Resveratrol Selectively Induces DNA Damage, Independent of Smad4 Expression, in Its Efficacy against Human Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: Alterations in Smad4 signaling and its loss cause genomic instability and head and neck squamous cell carcinoma (HNSCC), suggesting that agents that target both Smad4-dependent and -independent pathways could control HNSCC.

Experimental Design: Resveratrol efficacy was evaluated against the HNSCC cells FaDu, Cal27, Det562, and Cal27-Smad4 for viability, DNA damage, cell-cycle progression, and apoptosis, as well as γ-H2AX expression, and focus formation (γ-H2AX and Brca1). Resveratrol efficacy was also examined in nude mice for FaDu xenograft growth. Xenografts were analyzed for γ-H2AX and cleaved caspase-3.

Results: Resveratrol (5–50 μmol/L) suppressed viability and induced DNA damage in FaDu and Cal27 cells but not in normal human epidermal keratinocytes and human foreskin fibroblasts, showing its selectivity toward HNSCC cells; however, Det562 cells were resistant to resveratrol even at 100 μmol/L. Cal27 cells stably transfected with Smad4 showed similar resveratrol effects as parental Cal27, indicating that a lack of resveratrol effect in Det562 cells was independent of Smad4 status in these cells. Furthermore, resveratrol caused S-phase arrest and apoptotic death of FaDu and Cal27 cells together with induction of Brca1 and γ-H2AX foci. Resveratrol (50 mg/kg body weight) treatment also inhibited FaDu tumor growth in nude mice, and γ-H2AX and cleaved caspase-3 were strongly increased in xenografts from resveratrol-treated mice compared with controls.

Conclusion: Our findings for the first time showed antiproliferative, DNA damaging, and apoptotic effects of resveratrol in HNSCC cells independent of Smad4 status, both in vitro and in vivo, suggesting that more studies are needed to establish its potential usefulness against HNSCC. Clin Cancer Res; 17(16); 1–10. ©2011 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide, and the 5-year survival rates are among the lowest (<60%) of the major cancers. HNSCCs are mainly caused by alcohol consumption, tobacco intake (smoking and smokeless products, such as betel quid), poor oral hygiene, and infection to high-risk types of human papilloma virus (1). According to the statistical estimates given by the American Cancer Society, approximately 73,080 new cases of HNSCC would have been diagnosed in the United States in the year 2010 (2). Despite advancements in surgical procedures, chemotherapy, radiation therapy, as well as combination of these, the patient survival rates have not improved in the last several decades. Moreover, it has been shown that the high rate of morbidity is due to both locoregional recurrence and distant metastasis (1, 3).

TGF-β receptor (TGF-βR) II is the main pathway that is deregulated in HNSCC (4) in which aberrant signaling downstream of this receptor contributes to tumor growth. TGF-β regulates its pleiotropic biological activities through a complex formation between TGF-βRI and TGF-βRII. TGF-β receptors then transmit signal through the activation of the downstream Smad signaling pathways, which consists of the Smad family of proteins (Smad1–5; 4). Receptor-activated Smads (Smad2 and 3 or Smad1 and 5) associate with Smad4 and form a heterooligomeric complex, which translocates to the...
DNA repair processes, which are defective in many cancer cells, offer avenues for therapeutic intervention. Thus, preclinical clinical development of nontoxic chemopreventive agents that can selectively induce DNA damage in cancer cells is highly desirable. Supporting this rationale, results of the present study are highly significant with major implications wherein we report that the anticancer effect of resveratrol is predominately due to its ability to induce DNA damage selectively in head and neck squamous cell carcinoma (HNSCC) cells without affecting normal, repair-proficient cells. This study reports that resveratrol causes S-phase arrest and apoptotic death of FaDu, Cal27, and Cal27-Smad4 cells together with induction of γ-H2AX and Brca1 foci, which are independent of Smad4 expression. The resveratrol-mediated upregulation of γ-H2AX and cleaved caspase-3 protein expression is also observed in FaDu tumor xenografts in athymic nude mice. These preclinical results hereby warrant further clinical investigation to determine the efficacy of resveratrol against HNSCC.

### Translational Relevance

DNA repair processes, which are defective in many cancer cells, offer avenues for therapeutic intervention. Thus, preclinical clinical development of nontoxic chemopreventive agents that can selectively induce DNA damage in cancer cells is highly desirable. Supporting this rationale, results of the present study are highly significant with major implications wherein we report that the anticancer effect of resveratrol is predominately due to its ability to induce DNA damage selectively in head and neck squamous cell carcinoma (HNSCC) cells without affecting normal, repair-proficient cells. This study reports that resveratrol causes S-phase arrest and apoptotic death of FaDu, Cal27, and Cal27-Smad4 cells together with induction of γ-H2AX and Brca1 foci, which are independent of Smad4 expression. The resveratrol-mediated upregulation of γ-H2AX and cleaved caspase-3 protein expression is also observed in FaDu tumor xenografts in athymic nude mice. These preclinical results hereby warrant further clinical investigation to determine the efficacy of resveratrol against HNSCC.

### Cell culture and reagents

The human HNSCC cells FaDu, Cal27, and Det562 were taken from American Type Culture Collection (ATCC) in 2006 and were confirmed by DNA fingerprinting with the ABI Identifiler kit using the following identifier loci: D3S158, vWA, FGA, amelogenin, D8S1179, D21S11, D18S51, D13S317, D7S820, CSF1PO, D16S539, TH01, TPOX, D2S1338, and D19S433. All results matched the fingerprint profiles in the ATCC database. Normal human epidermal keratinocytes (NHEK) and human foreskin fibroblasts (HFF) were from Lonza Walkersville, Inc. Dulbecco’s modified Eagle’s medium (DMEM) and other cell culture materials were from Invitrogen Corporation. Antibodies for γ-H2AX and cleaved caspase-3, and anti-rabbit peroxidase-conjugated secondary antibody were from Cell Signaling. Smad4 and Brca1 antibodies were purchased from Santa Cruz Biotechnology. Annexin V/Vybrant Apoptosis Assay Kit was from Molecular Probes. Resveratrol and β-actin were from Sigma-Aldrich. FaDu, Cal27, Det562, and HFF cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. NHEK cells were cultured in KGM-Gold Bullet Kit (Lonza; catalogue # 00192060) under standard culture conditions.

### Generation of Cal27 vector control (pcDNA3) and Smad4-overexpressing stable cell lines

Following similar procedures (8), Cal27 cells were transfected with pcDNA Flag-Smad4M (plasmid 14959) or vector control pcDNA3 (Addgene), using Lipofectamine 2000. Stable transfectants were selected using G418 at 0.2 mg/mL in DMEM (10% FBS) for 4 weeks, and cells were pooled and maintained in the same selective medium.

### MTT assay

Cells were plated in 96-well plate under standard culture conditions, next day treated with resveratrol, and at the end of desired treatment time, 20 μL MTT stock solution (5 mg/mL) was added to each well and incubated for another 5 hours. Thereafter, media was aspirated, 200 μL of dimethyl sulfoxide (DMSO) was added to each well, and absorbance was measured at 570 nm.

### Comet assay

Following desired treatments, cells were harvested, washed with PBS, 50 μL cell suspension containing 5,000 cells mixed with 500 μL of 0.5% agarose at 37°C, and 75 μL of this mixture instantly added to comet slides (OxiSelect Comet Assay Kit; Cell Biolabs, Inc.). Embedded cells were immersed in lysis buffer at 4°C for 1 hour and then replaced with cold alkaline buffer and placed at 4°C for 30 minutes in dark followed by electrophoresis (35 V for 15 minutes at 4°C) in a horizontal electrophoresis chamber filled with neutral Tris borate EDTA (TBE) electrophoresis buffer. Thereafter, slides were transferred to a container filled with cold distilled water for 2 to 4 minutes and then placed in 70% ethanol for 5 minutes and air dried.

### Comet assay

Following desired treatments, cells were harvested, washed with PBS, 50 μL cell suspension containing 5,000 cells mixed with 500 μL of 0.5% agarose at 37°C, and 75 μL of this mixture instantly added to comet slides (OxiSelect Comet Assay Kit; Cell Biolabs, Inc.). Embedded cells were immersed in lysis buffer at 4°C for 1 hour and then replaced with cold alkaline buffer and placed at 4°C for 30 minutes in dark followed by electrophoresis (35 V for 15 minutes at 4°C) in a horizontal electrophoresis chamber filled with neutral Tris borate EDTA (TBE) electrophoresis buffer. Thereafter, slides were transferred to a container filled with cold distilled water for 2 to 4 minutes and then placed in 70% ethanol for 5 minutes and air dried.

### Comet assay

Following desired treatments, cells were harvested, washed with PBS, 50 μL cell suspension containing 5,000 cells mixed with 500 μL of 0.5% agarose at 37°C, and 75 μL of this mixture instantly added to comet slides (OxiSelect Comet Assay Kit; Cell Biolabs, Inc.). Embedded cells were immersed in lysis buffer at 4°C for 1 hour and then replaced with cold alkaline buffer and placed at 4°C for 30 minutes in dark followed by electrophoresis (35 V for 15 minutes at 4°C) in a horizontal electrophoresis chamber filled with neutral Tris borate EDTA (TBE) electrophoresis buffer. Thereafter, slides were transferred to a container filled with cold distilled water for 2 to 4 minutes and then placed in 70% ethanol for 5 minutes and air dried.

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overnight at room temperature. After staining with Vista Green for 15 minutes at room temperature, comets (both double- and single-strand DNA breaks) were observed by fluorescent microscopy (Olympus BH2) at 100× total magnification.

Cell-cycle distribution and apoptosis analyses

Cells were treated with resveratrol, trypsinized, and washed twice with ice-cold PBS, and pellets were incubated in 0.5 mL of saponin/prodipidum iodide (PI) solution at 4°C for 24 hours in dark as reported earlier (13). Alternatively, cells were subjected to Annexin V and PI staining using Vybrant Apoptosis Assay Kit 2 following the vendor protocol. Stained cells were analyzed by flow cytometry at the fluorescence-activated cell-sorting (FACS) Analysis Core of the University of Colorado Cancer Center (UCCC) for cell-cycle distribution and apoptotic cell populations, respectively.

Immunoblotting

At 60% to 65% confluency, cells were treated with resveratrol, cell lysates were prepared, 30 to 60 μg protein per sample was denatured with 2× sample buffer and subjected to SDS-PAGE on 12% or 16% gel, and separated proteins were transferred onto membrane by Western blotting as described (13). Membranes were blocked with blocking buffer for 1 hour at room temperature and probed with primary antibody overnight at 4°C followed by incubation with peroxidase-conjugated appropriate secondary antibody and enhanced chemiluminescence (ECL) detection.

Immunofluorescence staining and confocal microscopy

Cells were plated on cover slips overnight and following desired treatment, washed gently with PBS, fixed in 4% formaldehyde for 15 minutes, incubated with ice-cold 100% methanol for 10 minutes and rinsed twice with PBS, and blocked in 5% bovine serum albumin in PBS for 60 minutes. Thereafter, cells were incubated with γ-H2AX or Brca1 antibody overnight at 4°C, washed with PBS, incubated for 45 minutes with Texas Red goat anti-rabbit and Alexa Flour goat anti-mouse secondary antibodies, and counterstained with 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes. Cell images were captured at 1,000× magnification on a Nikon D Eclipse C1 confocal microscope and analyzed by EZ-C1 Free viewer software.

Tumor xenograft study

Exponentially growing FaDu cells were trypsinized, washed, and resuspended in serum-free and antibiotic-free DMEM. Athymic nu/nu male mice (6-week-old) were maintained under standard conditions with free access to water and AIN-76 diet, randomly divided into 5 groups (n = 8/group), and injected s.c. with 3 × 10^5 cells mixed with Matrigel (1:1) in the right flank to initiate tumor xenograft growth. Next day, mice in groups 2 and 3 were gavaged orally with 10 and 50 mg/kg body weight doses of resveratrol in 0.2 mL of 0.5% carboxy methyl cellulose (CMC) for 5 d/wk for 30 days, respectively, and those in group 1 (control) with CMC alone. For groups 4 and 5, xenograft was allowed to grow for 15 days and then mice were gavaged orally with 0.5% CMC and 50 mg/kg body weight resveratrol dose in CMC for 5 d/wk for 15 days, respectively. Xenograft growth was measured twice weekly in all groups of animals after seventh day of cell implantation with a digital caliper, and tumor volume calculated as reported recently (14). Body weight and diet consumption were recorded twice weekly throughout study. At experiment terminations, tumors were excised, weighed, and stored at −80°C. Animal care and experiments were in accordance with University of Colorado Denver–approved Institutional Animal Care and Use Committee protocol.

Immunohistochemical staining

Tumor tissues, fixed in 10% phosphate-buffered formalin for 12 hours at 4°C, were dehydrated in ascending concentrations of ethanol, cleared with xylene, embedded in paraffin, and tissue blocks were cut with a rotary microtome into 4-μm sections and processed for immunohistochemical (IHC) staining. Briefly, sections after deparaffinization and rehydration were treated with 0.01 mol/L citrate buffer (pH 6.0) in a microwave for 30 minutes for antigen retrieval, followed by quenching of endogenous peroxidase activity with 3% H2O2 in methanol (v/v) for 5 minutes. Next, sections were incubated with specific antibodies, γ-H2AX and cleaved caspase-3, in PBS for 2 hours at room temperature, followed by overnight incubation at 4°C in a humidified chamber. Negative controls were incubated only with universal negative control antibodies under identical conditions. Sections were then incubated with appropriate biotinylated secondary antibody (1:200–400 dilutions), followed with conjugated horseradish peroxidase–streptavidin (Dako) and 3,3′-diaminobenzidine (Sigma Chemical Co.) working solution and counterstained with hematoxylin (15). Microscopic images were taken by AxioCam MrC5 camera at 400× magnification and processed using Axiosvision 4.6 (Carl Zeiss microimaging). IHC staining was quantified as the number of positive cells (brown cells) × 100/total number of cells in 5 arbitrary selected 400× fields from each tumor.

Statistical analysis

Statistical significance of differences between control and treated samples was determined using Student’s t test (SigmaStat 3.5). The values of P < 0.05 were considered significant. The data in all cases are representative of at least 2 to 4 independent studies with reproducible results.

Results

Resveratrol inhibits cell viability and induces DNA damage selectively in HNSCC cells

First, we evaluated resveratrol efficacy in a panel of human HNSCC cell lines, namely, FaDu (homologous deletion of Smad4), CaL27 (nonsense mutation in Smad4),
and Det562 (wild-type Smad4) cells. Resveratrol at 50 μmol/L dose caused 83% (P < 0.001) and 63% (P < 0.001) decrease in viability of FaDu cells after 48 and 72 hours, respectively (Fig. 1A). Cal27 cells at 50 μmol/L resveratrol dose also showed up to 43% (P < 0.001) decrease in viability (Fig. 1A); however, such resveratrol treatments were not effective in Det562 cells, until a higher dose (100 μmol/L) and longer treatment of the agent (Fig. 1A). Several recent studies have shown that natural chemopreventive agents cause DNA damage in tumorigenic cells leading to apoptotic cell death (9), and accordingly, next we assessed resveratrol effect on DNA damage in HNSCC cells employing the alkaline comet assay. Compared with controls wherein DNA remained within the nucleus, in resveratrol-treated cells, damaged DNA exited nucleus and stained as a tail (Fig. 1B). Overall, comet assay results clearly showed DNA-damaged FaDu and Cal27 cells following resveratrol treatment; however, Det562 cells had less DNA damage induced by resveratrol (Fig. 1B), which is consistent with a lack resveratrol effect on Det562 cell viability (Fig. 1A), suggesting that the observed decrease in cell viability by resveratrol in FaDu and Cal27 cells is possibly due to its DNA damage inducing effect. Importantly, DNA damage was not observed in normal cells (NHEK and HFF) even at higher dose of resveratrol (Fig. 1C), showing its selectivity toward HNSCC cells. In Figure 1C, NHEK cells in the treatment groups are purposely added less in number to see the comet tail. Thus, cells are more in the control group than in resveratrol-treated group. Furthermore, we have found that resveratrol had no effect on the cell growth and cell number (data not shown) of NHEK cells.

**Resveratrol selectively causes cell-cycle arrest and apoptosis in HNSCC cells independent of Smad4**

On the basis of results in Figure 1 showing resveratrol caused decreased cell viability and DNA damage selectively in HNSCC cells carrying nonfunctional Smad4 (FaDu and Cal27), next we assessed its effect on cell-cycle progression and apoptosis in FaDu cells (deletion Smad4) and Cal27-Smad4 cells overexpressing Smad4 (Fig. 2A) to address whether a lack of resveratrol effect in Det562 cells is due to their wild-type Smad4 status. Resveratrol (10 μmol/L) arrested FaDu cells in S-phase (66%, P < 0.001) compared to controls with 34% S-phase population (Fig. 2B); however, higher resveratrol concentrations (25–50 μmol/L) for 24 hours caused G1 arrest (63-78%) compared to control (41%; Fig. 2B). For Cal27, compared to controls with 38% to 39% cells in S-phase, resveratrol (25 μmol/L) significantly induced S-phase arrest (53% and 56%; P < 0.001) in both vector control Cal27-pcDNA3 and Smad4-overexpressing (based on overexpressed Smad4 protein; Fig. 2A) Cal27-Smad4 cells, respectively (Fig. 2C). These findings revealed that resveratrol efficacy in Cal27 and a lack of efficacy in Det562 cells are independent of Smad4 expression. In other studies, resveratrol (50–100 μmol/L) treatments did not alter cell-cycle progression in NHEK cells (Fig. 2D), further supporting its selectivity...
toward HNSCC cells. Resveratrol (25–50 μmol/L) treatment also induced apoptosis in FaDu, Cal27-pcDNA3, and Cal27-Smad4 cells by 20- to 27-fold, 2.5- to 4-fold, and 3- to 5-fold (P < 0.001), respectively (Fig. 3). Similar to S-phase arrest results, Cal27 cells with overexpression of Smad4 retained sensitivity to resveratrol-induced apoptosis (Fig. 3C), suggesting that the effect on apoptosis is also independent of Smad4.

Resveratrol induces γ-H2AX and Brca1 focus formation in HNSCC cells

H2AX serine-139 phosphorylation (the so-called γ-H2AX) is essential for recruiting and localizing DNA repair proteins (Brca1 and Rad51) at the sites containing damaged chromatin and for checkpoint activation, which arrest cell-cycle progression (16). Brca1 is a tumor suppressor protein involved in maintaining genome integrity, and
following DNA damage, it accumulates in nucleus, where it concentrates in nuclear foci with other DNA double-strand breaks repair factors, including homologous recombination protein, RAD51, and end-joining RAD50–MRE11–NBS1 protein complex (17). Accordingly, to further dissect resveratrol mechanism of action, we assessed γ-H2AX and Brca1 focus formation. Resveratrol enhanced γ-H2AX levels in both FaDu and Cal27 cells but had no effect in Det562 cells. A higher resveratrol dose (100 μmol/L) also induced γ-H2AX in NHEK but to a lesser extent than in FaDu and Cal27 cells (Fig. 4A). We also compared γ-H2AX levels in parental Cal27 versus 2 independently derived Cal27-Smad4 cells (generated by us and the Wang laboratory; ref. 8) and found them to be comparable among these lines. Together, these results clearly indicated that resveratrol triggered DNA damage in HNSCC cells independent of Smad4 expression. Consistently, compared with controls, resveratrol also induced discrete nuclear γ-H2AX and Brca1 focus formation in FaDu cells where Brca1 colocalized with γ-H2AX at the sites of DNA damage (Fig. 4B).

Another important observation related to DNA damaging effect of resveratrol in FaDu cells is an S-phase arrest at 10 μmol/L, but a G1 phase arrest at higher concentration (25–50 μmol/L; Fig. 2A), and a higher γ-H2AX levels with increasing resveratrol concentration indicative of increasing levels of DNA damage (Fig. 4A), which is consistent with G1 or G1–S arrest at higher concentrations. Accordingly, we also determined cyclin levels in FaDu cells after resveratrol treatment and found that whereas cyclin D1 levels decreased, cyclin E levels increased (data not shown) simultaneously, a situation representing inhibition of DNA replication and a concomitant G1–S arrest and blockade to S-phase progression by resveratrol, as we had seen in ovarian cancer cells (9).

Resveratrol-induced γ-H2AX and Brca1 focus formation is independent of Smad4 in Cal27 cells

Recently, Smad4 loss was reported to cause genomic instability in mice, which correlated with less expression and function of genes encoding proteins in the Fanc/Brca in DNA repair pathway together with HNSCC development (8). Thus, we measured γ-H2AX and Brca1 focus formation in Cal27-pcDNA3 and Cal27-Smad4 cells. Cal27-pcDNA3 and Cal27-Smad4 cells showed almost parallel γ-H2AX focus formation after resveratrol (50 μmol/L) treatment for 24 hours (Fig. 5A). Mitomycin C (MMC; 20 ng/mL) treatment also induced the γ-H2AX focus formation in both the cell lines (Fig. 5A). However, MMC-treated cells displayed less intense staining than resveratrol-treated cells (Fig. 5A). Brca1 staining was also examined in both cell lines after resveratrol treatment. Resveratrol (50 μmol/L) treatment for 24 hours induced strong nuclear localization of Brca1 as compared with untreated control (Fig. 5A). However, Cal27-Smad4 cells showed Brca1 focus formation similar to Cal27-pcDNA3 control cells (Fig. 5B). As shown previously (8), Smad4 was required for the formation of Brca1 foci by MMC (Fig. 5B). Thus, unlike MMC, resveratrol can produce Brca1 foci independently of Smad4.

Resveratrol inhibits FaDu tumor xenograft growth together with in vivo DNA damage and apoptotic effects

We also extended studies to an in vivo xenograft model to establish biological significance of all our in vitro...
Figure 4. Resveratrol induces γ-H2AX and Brca1 focus formation in HNSCC cells. A, cells were treated with DMSO alone or resveratrol for 24 hours, total cell lysates were prepared and subjected to SDS-PAGE followed by immunoblotting, and membranes were probed with γ-H2AX and β-actin antibodies as detailed in Materials and Methods. B, resveratrol induces the γ-H2AX and Brca1 focus formation. FaDu cells were treated with DMSO (control) or resveratrol (25–50 µmol/L) for 24 hours followed by immunocytochemical staining for γ-H2AX and Brca1 by using specific antibody, cell images were captured at 1,000× magnification on a Nikon D Eclipse C1 confocal microscope, and images were analyzed by EZ-C1 Free viewer software. Red and green fluorescence represents staining for γ-H2AX and Brca1, respectively. Res, resveratrol; C, control.
findings. Resveratrol feeding by oral gavage resulted in a dose- and time-dependent inhibition in tumor growth, and at the end of the study (30 days), tumor volume/mouse decreased from 2.00 ± 0.25 in controls to 1.35 ± 0.12 and 1.01 ± 0.17 cm³ in 10 and 50 mg/kg body weight resveratrol-fed groups, respectively, accounting for 32% and 50% (P < 0.01) inhibition in tumor growth (Fig. 6A). A reduction in tumor weight/mouse further supported these results wherein resveratrol-treated groups showed 0.95 ± 0.15 and 0.76 ± 0.13 g/mouse tumor weight, respectively, as compared to controls with 2.00 ± 0.35 g/mouse tumor weight (Fig. 6A). In contrast, resveratrol (50 mg/kg body weight) feeding for 15 days had less effect that was not statistically significant (Fig. 6A). In both efficacy studies, compared with controls, we did not observe any significant changes in body weight and diet consumption in resveratrol-fed groups of mice throughout the experiments (data not shown), suggesting that the resveratrol doses and treatment regimens employed in this study are nontoxic.

The qualitative γ-H2AX and cleaved caspase-3 IHC analyses of tumor xenografts for DNA damage and apoptosis markers, respectively, clearly showed a strong increase in staining in both resveratrol-treated tumors and controls (Fig. 6B). Quantitative nuclear γ-H2AX immunostaining showed 25% (P < 0.001) and 32% (P < 0.001) induction in FaDu tumor xenografts from 10 and 50 mg/kg body weight resveratrol-treated mice, respectively, compared with 13% in controls (Fig. 6B, top). Similarly, cleaved caspase-3 staining also increased 11% to 16% (P < 0.001) in FaDu tumor xenografts from resveratrol-treated mice compared with 5% cells in controls (Fig. 6B, bottom).

Discussion

Following DNA damage, normal cells either repair the damage or undergo apoptotic death mostly in p53-dependent manner. However, DNA repair processes are defective in many cancer cells (18) that offer avenues for therapeutic intervention. Consequently, the present study
is highly significant, with major therapeutic implications in that the anticancer effect of resveratrol is predominately due to its ability to induce DNA damage selectively in HNSCC cells without affecting normal, repair-proficient cells.

Our detailed mechanistic studies revealed that resveratrol-induced DNA damage in HNSCC cells results in DNA strand breaks, as evident by the production of comet tails and "defective" γ-H2AX and Brca1 repair foci resulting in irreversible arrest of HNSCC cells either at the G1 to S-phase boundary or in S-phase, eventually leading to apoptotic death. Similar results have been seen in breast cancer cells lacking Brca2 (19) and in colon cancer cells lacking Cdc14 (20) where foci form after DNA damage but are repaired inefficiently. Because p53 is important for DNA repair mechanisms, the novelty of our study is that resveratrol effects were independent of p53 because all 3 HNSCC cell lines harbor mutant p53 mutations (21). Furthermore, sensitivity to resveratrol was directly proportional to extent of basal DNA damage. For example, FaDu cells having higher level of basal DNA damage (Fig. 1B) were more sensitive to resveratrol than Cal27 cells (Fig. 1A). Similarly, Det562 cells with no detectable γ-H2AX even in 50 μmol/L resveratrol were comparatively more resistant to treatment than the other 2 HNSCC cell lines. Because conventional cancer therapy damages DNA in normal and tumor cells (22), it is of considerable importance that resveratrol does not cause DNA damage and checkpoint activation in normal cells with efficient DNA repair pathways, thereby establishing the selectivity of resveratrol in affecting HNSCC cells.

The TGF-β/Smad signaling pathway is altered by genetic mutation that contributes to the carcinogenesis of HNSCC (23). Downregulation of the Smad4 tumor suppressor, the central mediator of TGF-β/Smad signaling pathway is common in malignant HNSCC and correlates with downregulated expression and an inefficient Fanc/Brca DNA repair pathway (8). Thus, DNA repair defects occur in HNSCC in which Smad4 is frequently mutated or poorly expressed. In this regard, our results are highly significant and show resveratrol efficacy in FaDu and Cal27 HNSCC cell lines, which both contain null mutations in Smad4. To determine whether the anticancer effects of resveratrol by the induction of DNA damage is dependent on Smad4-regulated DNA repair mechanisms, matching Cal27 and Cal27-Smad4 stable transfectants were used. Resveratrol treatment caused comparable DNA damage, "defective" repair foci, cell-cycle arrest, and apoptotic death in both cell lines irrespective of their Smad4 status, establishing the fact that Smad4 is dispensable for the anticancer effects of resveratrol in HNSCC. Perhaps, Smad4 is required to process MMC-induced damage and form foci (8), whereas it is not required to form the unreparable foci induced by resveratrol. Together, these results indicate that most of the human HNSCC tumors will be sensitive to resveratrol, even though more than 80% of them are defective in Smad4 (24), signifying strong translational potential of our findings.

An important aspect of our study is that the in vitro effects of resveratrol were reproducible in xenograft studies in both preventive and therapeutic treatment
regimens showing an inhibition in FaDu tumor xenograft growth in nude mice, which was associated with high levels DNA damage and a marked induction in apoptosis. Overall, our results clearly show strong resveratrol efficacy against the growth and proliferation of HNSCC in vitro and in vivo. Specifically, resveratrol produces these effects on HNSCC via its selective ability to induce DNA damage and apoptotic death. Importantly, the anticancer effects of resveratrol and all associated mechanisms are independent of Smad4 status, the mutation/absence of which is one of the primary causes of failed cellular DNA repair machinery in HNSCC. On the basis of these findings, more studies are needed in future to evaluate both anticancer and chemopreventive efficacy of resveratrol in relevant preclinical models to establish its potential usefulness against human HNSCC.

References

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