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Journal Title: Clinical Cancer Research
Volume: Volume 24, Number 4
Publisher: American Association for Cancer Research | 2018-02-15, Pages 858-869
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1158/1078-0432.CCR-17-0345
Permanent URL: https://pid.emory.edu/ark:/25593/tbggw

Final published version: http://dx.doi.org/10.1158/1078-0432.CCR-17-0345

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Accessed April 23, 2019 12:42 PM EDT
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Abstract

Purpose—Previous studies revealed diverging results regarding the role of survivin in squamous cell carcinoma of the head and neck (SCCHN). This study aimed to evaluate the clinical significance of survivin expression in SCCHN; the function of survivin in DNA damage repair following ionizing radiation therapy (RT) in SCCHN cells; and the potential of honokiol to enhance RT through downregulation of survivin.

Experimental Design—Expression of survivin in SCCHN patient primary tumor tissues (n=100) was analyzed and correlated with clinical parameters. SCCHN cell lines were used to evaluate the function of survivin and the effects of honokiol on survivin expression in vitro and in vivo.
Results—Overexpression of survivin was significantly associated with lymph nodes metastatic status (p = 0.025), worse overall survival (OS) and disease free survival (DFS) in patients receiving RT (n = 65, OS: p = 0.024, DFS: p = 0.006) and in all patients with SCCHN (n = 100, OS: p = 0.002, DFS: p = 0.003). In SCCHN cells, depletion of survivin led to increased DNA damage and cell death following RT, whereas overexpression of survivin increased clonogenic survival. RT induced nuclear accumulation of survivin and its molecular interaction with γ-H2AX and DNA-PKCs. Survivin specifically bound to DNA DSB sites induced by I-SceI endonuclease. Honokiol (which downregulates survivin expression) in combination with RT significantly augmented cytotoxicity in SCCHN cells with acquired radioresistance and inhibited growth in SCCHN xenograft tumors.

Conclusions—Survivin is a negative prognostic factor and is involved in DNA damage repair induced by RT. Targeting survivin using honokiol in combination with RT may provide novel therapeutic opportunities.

Keywords
Radioresistance; Survivin; Head and neck cancer; Honokiol

Introduction
Radiotherapy (RT) remains a major therapeutic choice for patients with SCCHN. However, despite the introduction of high-precision radiotherapy techniques and new systemic chemotherapeutic agents, a significant percentage of patients develop resistance to RT, and inevitably progress without cure. In patients diagnosed with Stage III and IV disease, the average five-year survival rate is 50% or less (1,2). Clinically, the identification and treatment of radioresistant SCCHN remains an unsolved problem. It is imperative to understand the molecular mechanisms involved in resistance to RT and to use this information to develop novel strategies to enhance the efficacy of RT in SCCHN patients.

Survivin is a multi-functional protein that participates in the regulation of cell cycle, apoptosis, and cellular stress response. A growing body of evidence suggests that survivin plays important roles in radiation-and chemo-resistance through inhibition of apoptotic signaling(3–5). Overexpression of survivin has been correlated with poor prognosis (6–8) and resistance to RT (9,10). Inhibition of this crucial survival factor has been shown to trigger apoptosis and sensitize cancer cells to RT. Previous studies suggest that survivin enhances tumor cell survival primarily through the inhibition of apoptosis(11), presumably by direct and indirect binding to caspase-9 (12,13). Survivin also induces mitochondrial fragmentation, reduces mitochondrial respiration, and represses BCL2L11/Bim, thereby preventing the accumulation of reactive oxygen species (ROS) and inhibiting apoptosis (14). However, there is increasing evidence to indicate that the role of survivin in response to RT may exceed the simple inhibition of apoptotic pathways, and involve broader cellular adaptation processes (15–17). It has been well documented that DNA double strand breaks (DSBs) are among the effects of RT that best correlate with its cytotoxicity (18). Recent studies have reported that survivin interacts with the non-homologous end-joining (NHEJ) DNA repair complex after RT, suggesting that survivin may be involved in DNA-damage repair (3,9). Several studies have reported that honokiol, a natural flavonoid, can inhibit survivin expression through downregulation of multiple pathways upstream of survivin, such
as EGFR, HSP90, JAK/STAT3, mTOR/NF-κB, and others (19–22). Honokiol also activates sirt3 and increases mitochondrial fusion and respiration (23), thereby antagonizing the protective effect of survivin on cell survival. Honokiol exhibits potent anticancer activities (20,24,25) in a variety of cancer models (19,21,24,26,27). These relatively wide-ranging anticancer capabilities and favorable safety profile make honokiol an attractive adjunct therapy to enhance RT in clinical settings.

In this study, we aimed to evaluate the clinical significance of survivin expression in primary SCCHN tumor tissues and to explore the function of survivin in DNA damage repair following RT in SCCHN cells. These studies may reveal a novel underlying mechanism by which survivin mediates radiation resistance. Finally, we tested the feasibility and efficacy of combination therapy consisting of honokiol and RT in SCCHN and explored the mechanism of action of honokiol in sensitizing SCCHN to RT in vitro and in vivo.

Material and methods

Patients and tissue samples

Archival paraffin-embedded tissue specimens from 100 primary SCCHN cases with (50 cases) and without (50 cases) corresponding lymph node metastases were used to analyze survivin protein expression by immunohistochemistry (IHC). The clinical characteristics of the patients were retrieved from the files in the Department of Pathology and Laboratory Medicine at Emory University. All tissue specimens and appropriate clinical information were obtained under the guidelines and approval of the Institutional Review Board of Emory University.

Cell culture

SCCHN cell lines: Tu212 cell line was kindly provided by Dr. Gary L. Clayman (University of Texas) in 2002, Tu686 cell line was kindly provide by Dr. Peter G. Sacks (New York University) in 2010, PCI-15A and JHU-022 cell lines were kindly provided by Dr. Robert Ferris (University of Pittsburgh) in 2012. All cells were routinely tested for mycoplasma by the MycoAlert Mycoplasma Detection Kit (Lonza Ltd., Allendale, NJ). The authenticity of cell lines were verified through the genomic short tandem repeat (STR) profile by the Research Animal Diagnosti Laboratory, University of Missouri (Columbia, MO) in 2009, and by Emory University Integrated Genomics Core (EIGC) in 2013. All cells were maintained in DMEM medium supplied with 5% fetal bovine serum at 37°C, 5% CO₂ and 95% humidity. Experiments were performed less than 2 months after thawing early passage cells. To generate acquired radioresistant cells (PCI-15A-IRR), we plated PCI-15A cells in 6-cm dishes and irradiated them with 4Gy of X-rays when cells approached 70% confluence. After 5 initial treatments to 4Gy RT, PCI-15A-RR cells were repeatedly treated with 6Gy RT 14 times over a period of 6 months. The parental cells were cultured and reseeded under the same conditions without RT.

Transfection with siRNA and plasmids

Survivin-specific small interfering RNA (siRNA) (SMART pool: a mixture of 4 siRNA) and control non-targeting siRNA were purchased from Thermo Scientific (Rockford, IL USA).
Transfection of PCI-15A cells with survivin siRNA (10 or 30nM) and control siRNA was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY USA) according to the manufacturer’s instructions. Ectopic expression of survivin in JHU-022 cells was performed by transfection with a human survivin cDNA plasmid or its control vector (pcDNA3) using Lipofectamine reagent, following the manufacturer’s protocol (Invitrogen).

**ChIP assay following DSB induced by I-SceI**

PDR-GFP plasmid (carrying the PDR-GFP homologous recombination reporter, in which expression of exogenous I-SceI introduces a single DSB in the cell’s genome (28) was transfected into PCI-15A cells followed by selection after 48h in 2µg/ml puromycin. Cells were expanded for 4 weeks. Then, pCBASceI plasmids were transfected into PCI-15A cells containing pDR-GFP as described (28). After 24h, chromatin immunoprecipitation (ChIP) was performed using Pierce Agarose ChIP Kit (Thermo Scientific, USA) according to the protocol provided by the manufacturer. Survivin-associated DNA was analyzed by PCR using primer 1 or primer2 for sequences upstream or downstream of the I-SceI site, respectively. Primer 1: forward, 5′-TAC AGC TCC TGG GCA ACG TG-3′; reverse, 5′-TCC TGC TCC TGG GCT TCT CG-3′. Primer 2: forward, 5′-CGT CCA GGA GCG CAC CAT CTT CTT-3′; reverse, 5′-ATC GCG CTT CTC GTT GGG GTC TTT-3′. The PCR products were analyzed. Additionally, survivin-associated DNA was quantified by real-time PCR using SYBR Green supermix (Bio-Rad, Hercules, CA) and the comparative CT (ΔΔCT) program. The input controls were used to normalize the DNA samples. Primer sequences used were: forward, 5′-GTG ACC ACC CTG ACC TAC GG-3′; reverse: 5′-AAG TCG TGC TGC TTC ATG TG-3′.

**In vivo anti-tumor efficacy assay**

The animal experiment was approved by the Institutional Animal Care and Use Committee of Emory University. Tu686 cells (1.5×10^6) were injected subcutaneously into 4–5 week-old female nude mice (athymic nu/nu, Taconic NY). When the tumors had developed to about 100mm^3, the mice were divided into four groups (n=6 per group) and treated as follows: 1) control treated with 20% intralipid (Baxter Healthcare), 2) honokiol (50mg/kg), 3) RT (1.5Gy every other day for 5 treatments), 4) honokiol (50mg/kg) plus RT (1.5Gy, every other day for 5 treatments). Honokiol was administered 3 times per week via intraperitoneal injection. For RT, mice were irradiated with 1.5Gy every other day for 5 treatments. The therapy was continued for 4 weeks. The body weight and tumor size were measured and calculated three times per week (29). The mice were sacrificed 4 weeks after the initiation of treatment. Tumor and organ tissues (liver, heart, lung, spleen, and kidney) were collected for H&E staining and immunostaining analyses.

**Statistical analysis**

Clinical parameters such as disease status, site, T-stage, N-stage, and treatment were treated as categorical variables and compared using Chi-square test and Fisher exact test. Analysis of variance (ANOVA) was employed to test the overall significance across different strata of each independent variable. Survival analysis was conducted according to the Kaplan-Meier method. The log-rank test was used to test the difference in survival between groups. COX modeling was used to estimate the adjusted effect of survivin expression on survival after
adjustment for other factors as well as the adjusted effects of other factors on survival. The hazard ratio and 95% confidence interval (CI) were calculated. The SAS statistical package (SAS Institute, Inc., Cary, North Carolina) was used for data management and analysis.

For in vitro studies, experimental data are presented as mean ± standard deviations from three or more independent experiments. Statistical significance was assessed using the Student’s t-test. For the in vivo anti-tumor efficacy study, a log-linear mixed model with random intercept was used to compare the significance of the mean tumor volumes among each group. The statistical significance of the effect of treatment on apoptosis and cell proliferation in xenograft tumor tissues was assessed using the Kruskal-Wallis test (one-way ANOVA). \( P < 0.05 \) was considered statistically significant in all analyses.

Results

Increased survivin expression correlates with worse survival in SCCHN patients

We analyzed the protein expression of survivin in the primary tumor tissue of 100 SCCHN patients by IHC and examined correlations with clinical parameters. A total of 99 tissue samples were available for analysis, of these, 51 patients (51.5%) had locoregional metastasis to cervical lymph nodes and 48 patients (48.5%) presented with disease confined to the primary site. Sixty-five patients received RT with or without chemotherapy (Supplementary Table 1). The median follow-up time, disease free survival (DFS), and overall survival (OS) for all patients were 10.42 (95% CI, 8.42–11.58), 12.8 (95% CI, 5-NA), and 7.17 (95% CI, 4.75–8.5) years, respectively. Survivin expression was detected in all primary tumor specimens as demonstrated by representative micrographs in Figure 1a–d. We assessed correlations between clinical data outcomes and survivin expression. Higher survivin expression levels were significantly correlated with cervical node metastasis (N-negative vs. N-positive \( p = 0.025 \), Supplementary Table 2), worse DFS (\( p = 0.003 \), Supplementary Table 3), and worse OS (\( p = 0.002 \), Supplementary Table 4) in univariate analysis for all patients (\( n = 99 \)). Multivariate analysis also revealed that increased survivin expression levels impacted OS (hazard ratio (HR) =1.79 (1.04–3.08), \( p = 0.036 \), Supplementary Table 5) and DFS (HR=2.94 (1.31–6.63), \( p = 0.009 \), Supplementary Table 6). In a sub-population univariate analysis of patients receiving RT (\( n = 65 \)), increased survivin expression significantly correlated with worse DFS (\( p = 0.006 \)) and OS (\( p = 0.024 \)). Kaplan-Meier survival curve shows that higher survivin expression by IHC \( (W1 \geq 1.46) \) was associated with decreased DFS and OS in all patients (Figure 1e) and in patients receiving RT (Figure 1f). Taken together, these results suggest a close correlation between higher survivin expression and poorer prognosis of SCCHN.

Overexpression of survivin promotes resistance to radiation

We examined the association between survivin expression and RT effect in four established SCCHN cell lines (PCI-15A, Tu212, Tu686 and JHU-022) with considerable differences in radiosensitivity. As shown in Figure 2a, the surviving fraction at 2Gy (SF2) was about 2.5-fold higher in PCI-15A and Tu212 cells than in JHU-022 cells. To evaluate whether survivin expression levels correlate with radioresistance of these cancer cells, Western blot analysis
of total cellular extracts was performed. We found that survivin protein levels were elevated in radioresistant cell lines (PCI-15A, Tu212 and Tu686) when compared with the radiosensitive cell line JHU-022 (Figure 2b).

To assess whether the presence of survivin is required for radioresistance, PCI-15A cells were transfected with survivin siRNA and tested for changes in radiosensitivity. As shown in Figure 2c, survivin siRNA markedly reduced endogenous survivin levels compared with control-siRNA, which was associated with increased radiosensitivity of PCI-15A cells as indicated by the survival curve. Conversely, overexpression of survivin in JHC-022 cells reduced radiosensitivity when compared with control, suggesting that survivin overexpression confers radioresistance to SCCHN cells (Figure 2d). Taken together, these results support an essential function of survivin in mediating the response to RT in SCCHN cells.

Given the apparent importance of survivin expression in the radioresistance of SCCHN cells, we examined the potential of using honokiol to attenuate survivin expression and reduce radioresistance. Consistently, treatment with 5µg/ml of honokiol markedly reduced protein expression of survivin at 24, 48 and 72 hours as seen by immunoblotting (Figure 2e), suggesting that honokiol is an inhibitor of survivin. When PCI-15A cells were pre-treated with 5µg/ml of honokiol for 24 h followed by RT, their clonogenic formation capability was inhibited (Figure 2e), suggesting the potential of honokiol as a sensitizing agent during RT of SCCHN cells.

**Radiation promotes survivin nuclear accumulation**

Since survivin has been reported to be a nuclear-cytoplasmic/mitochondrial shuttling protein (3,30,31), we evaluated whether radiation exposure promotes the nuclear accumulation of survivin in SCCHN cells. Prior to RT, survivin was predominantly present in the cytoplasmic fraction of PCI-15A cells (Figure 3a), but rapidly accumulated in the nuclei following RT. The RT-induced nuclear accumulation of survivin was also clearly demonstrated by the nuclear staining of survivin in PCI-15A cells at a time point 40 min post-exposure (Figure 3b).

Given the observation that honokiol could inhibit survivin expression in SCCHN cells (Figure 2e), we next investigated whether honokiol treatment may interfere with RT-induced nuclear shuttling of survivin. PCI-15A cells were treated with 5µg/ml of honokiol for 24 h followed by 4Gy of RT. Forty minutes after RT, cells were subjected to Western blotting and immunofluorescence staining. Compared to the control, RT alone induced a clear increase in nuclear accumulation of survivin, whereas SCCHN cells pre-treated with honokiol demonstrated reduced RT-induced nuclear localization of survivin (Figure 3b, 3c). Interestingly, when PCI-15A cells were pre-treated with 5µg/ml of honokiol for 1h followed by 4Gy RT, the RT-induced nuclear accumulation of survivin was uninhibited, suggesting that honokiol may act to downregulate survivin expression.
Radiation promotes the interaction of survivin with DNA double strand break response proteins

A recent study suggests that survivin may be involved in DNA double strand break (DSB) repair after RT (3). We postulated that following RT, nuclear survivin may physically interact with the DNA DSB repair machinery in SCCHN cells. Using co-immunoprecipitation (co-IP) assays, we examined the interaction between survivin and DNA DSB response and repair proteins including γ-H2AX and DNA-PKcs at 0, 20, 40, and 60 min after RT (4Gy) in PCI-15A cells. γ-H2AX is a recognized marker of DNA DSB. DNA-dependent protein kinase catalytic subunit (PKcs) binds to DNA DSB ends and is required for the NHEJ pathway of DNA repair, which also influences the HR repair pathway (32,33). As shown in Figure 4a, increased levels of γ-H2AX and DNA-PKcs were observed 20 minutes after RT and remained elevated in survivin immunoprecipitates as compared to the control. Reciprocal co-IP with anti-γ-H2AX antibody revealed increased expression of survivin after RT when compared with the control (Figure 4a), indicating a physical interaction between the two proteins. To further confirm this observation, PCI-15A cells were stained with anti-survivin (green), anti-γ-H2AX (orange) antibodies, and DAPI (blue) at 40 min after RT (4Gy). Confocal microscopy analysis revealed that survivin was co-localized with γ-H2AX in nuclear foci at sites of DNA DSB response and repair (Figure 4b).

To further evaluate whether survivin specifically binds the sites of DNA damage, we established a cellular system using PCI-15A cells carrying the DR-GFP homologous recombination reporter, in which expression of exogenous I-SceI (homing-endonuclease) introduces a single DSB in the cell’s genome (28). After induction of DSBS by I-SceI, ChIP assays were performed. Chromatin was immunoprecipitated from the cells with a survivin antibody. PCR was then performed to detect survivin aggregated at the induced DSB sites by using primers specifically designed for DSB site sequences. As shown in Figure 4c, increased survivin binding to the DSB site induced by I-SceI was observed compared to the signal from samples without DSB. Additionally, survivin-associated DNA was quantified by real-time PCR using ChIP samples, which revealed about a 22-fold increase in samples with induced DSB compared to that in non-DSB samples (Figure 4c), confirming the results obtained from immunofluorescence analysis and providing direct evidence that survivin specifically targets and binds to the DNA DSB site.

We next asked whether the binding of survivin to DNA DSB sites and complexation with NHEJ DNA repair factors is mechanistically significant to DNA repair. We performed a neutral single cell gel electrophoresis (comet) assay to evaluate the DNA DSB repair induced by RT in PCI-15A cells with survivin knockdown. Compared to control siRNA treated cells, survivin knockdown cells showed a statistically significant increase in tail DNA (non-repaired DNA DSB) (Figure 4d) indicating impaired DNA repair, suggesting that survivin is involved in the DNA DSB repair process in SCCHN cells.

When PCI-15A cells were pre-treated with honokiol (5µg/ml for 24 h) followed by treatment with 4Gy RT, we observed a reduction in the level of survivin in the nuclei and decreased co-localization with γ-H2AX by immunofluorescence staining (Figure 4e). A comet assay was performed after treatment with honokiol (5µg/ml for 24 h) in combination with RT.
(4Gy). Compared to untreated cells, those treated with either RT or honokiol alone showed a mild increase in tail DNA. In contrast, PCI-15A cells treated with the combination of honokiol and RT showed a statistically significant increase in tail DNA (Figure 4f), suggesting that honokiol may affect the DNA DSB repair process in SCCHN cells and sensitize DNA to RT. Taken together, these results indicate that while RT promotes the complex formation between survivin and DNA DSB response and repair proteins, honokiol can antagonize the RT-induced nuclear accumulation of survivin, presumably through the downregulation of survivin.

Radiation-induced survivin upregulation may contribute to acquired radioresistance

As a survival factor that is responsive to stress, survivin could be induced by radiation or chemotherapeutic agents. We measured the expression of survivin after RT. As shown in Figure 5a, a single dose of RT (4Gy or 8Gy) increased survivin expression in PCI-15A cells at 2 hours and an elevated level was maintained at all time points when compared with the control. Consistently, a similar effect of RT on survivin induction was observed in RT-treated Tu686 cells (Supplementary Figure S1). Treatment with honokiol (5µg/ml) abolished the RT-induced increase in survivin expression in PCI-15A cells, and also downregulated survivin upstream signaling components, including EGFR and STAT3 (Figure 5a).

Previous studies have suggested that cancer cells may acquire resistance to RT-induced apoptosis through the dynamic interplay and regulation of multiple pro-survival factors. Considering our observations and the biological functions of survivin in cell survival, we hypothesized that RT-induced overexpression of survivin could be one of the major factors in the acquisition of radioresistance. To further test this hypothesis, we established a PCI-15A cell line with acquired resistance to RT, named PCI-15A-RR, through repeated radiation exposure. Clonogenic assay confirmed that PCI-15A-RR had acquired increased resistance to RT (SF2: 0.79±0.02), whereas the parental PCI-15A (PCI-15A-P) cell line maintained its sensitivity to RT (SF2: 0.40±0.09). Importantly, a significant increase in the expression of survivin protein as well as EGFR and HSP90 (two proteins involved in regulating survivin expression) suggests that RT-induced survivin upregulation may contribute to acquired radioresistance (Figure 5b). In addition to survivin, we also examined the expression of two important anti-apoptotic proteins, Bcl2 and Mcl-1, whose overexpression has been implicated in RT resistance. Interestingly, increased expression of Mcl-1, but not of Bcl-2, was observed (Figure 5b).

To examine the ability of honokiol to attenuate acquired radioresistance via the inhibition of survivin, PCI-15A-RR cells were treated with honokiol and Western blot analysis revealed a markedly reduced survivin expression level in honokiol-treated cells compared with the control cells (Figure 5c). Consistently, clonogenic assay in PCI-15A-RR cells showed that pre-incubation with honokiol (5µg/ml) for 24 h and then treatment with RT (2Gy) resulted in a significantly decreased survival rate when compared to treatment with RT (2Gy) alone (Figure 5d), indicating the potential of honokiol as a natural agent to overcome acquired radioresistance in SCCHN cells.
Honokiol downregulates survivin and improves the efficacy of radiation in SCCHN xenograft tumors

We evaluated the antitumor efficacy of treatment with honokiol, RT, and their combination for 4 weeks in the Tu686 xenograft model. As shown in Figure 6a, treatment with honokiol alone (50mg/kg, 3 times per week, via i.p.) slightly suppressed tumor growth, although the difference was not statistically significant (p=0.778) (Figure 6a). At the dose of 1.5Gy delivered every other day for 5 doses, RT alone moderately inhibited the growth of Tu686 subcutaneous tumors, when compared to the vehicle control (p=0.192). In contrast, the combination treatment significantly inhibited the growth of Tu686 tumors, when compared with any other groups (vs. control: p=0.037; vs. honokiol: p=0.030; vs. RT: p=0.007). The combination of honokiol and RT demonstrated an additive effect on tumor growth inhibition (p=0.969). The average tumor volume and weight in each treatment group at the end point were 3267.2±1316.7 mm$^3$ and 2.26±0.86g (control), 2597.9 ± 939.4 mm$^3$ and 1.26±0.42g (honokiol), 1169.7±109.9 mm$^3$ and 0.64±0.07g (RT), and 487.9 ±164.0mm$^3$ and 0.28±0.11g (combination), respectively (Figure 6a, b). These results indicate that honokiol may improve the antitumor activity of RT in SCCHN xenograft tumors.

We evaluated the systemic toxicity of honokiol, RT and the combination treatment in the xenograft model. Compared with the control group, the body weights of mice in all three treatment groups were similar, with negligible toxicity profiles observed under the tested conditions (Figure 6c). Consistently, histopathologic analyses did not find any significant tissue damage in the major organs (including liver, spleen, kidney, heart and lung) collected from any treatment groups, including the combination treatment (Figure 6d).

We performed IHC analysis to examine the in vivo effect of each treatment on the expression of survivin and Ki67, which is a proliferation marker. IHC analysis revealed a markedly reduced survivin expression in tumors treated with honokiol, either alone or in combination with RT, compared with the control and radiation treated tumors. The combined treatment significantly reduced the tissue level of Ki-67 (percentage of Ki-67 positive cells: 4.00 ± 1.15), when compared to that in the control group (15.83 ± 3.36; p<0.001), honokiol group (11.14 ± 3.44; p<0.001), or radiation group (7.73 ± 3.66; p=0.041) (Figure 6e). Consistently, TUNEL assay revealed a significant increase in apoptotic tumor cells in the tumor tissues from the combination group (9.56 ± 2.96) when compared with that in the control group (2.11 ± 1.27; p=0.002), honokiol group (3.11 ± 1.36; P=0.004) and RT group (6.33 ± 2.45; P=0.04) (Figure 6e). These results indicate that honokiol can enhance RT efficacy in vivo through the downregulation of survivin.

Discussion

Overexpression of survivin has been frequently associated with radioresistance (9,10), metastasis (34,35), and poor prognosis (4,9,36) in many cancers including lung, colon, breast, prostate, gastric and pancreas. However, the clinical implication of survivin expression in the prognosis of SCCHN remains controversial. Overexpression of survivin has been reported as a negative prognostic factor in SCCHN (7,37,38). It also has been reported that survivin nuclear accumulation was an independent predictor of poor survival in patients with oropharyngeal squamous cell carcinoma (SCC) (39). However, contradictory
results have also been reported. For example, one study showed that high survivin was correlated with favorable overall survival in patients with oral SCC (40). In another study, survivin was not found to be a prognostic factor for oral SCC (41). These diverging observations point to a need to clarify the role of survivin in the progression of SCCHN. In this study, we present clinical data supporting that increased survivin expression is significantly associated with metastasis and worse overall and disease free survival in SCCHN patients receiving RT and in all patients. Further inclusion of larger numbers of patient specimens is necessary to confirm the clinical significance of survivin in SCCHN. Mechanistically, we demonstrated that overexpression of survivin contributes to radioresistance in SCCHN cells by facilitating DNA damage response and repair.

Although many studies including our own have documented a correlation between overexpression of survivin and tumor aggressiveness, radiation-resistance, and poor survival in several cancer types (9,10,34,42), the underlying mechanisms are not fully understood. Previous studies suggested that survivin enhances the survival of tumor cells primarily through the suppression of apoptosis by inhibition of caspases (11). Recent studies have suggested a link between survivin and radiation-induced DNA damage response. Accordingly, interference with survivin expression or function resulted in reduced DNA repair (3,43), and therapeutic targeting of survivin with the small molecule transcriptional suppressor YM155 delayed DSB repair (44). Despite the suggestion that survivin may potentially control the expression of the DNA repair protein Ku70 (45), the molecular basis for a direct involvement of survivin in DNA damage response and repair remains to be elucidated.

It has been well documented that exposure to RT induces the formation of DNA DSBs, the most cytotoxic of DNA lesions. DNA DSBs are mainly repaired by two mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). Current models of the NHEJ mechanism implicate a rapid binding of the heterodimeric Ku proteins (Ku70/Ku80) to double-stranded DNA ends, and the recruitment of DNA dependent protein kinase (DNA-PKcs), generating a DNA-PK holoenzyme complex and initiating further steps in DSB repair. We found that RT stimulates rapid nuclear accumulation of survivin and its interaction with DNA-PKcs and γ-H2AX, suggesting that survivin may participate in DNA DSB repair following RT. To further test whether the function of survivin in DNA DSB repair is dependent on its interaction with H2AX proteins, we examined the effect of survivin siRNA and H2AX siRNA in PCI-15A cells at 40 min following radiation. Interestingly, H2AX depletion resulted in a reduction of survivin foci formation, whereas survivin siRNA transfection did not have a significant effect on H2AX foci formation (Supplementary Figure 2). These results suggest that although both survivin and H2AX contribute to DSB repair and physically associate with each other, they may exert their functions in a relatively independent manner.

Utilizing PCI-15A cells carrying the DR-GFP homologous recombination reporter, in which expression of exogenous I-SceI (homing-endonuclease) introduces a single DSB in the cell’s genome, our data provide direct evidence that survivin specifically binds to DNA DSB sites. Consistent with our observations, a study reported that the complexation of survivin with DNA-PKcs and other DSB repair proteins facilitates DNA-PKcs enzymatic activity
following radiation exposure in a glioblastoma model (3). These data from our group and others suggest a newly identified function of survivin in directly mediating DSB repair through the stimulation of NHEJ, in addition to its well-characterized roles in the regulation of apoptosis.

Significant efforts have been made to develop strategies to target survivin therapeutically in a variety of cancers, and several agents have been shown to modulate the expression/activation of survivin, such as an antisense molecule (LY218130B) and transcriptional repressors (YM155 and EM-1421). Several phase I and II clinical trials of YM155 combined with chemotherapeutic agents have been conducted in patients with breast, prostate, non-small-cell lung cancer, non–Hodgkin lymphoma, and colorectal cancer (46,47). Nonetheless, the portfolio of survivin antagonists or suppressors available for clinical testing, particularly in SCCHN, is very limited. In this study, we demonstrated the potential utility of a natural compound, honokiol, in targeting survivin and enhancing RT. Also, honokiol exhibits a favorable safety profile in preclinical (48,49) and clinical study (50). These observations lay the foundation for the future use of honokiol as a novel therapeutic to improve standard treatment in SCCHN.

A major challenge affecting the outcomes of patients with SCCHN is the development of acquired radioresistance. To test whether honokiol could overcome acquired radioresistance of SCCHN, we established a SCCHN cell model to mimic acquired radiationresistance. An elevated level of survivin expression was observed in the resistant cells, suggesting that this upregulation could, at least in part, contribute to acquired radioresistance. Importantly, honokiol downregulated survivin and reversed radiation resistance in SCCHN cells with acquired radiationresistance. In addition to survivin, we also examined the expression of two important anti apoptotic proteins, Bcl2 and Mcl-1, whose overexpression has been linked to resistance to RT. Increased expression of Mcl-1 but not Bcl-2 was observed in the resistant cell model. Consistent with our previous study and others, Mcl-1 expression was decreased by honokiol, thus, downregulation of Mcl-1 may also contribute to overcoming radiation resistance in this model. Our findings could provide a basis for identifying targets to increase susceptibility to radiation and the subsequent development of target-based radiosensitizers.

In conclusion, our data support that survivin is a negative prognostic factor in patients with SCCHN, and plays a crucial role in RT resistance in SCCHN cells. Beyond the well characterized function of survivin as an apoptosis inhibitor, we demonstrated that survivin is involved in RT-induced DNA damage repair, which may contribute to RT resistance. Moreover, honokiol treatment downregulates survivin and sensitizes resistant SCCHN cells to RT. Thus, the combination of honokiol and RT appears to have promise to improve clinical outcomes in SCCHN.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

Author’s Financial Support: Dong M. Shin, NIH/NCI P50CA128613, Jack L Arbiser, NIH, RO1AR47901; Xingming Deng, NIH/NCI, R01CA136534

The authors thank Dr. Anthea Hammond for her critical reading and editing of the article.

Abbreviations List

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SCCHN</td>
<td>squamous cell carcinoma of the head and neck</td>
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<td>RT</td>
<td>radiationtherapy</td>
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<td>OS</td>
<td>overall survival</td>
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<td>DFS</td>
<td>disease free survival</td>
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<td>DSBs</td>
<td>double strand breaks</td>
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<td>NHEJ</td>
<td>non-homologous end-joining</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>HR</td>
<td>hazard ratio</td>
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<td>co-IP</td>
<td>co-immunoprecipitation</td>
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<tr>
<td>PKcs</td>
<td>kinase catalytic subunit</td>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
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<td>HR</td>
<td>homologous recombination</td>
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<td>WI</td>
<td>weighted index</td>
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References


### Statement of Translational Relevance

Radiotherapy remains one of the major treatment approaches for squamous cell carcinoma of the head and neck (SCCHN) and radioresistance is a major challenge affecting patient outcomes. This study demonstrates that overexpression of survivin is associated with radioresistance in preclinical models and with poor clinical outcome in SCCHN patients. Beyond the well characterized function of survivin as an inhibitor of apoptosis, we have demonstrated that survivin is involved in radiation-induced DNA damage repair, thus contributing to radioresistance. Recent studies have shown that honokiol (a natural compound isolated from Magnolia tree bark) inhibits survivin expression in certain human cancer cell lines. We evaluated the potential of using honokiol to mitigate radioresistance, and found that in SCCHN models, honokiol effectively downregulated survivin expression to reduce radioresistance. These clinical and preclinical observations provide a strong rationale to target survivin to enhance the therapeutic efficacy of radiation in SCCHN as well as other cancers.
Figure 1.
(a–d) Representative images of survivin expression in SCCHN exhibiting weak (b), medium(c), and strong (d) IHC staining, (a) negative control. (e) Kaplan-Meier survival curve shows the overall survival (OS, left) and disease free survival (DFS, right) according to survivin expression measured as weighted index (WI) in all patients. The WI cut-off value is 1.46, which is the median for survivin expression. (f) Kaplan-Meier survival curve shows the OS (left) and DFS (right) according to survivin WI in patients receiving RT. The cut-off value is 1.48, the median survivin WI.
Overexpression of survivin promotes resistance to RT. (a) The intrinsic radiosensitivity of four SCCHN cell lines was measured as the surviving fraction at 2Gy (SF2). (b) Expression of survivin in four cell lines. (c) PCI-15A cells were transfected with siRNA specific for survivin or control siRNA. Forty-eight hours after transfection, survivin expression was examined and clonogenic survival assays were performed. (d) JHU-022 cells were transfected with a human survivin cDNA plasmid or its control vector (pcDNA3). (e) Survivin expression was detected in PCI-15A cells treated with 5µg/ml of honokiol for 24, 48 and 72 h. For clonogenic survival assay, PCI-15A cells were first treated with 5µg/ml of...
honokiol (HNK) for 24 h followed by 2, 4, 6, or 8 Gy RT. Data are displayed as the mean ±SD from three independent experiments.
Figure 3.
RT promotes survivin nuclear accumulation. (a) PCI-15A cells were irradiated (4Gy). Survivin expression in the cytoplasmic and nuclear fractions was detected at 20, 40, and 60 min after RT. (b) PCI-15A cells were treated with 5µg/ml of honokiol for 24 h followed by 4Gy RT. Forty minutes after RT, cells were subjected to immunoblotting and (c) immunofluorescence staining analysis for survivin (green). Nuclei were counter stained with DAPI (blue) (magnification 400×). One representative out of three independent experiments is displayed.
Figure 4.
RT promotes complexation of survivin with DNA DSB response and repair proteins. (a) PCI-15A cells were irradiated (4Gy). Co-IP with anti-survivin (left) antibody was performed using the subcellular extract nuclear fraction, and associated γ-H2AX and DNA-PKCs (left) were detected. Reverse IP with anti-γ-H2AX antibody (right) was performed and followed by survivin detection; and (b) cells were stained with anti-survivin, anti-γ-H2AX, and DAPI (magnification 400×). (c) Recruitment of survivin at DSB sites. Examples of ChIP analysis by PCR of survivin on a DSB induced by I-SceI, where input demonstrates equal amounts of DNA used for ChIP. (d) Cells were transfected with siRNA specific for survivin or control.
siRNA (for 48h) followed by 4 Gy RT. Comet assay was performed to evaluate DNA damage. (e) Cells were pretreated with honokiol (5µg/ml for 24 h) followed by 4Gy RT. Forty minutes after RT, cells were stained with anti-survivin, anti-γ-H2AX, and DAPI, and (f) Comet assay was performed to evaluate DNA damage.
Figure 5.
Radiation induces survivin expression. (a) Upper: Effect of RT on survivin expression in PCI-15A cells at the indicated time points after RT (4 or 8 Gy). Lower: cells were pretreated with honokiol (5µg/ml for 24 h) followed by 4 Gy RT for 24 or 48 h. (b) Expression of survivin and other indicated proteins in PCI-15A parental and radioresistant cells (PCI-15A-RR) (left). The radiosensitivity was measured as SF2 (upper). (c) Honokiol downregulates survivin expression and inhibits the EGFR-STAT3 signaling pathway in PCI-15A-RR cells. Cells were treated with honokiol (5µg/ml) for 24 and 48 h. (d) PCI-15A-RR cells were treated with RT (2Gy) or honokiol (5µg/ml) alone or in combination then clonogenic assay was performed. All experiments were repeated three times, and representative data are presented.
Figure 6.
Inhibition of tumor growth by the combination of honokiol and radiation in a xenograft model. (a) The tumor growth was significantly inhibited in the combination treated group, when compared with any other groups (vs. control: p=0.037; vs. honokiol: p=0.030; vs. radiation: p=0.007). Tumor weights (b) and body weights (c) of mice in all groups. (d) Histopathologic analyses of major organs from control and combination treatment group. (e) Effects of combination treatment of honokiol and radiation on survivin expression, cell
proliferation, and apoptosis \textit{in vivo}. Apoptotic cells are shown in green; DNA counter stained with DAPI in blue (magnification 200x).