analysis was performed using SPSS software (Release 17.0; SPSS Inc., San Rafael, CA, USA). Data were considered significant if  $P < 0.05$ .

## Results

**Expression of YY1 is upregulated in esophageal cancer tissues.** To test the hypothesis that normal and cancerous esophageal tissues express a different subset of the *YY1* gene, we measured the YY1 mRNA level using real-time PCR. The histopathological features of the 24 normal esophageal tissues



**Fig. 1.** Upregulation of Ying Yang 1 (YY1) in esophageal squamous cell carcinoma (ESCC) samples. (a) Quantitative analyses of relative mRNA levels of YY1. YY1 mRNA expression in 24 normal esophageal tissue specimens and 60 ESCC tissue samples were analyzed by real-time PCR. mRNA levels are normalized to *GAPDH*,  $*P < 0.05$ . (b) Representative immunohistochemistry (IHC) analysis of YY1 expression in normal esophageal tissue specimen and ESCC tissues (magnification,  $\times 100$ ). (c) Box plot representing the range of YY1 IHC staining score in normal esophageal tissues ( $n = 15$ ), ESCC tissues ( $n = 102$ ) and tumor adjacent tissues ( $n = 52$ ),  $*P < 0.05$ .



**Fig. 2.** Effect of Ying Yang 1 (YY1) on cell proliferation and focus formation. (a) and (b) TE-1 cell lines were seeded in a 96-well plate and incubated for 48 h. Cell proliferation was measured by MTT. (b) and (d) Cells were stably transfected with indicated vectors. One thousand cells were seeded onto each plate. After 10 days, cells were stained with crystal violet. Colonies consisting of more than 50 cells were counted. Data are presented as the means  $\pm$  SEM and were normalized to the control cells, \* $P < 0.05$ ; \*\* $P < 0.01$ . (e) and (f) TE-1 cells were transfected with indicated vectors for 48 h. Cell extracts were subjected either to Western blot analysis for YY1, P21, CDK4 or Cyclin D1, or co-immunoprecipitation (IP) with p21 antibodies followed by blotting for CDK4 or Cyclin D1.

and 60 ESCC samples are summarized previously.<sup>(19)</sup> The relative expression of YY1 in 24 normal esophageal tissues and 60 ESCC tissues is shown in Figure 1(a). Statistical analysis revealed significant differences in YY1 gene expression between cancerous and normal tissues ( $0.88 \pm 0.24$  vs  $1.93 \pm 0.38$ ,  $P < 0.05$ , Fig. 1a).

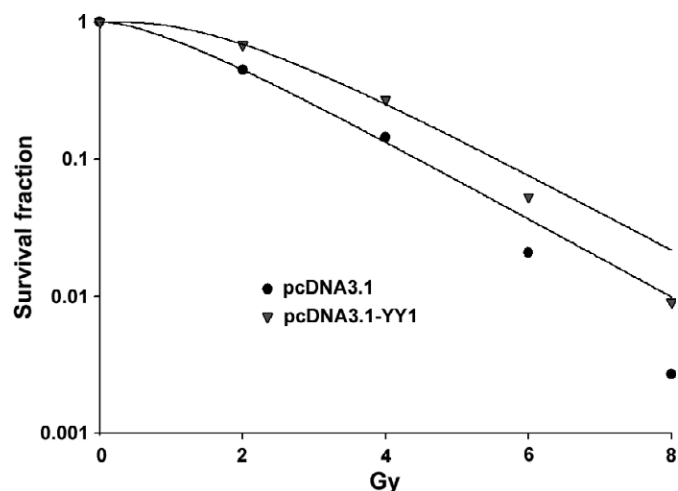
To confirm the upregulation of YY1 on the protein level, immunohistochemistry staining was performed in 15 paraffin-embedded normal esophageal tissues and 102 ESCC tissues, 52 of which had corresponding adjacent tumor tissues. The histopathological features of the patients are summarized in Table S1. Corresponding with the real-time PCR analysis, an overall stronger staining for YY1 was frequently observed in the nucleus of ESCC tissues, whereas

very weak staining of YY1 was observed in the normal samples (Fig. 1b). However, there was heterogeneity within the tumor samples. The difference in the YY1 expression pattern within tumor samples may be attributed to the heterogeneity of the ESCC background. When the samples were scored by a pathologist, as described in the Materials and Methods section, YY1 expression in normal esophageal tissues was similar to its expression in adjacent tumor tissues; however, there were significant differences in its expression among tumor tissues and between tumor tissues and adjacent tumor tissues ( $P = 0.003$  and  $P = 0.001$ , respectively; Fig. 1c). These results demonstrated an increase in the transcription of YY1 in esophageal carcinoma, suggesting a characteristic of this malignancy.

**YY1 suppressed esophageal cancer cell growth *in vitro*.** To explore the role of YY1 in esophageal cancer cell growth, cells were first transfected with a YY1-overexpressing vector (pcDNA3.1-YY1) or a control vector (pcDNA3.1), and the resulting growth was measured with MTT and colony formation. The results showed that increased YY1 expression suppressed cell growth and focus formation (Fig. 2a,b). We further knocked down YY1 expression using an shRNA. The downregulation of YY1 promoted cell proliferation and colony formation (Fig. 2c,d).

It has been reported that YY1 inhibits P21 expression and interfere with p21-CDK4-CyclinD1 cell cycle regulatory complex,<sup>(10)</sup> which is implicated in cell cycle progression,<sup>(23,24)</sup> We therefore determined the effect of YY1 expression on p21-CDK4-CyclinD1 complex formation. As shown in Figure 2e, YY1 overexpression reduced p21 expression in TE-1 cells. Moreover, co-immunoprecipitation studies revealed that YY1 reduced intracellular levels of p21-CDK4 and p21-CyclinD1 complexes (Fig. 2e). Conversely, knock-down of YY1 by shRNA upregulated P21 expression and increased its binding to CDK4 and Cyclin D1 (Fig. 2f), indicating that YY1 inhibits ESCC cell growth and inhibits formation of a cell cycle regulatory complex. In contrast, YY1 overexpression or underexpression did not affect total levels of CyclinD1 and CDK4 (Fig. 2e,f). Taken together, these results indicate that YY1 suppresses esophageal tumor growth *in vitro*.

**YY1 overexpression conferred radioresistance to TE-1 cells.** To investigate the effect of YY1 on the radiosensitivity of TE-1 cells, we performed an *in vitro* clonogenic cell survival assay. TE-1 cells that were stably transfected with pcDNA3.1-YY1 exhibited higher clonogenic survival rates than cells that were treated with radiation alone. The sensitizer enhancement ratio (SER) was 0.66 for cells transfected with pcDNA3.1-YY1, compared to cells transfected with pcDNA3.1 (Fig. 3). The data were further analyzed using two-way ANOVA to test the interaction effect between YY1 expression and radiation. The relationship between YY1 expression and radiation was statistically significant ( $P < 0.001$ ) in TE-1 cells. This result demon-



**Fig. 3.** Effect of Ying Yang 1 (YY1) on cell radiosensitivity in TE-1 cells. Clonogenic cell survival curves were generated for TE-1 cells that were stably transfected with the indicated vectors and were then exposed to 2, 4, 6 or 8 Gy X-ray irradiation. The survival data were normalized to those of the non-irradiated control group. The SER was calculated for TE-1 cells that were transfected with the YY1 overexpression vector (pcDNA3.1-YY1). The values shown are for three independent experiments.

strates that YY1 overexpression conferred radioresistance to TE-1 cells.

**YY1 positively regulates HO-1 expression.** As a multifunctional transcription factor, YY1 harbors a variety of targets. YY1 was recently reported to activate HO-1 in rat aortic smooth muscle cells.<sup>(15)</sup> To investigate whether YY1 targets HO-1 in esophageal cancer cells, we transfected TE-1 cells with a YY1-overexpressing vector (pcDNA3.1-YY1). The results revealed that the transfection of YY1 cDNA increased both YY1 and HO-1 proteins (Fig. 4a). The effective knockdown of YY1 by shRNA was confirmed by Western blot and reduced HO-1 expression (Fig. 4b).

HO-1 expression was also measured using a luciferase construct containing the *HO-1* promoter (pGL3-HO1). The co-transfection of pGL3-HO1 with the YY1 overexpression vector significantly increased the reporter activity, indicating that YY1 stimulates YY1 transcription (Fig. 4c). Conversely, the co-transfection of the luciferase reporter with shRNA-YY1 significantly decreased luciferase activity (Fig. 4d). These results demonstrate that YY1 positively regulates HO-1 transcription in esophageal cancer cells. It was of interest to explore the possibility that YY1 mediated biological function through HO-1. We found that the cell proliferation and focus formation inhibited by YY1 overexpression were both reversed by silencing of HO-1 (Fig. 4e,f, and Fig. S1). These results together indicate that YY1 acts through HO-1 to suppress cell growth in TE-1 cells.

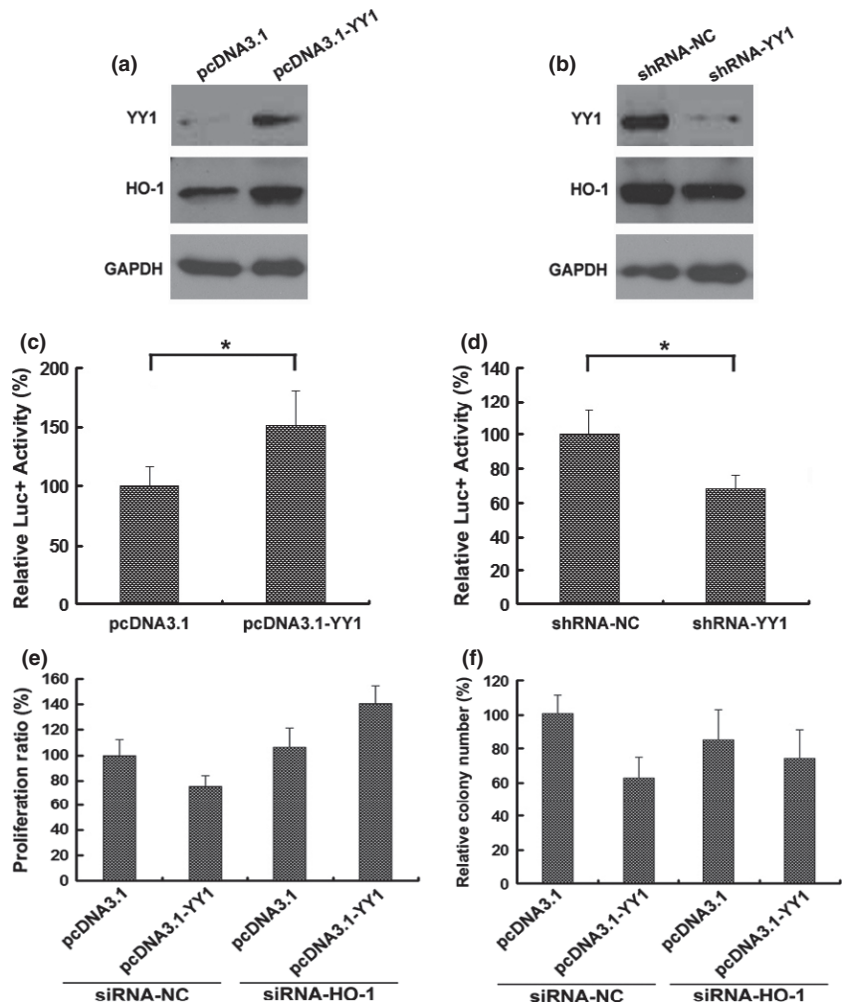
**Expression of YY1 correlates that of HO-1 in ESCC tissues.** We next performed a correlation analysis between YY1 and HO-1 expression in tissue samples. We detected the expression of YY1 and HO-1 using IHC in 102 ESCC samples and 52 adjacent tumor esophageal tissues. Representative IHC staining of both YY1 and HO-1 in the same tissue is shown in Figure 5(a). Tissue samples with strong YY1 staining in the nucleus exhibited positive HO-1 staining, whereas weak YY1 expression showed negative HO-1 staining. Statistical analysis revealed that the protein levels of the two genes correlated with each other ( $\rho = 0.505$ ,  $P < 0.01$ ; Fig. 5b), indicating that the expression of YY1 correlates with the expression of HO-1 in human tissues.

**HO-1 overexpression inhibited esophageal cancer cell growth.** The effect of HO-1 in esophageal cancer cell growth remains unknown. To explore its role in proliferation, cells were infected with the HO-1-overexpressing virus, Ad-HO-1 or control virus, Ad-EGFP. Cells infected with Ad-HO-1 showed no significant difference in cell growth after 48 h of incubation, compared with cells infected with mock virus or Ad-EGFP (data not shown). However, as shown in Fig. S2 (a,b), the overexpression of HO-1 by a vector or adenovirus caused a significant reduction in TE-1 cell colony size but not colony number, indicating that HO-1 could moderately suppress proliferation long term. These results suggest that HO-1 is a YY1 target that plays a role in regulating cell growth in esophageal cancer cells.

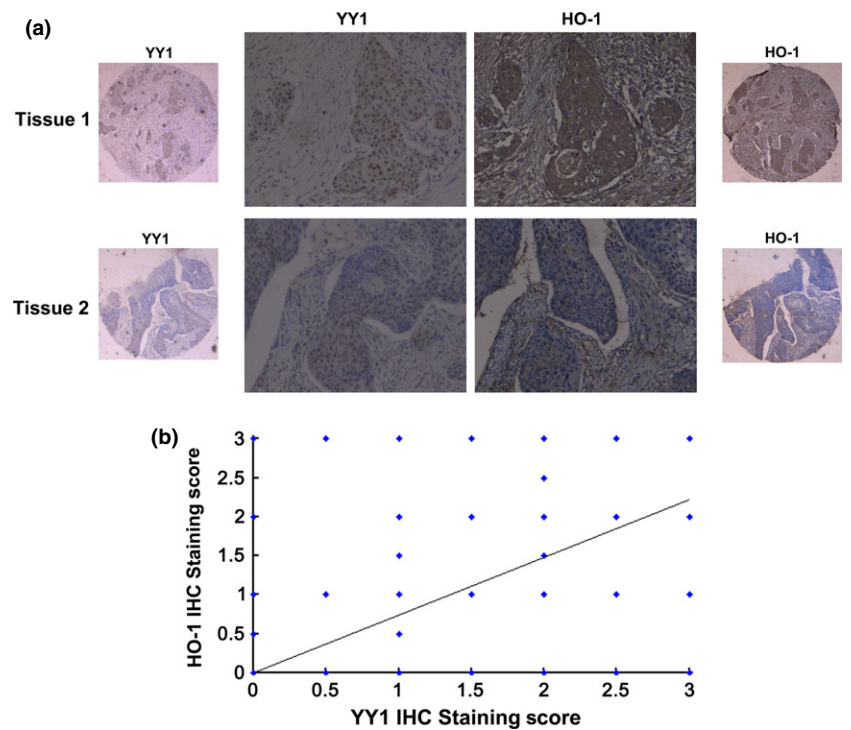
## Discussion

Ying Yang 1 has exhibited the multifunctional ability to regulate various biological processes.<sup>(5-8)</sup> Transcription factor YY1 is capable of either repressing or stimulating gene transcription.<sup>(25,26)</sup> It was estimated that over 7% of vertebrate genes contained YY1 binding sites, suggesting a ubiquitous regulation of YY1 in multiple biological processes, including proliferation, apoptosis, development and mitochondrial function.<sup>(27,28)</sup>

Ying Yang 1 is overexpressed in most cancer types, such as prostate carcinoma,<sup>(29)</sup> cervical cancer,<sup>(30)</sup> colon cancer,<sup>(31)</sup> breast cancer,<sup>(32)</sup> ovarian cancer,<sup>(33)</sup> and gliomas.<sup>(34)</sup> In the present study, we found that the mRNA and protein levels of



**Fig. 4.** Ying Yang 1 (YY1) positively regulates heme oxygenase-1 (HO-1) in esophageal cancer cells. (a) TE-1 cells were stably transfected with YY1 overexpression vector (pcDNA3.1-YY1) or control vector (pcDNA3.1). YY1, HO-1, P21 and GAPDH protein levels were detected by Western blot. (b) Cells were transfected with an shRNA targeting YY1 (shRNA-YY1) or control shRNA (shRNA-NC) for 24 h. HO-1, YY1 and GAPDH protein levels were detected by Western blot. (c) A luciferase reporter containing the *HO-1* promoter was co-transfected with indicated vectors or shRNAs into TE-1 cells. After 24 h of incubation, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). TE-1 cell lines were co-transfected with indicated siRNA and vector, and evaluated for cell proliferation (e) and focus formation (f). \* $P < 0.05$ , compared with pcDNA3.1 transfected cells.



**Fig. 5.** Correlation analyses between Ying Yang 1 (YY1) and heme oxygenase-1 (HO-1) expression in tissue samples. (a) Representative immunohistochemistry (IHC) staining of both YY1 and HO-1 in the same tissue. (b) IHC staining of YY1 protein showed a significant positive correlation with HO-1.

YY1 in ESCC tissues were significantly increased compared with normal esophageal tissues or adjacent tumor tissues. Using a human ESCC TE-1 cell line, we found that YY1 inhibits cell proliferation but confers cell radioresistance. Our study supports previous findings demonstrating that in addition to P21, YY1 also stimulated HO-1 in ESCC cells. The expression of YY1 significantly correlated with that of HO-1 in ESCC tissues.

The role of YY1 in tumorigenesis and cancer progression is still a matter of intense debate.<sup>(35)</sup> The controversial results indicate that YY1 exhibits distinct effects on tumorigenesis, which may represent different phases of the multi-step carcinogenesis process. It may depend on the type of tumor or other still-undefined factors. Here, we found that YY1 inhibits TE-1 cell proliferation by enhancing the binding of P21 to Cyclin D1 and CDK4, a protein complex known to mediate cell cycle progression. YY1 controls a plethora of genes, including c-Myc, c-Fos, Her2, VEGF and cyclooxygenase-2.<sup>(36)</sup> Here, we also identified HO-1, the rate-limiting enzyme in heme degradation, as a novel downstream target of YY1 in esophageal cancer cells. Cell proliferation and focus formation inhibited by YY1 overexpression were both reversed by silencing of HO-1, suggesting that YY1 mediates biological function through HO-1. However, the consensus binding sequence for YY1 was not found in the *HO-1* promoter, suggesting that YY1 may act as a co-activator to facilitate HO-1 transcription. The overexpression of YY1 induces the expression of HO-1 in rat aortic smooth muscle cells (RASMCs) *in vitro*, and the interaction between YY1 and HO-1 is complex and may involve a feed forward amplification.<sup>(15)</sup>

The role of HO-1 in tumor growth is controversial. HO-1 inhibits cell proliferation, migration and invasion in prostate

cancer cells and impairs tumor growth *in vivo* by repressing MMP-9.<sup>(37,38)</sup> In breast cancer, HO-1 may inhibit cell proliferation.<sup>(18)</sup> The overexpression of human HO-1 after its retroviral transfer into Panc-1 cells did not interfere with tumor growth *in vitro*, while the development of tumors *in vivo* was accelerated upon transfection with HO-1.<sup>(39)</sup> However, the overexpression of HO-1 enhances the proliferation and increases angiogenic potential of murine and human melanoma cells. Moreover, the increased production of HO-1 in B16 tumor cells shortens the survival of mice.<sup>(40)</sup> In the present report, we found that HO-1 inhibited ESCC proliferation using an HO-1-overexpression vector and an HO-1-overexpression adenovirus. Smaller and fewer colonies were found in the HO-1-overexpressed cells.

In summary, the expression of *YY1* is upregulated in ESCC tissues and modulates cell proliferation through HO-1 in esophageal cancer cells. Thus, YY1 may be involved in the progression and pathogenesis of ESCC.

## Acknowledgments

This work is supported by the National Natural Science Foundation of China (81102078 and 81172597), Natural Science Foundation of Jiangsu Province (BK2012561), Innovative Project of Jiangsu Province (BL2012046), Changzhou Social Development (CE20125026) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

## Disclosure

The authors have no conflict of interest.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Effect of heme oxygenase-1 (HO-1) on cell focus formation.

**Fig. S2.** Ying Yang 1 (YY1) mediated focus formation through heme oxygenase-1 (HO-1).

**Table S1.** Patient demographics for the IHC analysis.