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Primary Vestibular Schwannoma Cells Activate p21 and RAD51-Associated DNA Repair Following Radiation-Induced DNA Damage

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Abstract

Hypothesis: Vestibular Schwannoma (VS) can avoid cell death following radiation injury by entering cell cycle arrest and activating RAD51-related DNA repair.

Background: Although the radiobiology of various cancers is well-studied, the radiobiological effects in VS are poorly understood. In this study, we describe how VS cells enter cell cycle arrest (through p21 expression), activate DNA repair (through RAD51 upregulation), and avoid cell death after radiation-induced double-stranded breaks (DSB) in DNA (as measured by γ-H2AX).

Methods: Primary human VS cells were cultured on 96-well plates and 16-well culture slides at 10,000 cells/well and exposed to either 0 or 18 Gray of radiation. Viability assays were performed at 96 hours in vitro. Immunofluorescence for γ-H2AX, RAD51, and p21 was performed at 6 hours.

Results: Radiation (18 Gy) induced the expression of γ-H2AX, p21, and RAD51 in 6 cultured VS, suggesting that irradiated VS acquire DSBs, enter cell cycle arrest, and initiate RAD51 DNA repair to evade cell death. However, viability studies demonstrate variable responses in individual VS cells with 3 of 6 VS showing radiation resistance to 18 Gy. On further analyses, radiation-resistant VS cells expressed significantly more p21 than radiation-responsive tumors.

Conclusions: In response to radiation-induced DNA damage, primary VS cells can enter cell cycle arrest and express RAD51 DNA repair mechanisms to avoid cell death. Radioresistant VS

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cells may mount a more robust p21 response to ensure sufficient time for DNA repair. Further investigation into DNA repair proteins and cell cycle checkpoints may provide important insight on the radiobiology of VS and mechanisms for resistance.

Keywords

vestibular schwannoma; VS; radiation; resistance; H2AX; p21; RAD51; cell cycle arrest

Introduction:

Vestibular schwannomas (VS) are benign intracranial tumors arising from the Schwann cells of the cochleovestibular nerve and account for 5-9% of all brain tumors [1]. VS result from inactivating mutations in the *NF2* gene, which codes for merlin, a tumor suppressor protein [2]. Due to their location at the cerebellopontine angle and internal auditory canal, VS can cause hearing loss, tinnitus, and vertigo. Depending on symptoms and tumor size, treatment options for VS can include initial observation, stereotactic radiosurgery (SRS), or microsurgical resection [3]. The use of SRS as an initial treatment approach for VS has increased to approximately 25% in the past ten years [4,5].

The main goal of radiation therapy for VS is to halt tumor growth while minimizing radiation toxicity to surrounding structures such as the cochlea and brainstem. Although the overall progression-free survival rate after SRS ranges from 88 to 94%, a subset of VS exhibit resistance to radiation therapy and continue to grow after SRS [6,7]. Unfortunately, the biological mechanisms responsible for radiation resistance in VS are not well understood. An improved understanding of the radiobiology of VS will provide insight into the development of methods for preventing radiation resistance in the future.

It is well established that ionizing radiation (IR) results in DNA damage in the form of single and double stranded breaks (DSB). Radiation-induced DSBs activate the ataxia-telangiectasia mutated (ATM) protein, which coordinates the DNA Damage Response (DDR) in part by phosphorylating the Ser-139 residue of the minor histone H2A variant to form γ-H2AX; thus, γ-H2AX is a sensitive and specific marker of DNA DSBs [8,9]. If this DNA damage goes unrepaired, cell cycle checkpoints are activated [10]. Subsequent cell cycle arrest, which is mediated by cell cycle arrest proteins p21 (cyclin dependent kinase inhibitor CDKN1A) and p53, allows the cell to sense and repair DNA damage through a variety of DNA repair methods, including homologous recombination (HR). In HR, RAD51 serves as the main repair protein that identifies homologous strands and recruits other enzymes in the repair of DSBs [10,11]. If DNA is still not adequately repaired, normal cells initiate cell death mechanisms [12,13]. However, there is strong evidence that various tumors display radiation resistance through upregulation of DNA repair and cell cycle arrest proteins [14-17]

A recent in vitro study showed that merlin-deficient Schwann cells (MD-SCs) displayed upregulation of the DNA repair protein RAD51 in response to IR-induced DNA damage when compared to normal human Schwann cells [18]. However, little is known about the activity of RAD51 in response to radiation-induced DSBs in VS. In this study, we aimed to (1) describe the activation of RAD51 in response to increased levels of radiation-induced DNA DSBs (as measured by γ-H2AX) and (2) describe how VS may enter cell cycle arrest

through upregulation of cell cycle arrest protein p21 to avoid cell death following radiation injury.

Materials and Methods:

Tumor Harvesting

Six patients undergoing VS surgery at University of Miami / Jackson Memorial Hospital were consented for tumor harvesting using a University of Miami Institutional Review Board-approved protocol (#20150637). Fresh tumor was collected at the time of surgery and placed in chilled Dulbecco's Modified Eagle Medium (DMEM; Gibco). Subsequently, the tumor was enzymatically dissociated and cultured in flasks pre-coated with 0.01% poly-L-ornithine (PLO; Sigma-Aldrich) and 25 μg/mL of laminin (ThermoFisher Scientific). VS cells were incubated in Schwann cell media (ScienCell) at 37 degrees Celsius and 5% CO_2.

Retrospective Chart Review

A retrospective chart review was performed for the six VS patients for: (1) age, (2) gender, (3) hearing status, (4) tumor volume, and (5) extent of tumor resection (Table 1). The American Academy of Otolaryngology Head and Neck Surgery (AAO-HNS) Hearing Classification Scale was used to stratify patients into serviceable hearing (Grade A/B) and non-serviceable hearing loss (Grade C/D). Tumor volumes were measured on magnetic resonance imaging with axial T1 sequences with contrast and MIM software (MIM Software, Inc.).

Radiation Delivery

Radiation was delivered with an RS 2000 biological cabinet X-Irradiator (Rad Source Technologies). All cells were irradiated at room temperature (RT) and received a radiation dose of either 0 or 18 Gray (Gy) at 160kV and 25.0 mA, delivered at a dose rate of 1.85 Gy/min. The 18 Gy radiation dose was chosen because we wanted to analyze a supratherapeutic radiation dose for VS.

Cell Viability Assays

Primary human VS cells were cultured at 10,000 cells per well on 96-well plates precoated with 0.01% PLO and laminin (25 μg/mL) in Schwann cell media (ScienCell) at 37 degrees Celsius and 5% CO2. Passages 1 or 2 were utilized for assays. After 24 hours in vitro, plates were switched to maintenance media, which consists of DMEM, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cells were then exposed to either 0 or 18 Gy of IR and the culture media was replaced with fresh maintenance media. Cell viability assays (CellTiter-Glo, Promega) were performed at 96 hours in vitro using the manufacturer's recommended protocol and measured with the Glomax luminometer (Promega). Mean fold change (MFC) in cell viability was calculated relative to the 0 Gy condition for all samples.

Immunofluorescence (IF)

Primary human VS cells were cultured on 0.01% PLO and laminin (25 μg/mL)-coated 16-well culture slides at 10,000 cells per well. After 24 hours in vitro, cells were switched to maintenance media and then exposed to either 0 or 18 Gy of IR. Cells were fixed overnight with 4% paraformaldehyde at 6 hours following radiation exposure. Cells were permeabilized and blocked with 5% donkey serum (Sigma) and 1% Triton X-100 in phosphate buffered saline (PBS) for 2 hours at RT. Subsequently, cells were incubated with primary antibody overnight at 4 degrees Celsius. The primary antibodies were anti-γ-H2AX (1:200) (GT231, Invitrogen), anti-RAD51 (1:1,000) (ab63801, Abcam), and anti-p21 (1:100) (MA5-14949, ThermoFisher Scientific). Cells were washed and incubated with fluorescence-conjugated secondary antibodies (donkey anti-mouse-Alexa 488, ThermoFisher Scientific, 1:200; donkey anti-rabbit-Alexa 594, ThermoFisher Scientific, 1:200) for 2 hours at RT. Cultures slides were then washed with PBS, stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei (ab104139, Abcam) for 10 minutes at RT, and cover-slipped with anti-fade mounting medium (Sigma). Representative images were obtained using a confocal microscope and 40X oil immersion lens (Leica SP5 Inverted Confocal Microscope). Nuclear foci counts and fluorescence intensities (corrected total cell fluorescence) for γ-H2AX and RAD51 were calculated using ImageJ software (National Institutes of Health, Baltimore, MD, USA). For p21 nuclear expression, nuclear counts for positive staining and fluorescence intensities (corrected total cell fluorescence) were measured.

Statistical Analysis

MFC in viability was analyzed with two-way ANOVA with Tukey post-hoc testing for radiation dosage and individual VS. γ-H2AX and RAD51 nuclear foci and fluorescence intensity values were analyzed using Mann Whitney U tests. p21 nuclear expression was analyzed using Fisher's Exact Test. Friedman's two-way analysis of variance with Tukey post-hoc testing was performed to determine differences in p21 expression between radiation dosages and radiation response. Pearson's correlation coefficients were calculated to assess the strength of linear correlations between γ-H2AX and RAD51 nuclear foci per cell, as well as between γ-H2AX foci and MFC in viability. Significance was set at p-value less than 0.05.

Results:

Clinical Characteristics of Patients with Vestibular Schwannoma (VS)

The study included six patients (two female, four male) who underwent microsurgical resection VS (Table 1). All tumors were non-irradiated. Of the six tumors, only VSA60 and VSA73 had radiographic evidence of progression at time of surgery, while the remaining had no prior imaging studies for comparison. Of note, VSA73 was a revision surgery for tumor progression. The mean age at surgery was 43.5 years old (range: 28-72 years old). The mean tumor volume was 6.46 cm^3 (range: 0.55-15.25 cm^3). Gross total resection was achieved in four of the six tumors, while near total resection was achieved in VSA69 and VSA73. Only one patient had serviceable hearing (AAO-HNS Grade A/B) while the remaining five patients had non-serviceable hearing (AAO-HNS Grade C/D). Axial

T1-weighted MRI images for the six patients are displayed in Figure 1. Four of the tumors were right-sided and two were left-sided.

Cell Viability by Radiation and Individual VS

Primary human VS cells were irradiated at either 0 or 18 Gy. Cell viability assays were performed at 96 hours after radiation exposure in vitro. Overall, radiation (18 Gy) resulted in a significant reduction in cell viability of approximately 20% in primary VS cells when compared to non-irradiated cells ($p<0.0001$) (Figure 2A). Additionally, analysis of MFC in viability by individual VS revealed disparities in viability losses in response to radiation (Figure 2B). Two statistically significant groups emerged: VSA59, VSA60, and VSA69 had greater viability losses than VSA58, VSA62, and VSA73; thus, VSA59, VSA60, and VSA69 were more radiation-responsive, while VSA58, VSA62, and VSA73 were more radiation-resistant ($p<0.05$). The dotted line in Figure 2B importantly distinguishes these two groups of VS at 80% MFC in viability; this delineation is relevant as changes in viability that fall below this value are defined in clinical trials as treatment success. Overall, these findings correlate with the current understanding that a subset of VS is known to display radiation resistance.

Immunofluorescence After Irradiation: DNA Damage and Repair

Immunofluorescence for RAD51, γ-H2AX, and p21 was performed on primary human VS cells 6 hours after exposure to either 0 or 18 Gy of IR (Figure 3). Radiation resulted in significant DNA damage in the form of DSBs as indicated by increased nuclear foci counts and fluorescence intensity for γ-H2AX in the 18 Gy condition as compared to non-irradiated cells ($p<0.0001$; Figure 3A, 3C). In response to this DNA damage, irradiated VS cells initiated RAD51-associated DNA repair, as is evident from increased RAD51 nuclear foci counts and fluorescence intensity for the 18 Gy condition ($p<0.0001$; Figure 3B, 3D).

Representative confocal images (40X) are provided for two VS samples: one radiation-responsive VS (VSA69) and one radiation-resistant VS (VSA73) (Figure 3E-F). Both VS display increased RAD51 DNA repair and γ-H2AX as a marker of radiation-induced DNA DSBs when exposed to radiation (18 Gy). Notably, the radiation-responsive VSA69 displayed increased γ-H2AX nuclear foci counts and fluorescence intensity compared to the radiation-resistant VSA73, suggesting that radiation-responsive VS have greater viability losses due to more extensive DNA damage. However, when the γ-H2AX and RAD51 nuclear foci counts and fluorescence intensities were compared between the 3 radiation-responsive and 3 radiation-resistant tumors, we found no statistically significant differences in γ-H2AX and RAD51 (data not shown).

Radiation also resulted in significant upregulation of cell cycle arrest protein p21, indicating that human VS cells enter into cell cycle arrest following radiation exposure ($p<0.0001$; Figure 4A-C). Taken together, these results suggest that human VS cells may enter cell cycle arrest following irradiation in order to repair damaged DNA through RAD51-related pathways like HR. Interestingly, the radiation-resistant VS (VSA58, VSA 62, and VSA73) displayed greater p21 fluorescence intensity than the radiation-responsive VS (VSA 59,

VSA60, and VSA69); these results may suggest that a more robust cell cycle arrest response (i.e. a greater level of p21 upregulation) allows for more extensive DNA repair and the avoidance of cell death activation (Figure 4C-E).

All six VS cells displayed upregulations of γ-H2AX, p21, and RAD51 when exposed to radiation (18 Gy), suggesting that radiation injury in VS results in DNA DSBs, p21 activation leading to cell cycle arrest, and subsequent RAD51-associated DNA repair. Overall, there were no significant differences in the nuclear foci counts or fluorescence intensities of γ-H2AX and RAD51 between the radiation-resistant and radiation-responsive groups at 6 hours. However, the fluorescence intensities of p21 in radiation-resistant VS were significantly higher than in radiation-responsive VS at 18 Gy (Figure 4C), indicating that radiation-resistant VS may display an overexpression of p21 and thus a robust cell cycle arrest period consistent with sufficient time for repair of radiation-induced DNA damage.

Relationship between Double-Strand Breaks, DNA Repair, and Cell Viability

There is a moderate, negative linear association between number of γ-H2AX nuclear foci and cell viability ($R = -0.5725$, $p<0.0001$), indicating that radiation-induced DNA damage yields greater viability losses (Figure 5A). A strong, positive linear association exists between γ-H2AX and RAD51 nuclear foci per cell ($R = 0.6994$, $p<0.0001$), supporting the theory that irradiated VS cells with more DNA DSBs activate more RAD51 for DNA repair (Figure 5B).

The γ-H2AX to RAD51 nuclear foci ratio was calculated for the 0 and 18 Gy conditions. At baseline (0 Gy), the mean γ-H2AX to RAD51 foci ratio was 0.71 (95% CI [0.45, 0.971]), while the mean ratio at 18 Gy was 1.84 (95% CI [1.54, 2.14]) (Figure 5C). These findings in the 18 Gy condition indicate that there are elevated radiation-induced DNA DSB levels as compared to the amount of RAD51 upregulation; this imbalance suggests that high doses of radiation (i.e. 18 Gy) can induce cell death when levels of DNA damage exceed the amount of RAD5-associated DNA repair upregulation. All together, these findings may also indicate that although RAD51 is a major part of the DDR in VS, other DNA repair proteins may contribute to the radiation-induced repair process.

Discussion

The main goal of SRS when treating VS is to halt tumor growth while attenuating damage to nearby structures. Although the tumor control rate after SRS is good, approximately 6 to 12% of patients will have VS progression with time [6,7,19]. In addition, tumor response rates decline further to approximately 83-87% in patients with large VS tumors or Neurofibromatosis Type 2, a genetic tumor disorder causing bilateral vestibular schwannoma [20-22]. In a recent study, Langenhuizen et al. found that fast growing tumors demonstrated significantly worse tumor control rates at 5- and 10-years (85.5% and 67.6%, respectively) than slow growing tumors (97.3% and 86.0%, respectively) on Kaplan-Meier analyses [23]. The mechanisms involved with radiation resistance in VS are largely unknown.

While the current understanding of VS radiobiology is limited, several studies have proposed possible mechanisms for radiation resistance in VS. Langenhuizen et al. postulated

that highly efficient DNA repair mechanisms may allow fast-growing tumors to exhibit radiation resistance [23]. Other studies suggest that radiation resistance in fast-growing tumors is related to a lack of proper vasculature and subsequent tumor hypoxia because the formation of reactive oxygen species is an important mechanism for radiation injury [24,25]. Others propose that alterations in the expression of a variety of oncogenes and tumor suppressor genes may contribute to radiation resistance in VS [26,27]. Gene expression studies in recurrent or radiation-resistant VS revealed an upregulation in the human growth kinase mTOR (mammalian target of rapamycin) and a downregulation in the tumor suppressor protein PTEN (phosphatase and tensin homolog), which would allow VS to grow and avoid normal cell cycle checkpoints (Gugel et al., 2020). However, it is clear that these diverse mechanisms do not provide a cohesive explanation for the radiation resistance seen in some VS.

RAD51-associated DNA repair in response to radiation has been studied in normal human Schwann cells and merlin-deficient Schwann cells (MDSC). In a recent in vitro investigation by Cohen et al., human MDSCs exhibited high levels of γ-H2AX 6 hours after exposure to 6, 12, and 18 Gy of single fraction radiation (Cohen et al., 2021). Although the levels of γ-H2AX were similar across the three radiation groups, MDSCs demonstrated a dose-dependent loss in cell viability at 96 hours. The authors show that a lower dose of radiation (i.e. 6 Gy), MDSCs can initiate a robust RAD51 DNA repair response to evade cell loss. These findings beg the question whether differential induction of γ-H2AX, initiation of cell cycle arrest to repair DNA, and a robust RAD51 adaptive response may contribute to radiation response in VS cells.

Thus, our study aimed to assess the relationships between DNA damage, RAD51 DNA repair, p21 activation of cell cycle arrest, and viability in six primary human VS cells. With our viability assays, we showed that 18 Gy of radiation significantly decreased MFC in viability by approximately 20% across all 6 VS, when compared to 0 Gy (Figure 2A); however, on further analyses, we found that three VS (i.e. VSA59, VSA60, and VSA69) had greater radiation responses to 18 Gy, with MFC in viabilities decreasing more than 20% at 96 hours ($p<0.0001$; Figure 2B). MFC in viability in the remaining 3 VS cells (i.e. VSA58, VSA62, and VSA73) were not significantly different between 0 and 18 Gray of radiation, indicating these cells were more radiation resistant.

All of our VS cells demonstrated significant upregulation of γ-H2AX, p21, and RAD51 at 18 Gy, suggesting that radiation can initiate DSBs in DNA, activate p21 (an important regulator of cell cycle arrest), and subsequently induce RAD51-dependent DNA repair (Figures 3-4). Our study also showed that there was a positive and strong correlation between γ-H2AX and RAD51 nuclear expression, suggesting that more γ-H2AX can induce a more robust RAD51 response (Figure 5B). Furthermore, we demonstrated that greater levels of γ-H2AX had a moderate, albeit significant, negative correlation with viability, indicating that an increase in DSBs was associated with more cell loss (Figure 5A). When we analyzed the relationship between radiation dosage and the relative γ-H2AX/RAD51 ratio, we found that VS cells exposed to 18 Gy of radiation demonstrated higher γ-H2AX/RAD51 ratios (Figure 5C). These findings suggest that irradiated VS cells may

undergo cell death at 18 Gy because the nuclear γ-H2AX levels exceeds RAD51 DNA repair.

Although we expected to find some differences, the levels of γ-H2AX and RAD51 were not significantly different between our radiation-resistant and radiation-responsive VS cells at the 6-hour time point tested. However, there were significant differences in p21 nuclear expression between the two groups, with radiation-resistant VS cells expressing significantly more p21 (Figure 4). These findings suggest that radiation-resistant VS cells may mount a strong p21 response to radiation that allows cells to enter cell cycle arrest and have sufficient time to repair DNA damage. Of the six VS tumors in our study, only VSA60 and VSA73 had radiographic evidence of progression at time of surgery, while the remaining had no prior imaging studies for comparison. Although both these VS tumors were fast-growing (i.e. demonstrating more than 2 mm of growth in diameter per year), VSA60 was radiation-responsive, while VSA73 was radiation-resistant in our in vitro study. Upon further analyses, no significant difference was found in the levels of γ-H2AX and Rad51 between these two tumors but higher levels of p21 was demonstrated in VSA73. Although these results contradict Langenhuizen et al. study that state that fast growing tumors are less radiation responsive, our study was the first to find an association between radiation response and cell arrest mechanisms in primary VS cells [23].

p21, also known as cyclin dependent kinase inhibitor CDKN1A, is an essential regulator of the cell cycle due to its inhibitory control over cyclin-dependent kinases 1 and 2 (CDK 1 and CDK2, respectively) and its ability to promote cell cycle arrest [28]. Interestingly, p21 has been characterized as a tumor suppressor gene or an oncogene depending on the circumstances under which it is dysregulated [29]. p21 has also been linked to radiation resistance in several cancers. Hotte et al. described radiation resistance in esophageal adenocarcinoma cells through a p21-mediated cell cycle arrest pathway [17]. Similarly, Tian et al. demonstrated that p21 overexpression was associated with radiation resistance in colon cancer cells. They also showed that radiation resistance can be overcome by inhibition of radiation-induced p21 gene expression using antisense oligonucleotide treatment [16]. With respect to CNS tumors, Kokunai et al. found that p21 overexpression enhanced cell survival by promoting G1 arrest and preventing apotposis in irradiated glioma cells [30]. In primary glioblastomas, which are known to be highly radioresistant, Gross et al. demonstrated that irradiation with 10 Gy can induce p21 expression in a subset of tumors [31]. Taken together, our findings emphasize the importance of investigating the role of p21 in radiation resistance in VS. Because p21 is modulated by the tumor suppressor protein p53, future investigations will involve the testing of p53 status in VS and determining biomarkers for altered p21 and p53 expression in VS [32].

A limitation of our study is that our *in vitro* model does not fully replicate the normal tumor microenvironment, which consists of a rich network of vessels and multiple cell types, including macrophages and fibroblasts. Thus, we were not able to assess radiation-induced damage to all supporting cells and effect on tumor vasculature [33]. Other factors that we could not evaluate using our model include tumor size and hypoxia, which may play a role in radiation resistance [34].

Although an advantage of this study is the use of primary human VS cells from six different patients, we chose to evaluate only 0 and 18 Gy of radiation. A common radiation dosage protocol for VS is ~11 to 13 Gy at the margin of the tumor (or the 50% isodose line). However, with this protocol, the center of the tumor can receive higher dosages of radiation, with maximal dosages up to 24-26 Gy [35,36]. Thus, we chose to test 18 Gy because it is a dosage that is considered toxic to cells in the radiobiology literature. In addition, it would serve as a positive control for understanding the effects of ~12 Gy and other hypofractionated protocols in the future.

Future investigations will include testing at multiple time points, intermediate radiation dosages (e.g. 6 and 12 Gy), and hypofractionated protocols to provide additional information on the radiobiological response of VS. Furthermore, studying additional DNA repair mechanisms, the expression of other cell cycle arrest proteins, and the activation of cell death pathways will be necessary to elucidate mechanisms of radiation resistance in VS tumors. In addition, understanding how radiation may affect a cell's ability to form colonies will provide important insight on how radiation affects cell survival long term. Because RAD51 may be an important mechanism of radiation-induced DNA repair in VS, the testing of radiosensitizers such as RAD51 inhibitors to prevent RAD51-associated DNA repair may improve overall progression-free survival in patients with VS. In addition, a greater understanding of the mechanisms behind radiation resistance in some VS tumors will allow for the optimization of treatment protocols on an individualized basis. The differential response of VS to radiation therapy opens the door to future applications of precision medicine for each tumor treated.

Conclusion

Individual VS cells respond differently to radiation, with some tumors demonstrating more radioresistant properties. In our study, we describe how VS cells may enter cell cycle arrest after radiation-induced DNA damage and upregulate RAD51 in order to repair DSBs. In addition, we describe how a robust p21 response may promote radiation resistance in some VS cells. An improved understanding of the radiobiology and mechanisms of radiation resistance in VS can help optimize SRS protocols and identify important targets for therapeutic intervention to improve tumor control rates in patients with VS.

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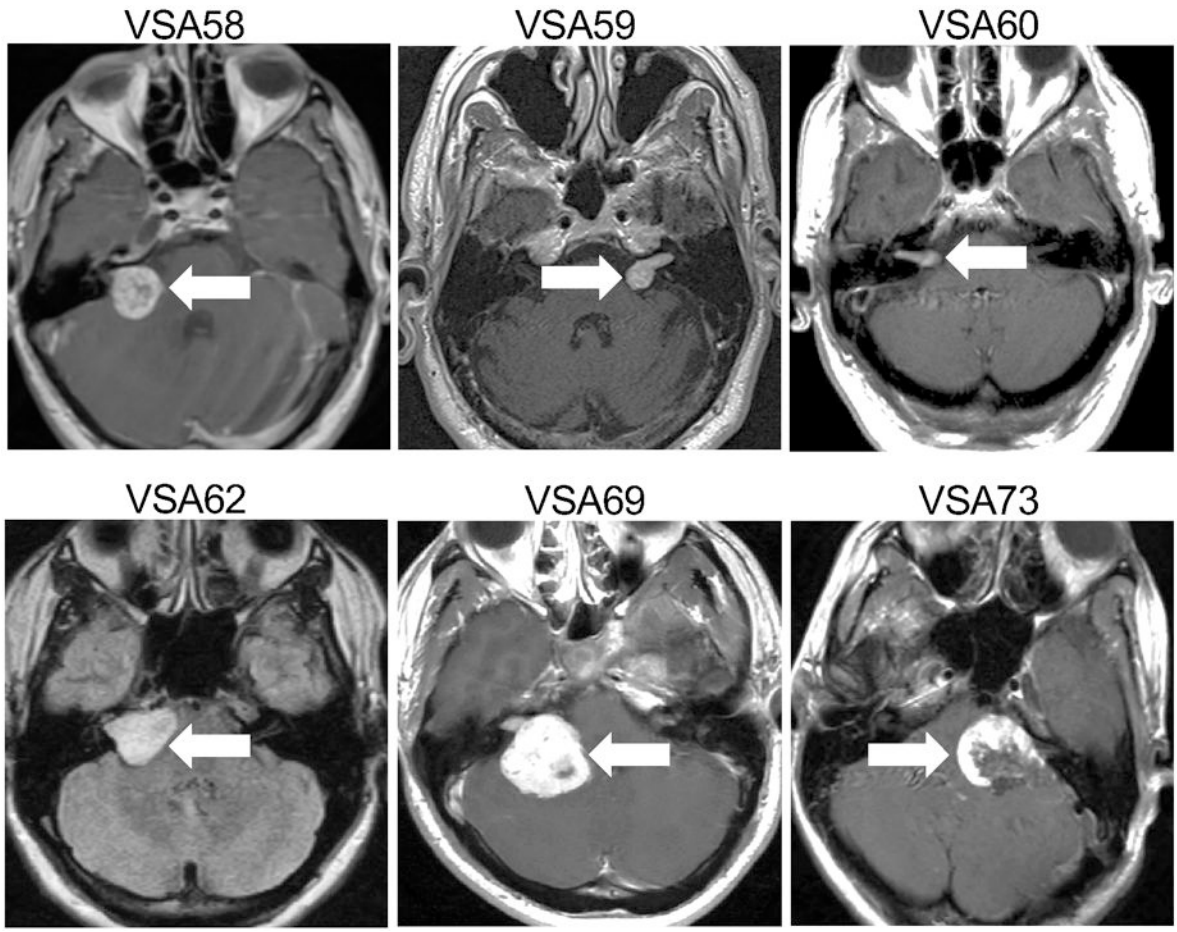

Figure 1. Magnetic Resonance Imaging (MRI) of Vestibular Schwannoma (VS).

Axial T1-weighted MRI images with contrast demonstrating location and size of VS from the 6 patient-derived VS cells. White block arrow points to VS.

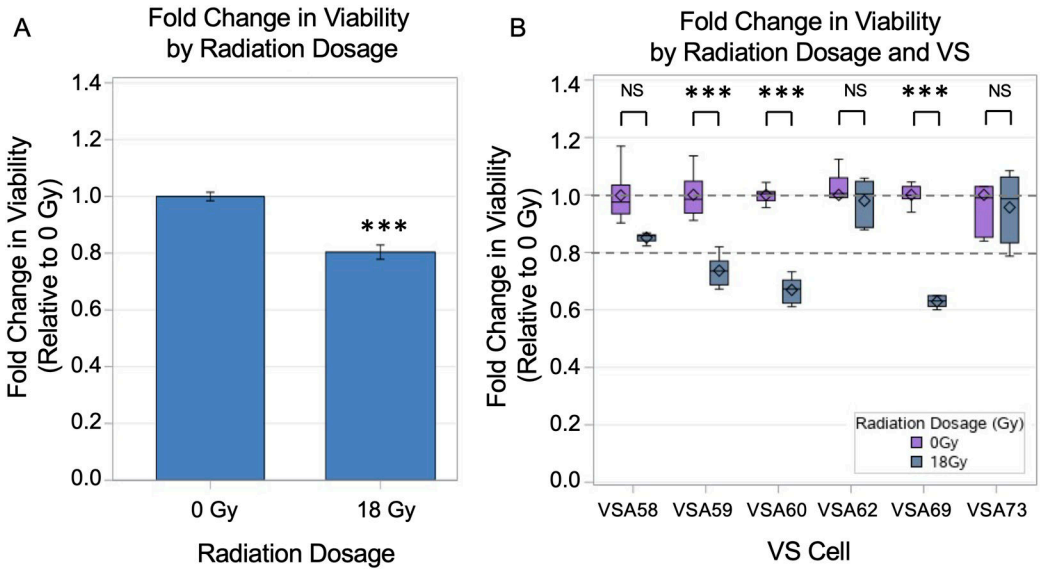

Figure 2. Fold Change in Viability by Radiation Dosage and Vestibular Schwannoma (VS).

[A] Mean Fold Change (MFC) in Cell Viability by Radiation. Radiation (18 Gy) initiated a significant reduction in cell viability of ~20% in primary VS cells, when compared to non-irradiated cells (0 Gy). Bar represents MFC. Error bar shows standard error mean. [B] MFC in Cell Viability by Radiation and VS. Further analysis revealed two groups of irradiated VS. VSA59, VSA60, and VSA69 were more radiation-responsive at 18 Gy ($p<0.0001$) compared to 0 Gy, while VSA58, VSA62 and VSA73 were more radiation-resistant ($p<0.05$). Boxplot = 25-50-75th percentiles. Diamonds = mean. Error bar represents minimum and maximum values. ***$p<0.0001$.

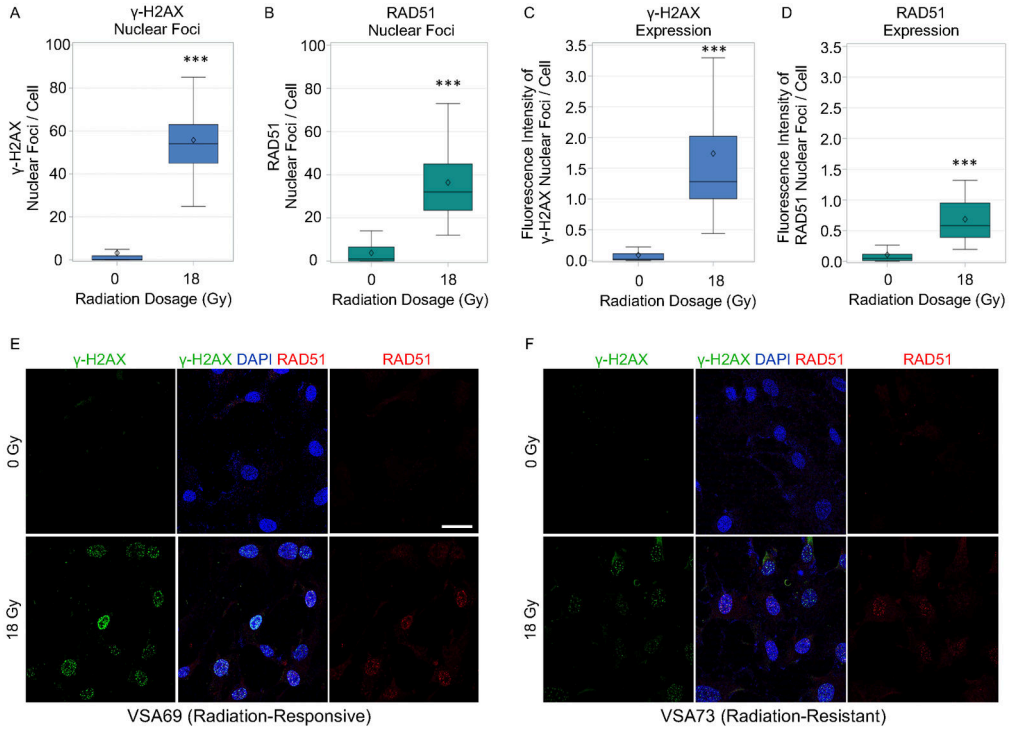

Figure 3. γ-H2AX and RAD51 in Irradiated Vestibular Schwannoma (VS).

[A-B] γ-H2AX and RAD51 Nuclear Foci By Radiation. The numbers of nuclear foci for γ-H2AX and RAD51 per cell were significantly higher in VS cells that received 18 Gy of radiation when compared to 0 Gy. These findings suggest that radiation induced DNA double-stranded breaks in VS cells and expression of DNA repair protein RAD51. [C-D] Fluorescence Intensities of γ-H2AX and RAD51 by Radiation Dosage. The fluorescence intensities of nuclear γ-H2AX and RAD51 were measured and displayed as corrected total cellular fluorescence per cell. The fluorescence intensities per cell for γ-H2AX and RAD51 were significantly higher in VS cells that received 18 Gy of radiation when compared to 0 Gy, further indicating that irradiated VS cells activate RAD51 to repair radiation-induced DNA damage. Boxplot = 25-50-75th percentiles. Diamonds = mean. Error bar represents minimum and maximum values.***p<0.0001 [E-F] Representative confocal images of one radiation-resistant VS (i.e. VSA73) and one radiation-responsive VS (i.e. VSA69) demonstrate γ-H2AX (green) and RAD51 (red) activation after exposure to 18 Gy of radiation. Furthermore, the radiation-responsive VS showed more γ-H2AX nuclear foci than the VS that was radiation-resistant. DAPI nuclear stain is represented by blue. White bar represents 25 μm.

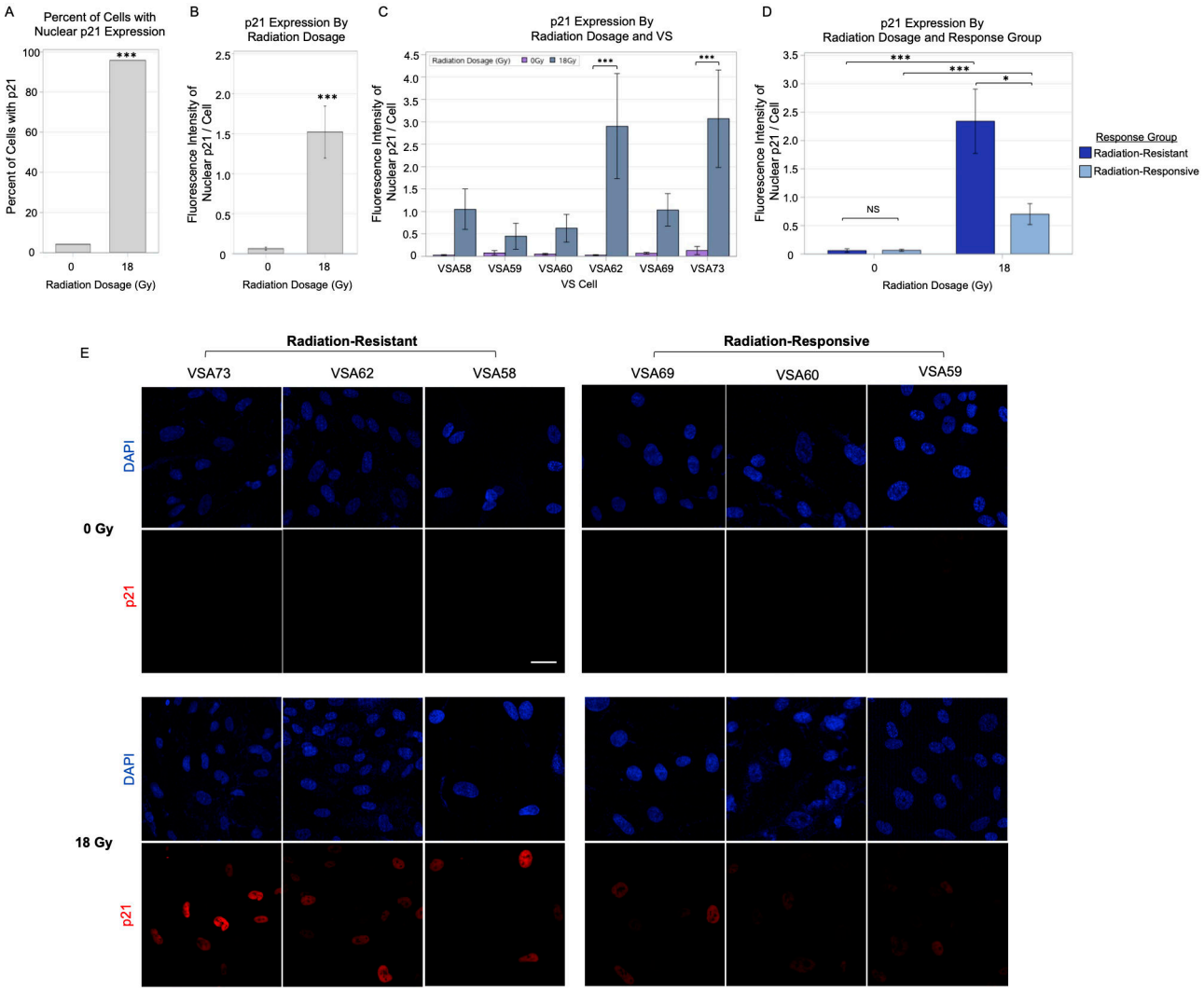

Figure 4. p21 Nuclear Expression in Irradiated Vestibular Schwannoma (VS).

[A] The percentage of cells with p21 nuclear expression increased significantly in VS cells at 18 Gy when compared to 0 Gy, suggesting that VS cells enter into cell cycle arrest after radiation in order to repair DNA damage. ***p<0.0001. [B] The fluorescence intensities of nuclear p21 were measured and displayed as corrected total cellular fluorescence per cell. The p21 fluorescence intensities per cell were significantly higher in VS cells that received 18 Gy of radiation when compared to 0 Gy, further indicating that irradiated VS cells may activate p21-related cell cycle arrest. [C-D] At 18 Gy, the radiation-resistant VS cells expressed significantly higher levels of p21 fluorescence intensities per cell, when compared to the radiation-responsive VS cells. These findings suggest that radiation-resistant VS cells may try to evade cell death through more robust expression of p21, allowing them to enter cell cycle arrest and repair radiation-induced DNA damage. Bar = mean. Error bars represent standard error mean. ***p<0.0001 [E] Confocal images using a 40X lens show more intense p21 nuclear expression (red) in radiation-resistant VS cells (i.e. VSA 58, VSA62, and VSA73), compared to radiation-responsive cells (i.e. VSA59, VSA 60, and VSA 69). DAPI nuclear stain is represented by blue. White bar represents 25 μm.

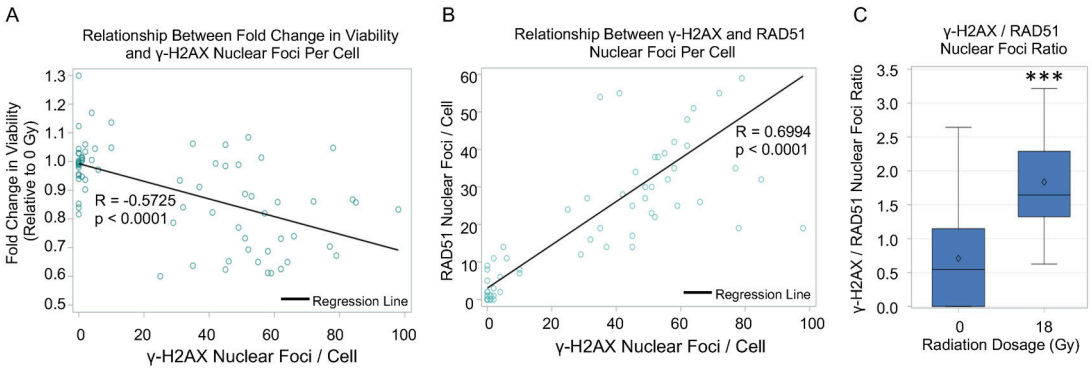

Figure 5. Relationships between viability, γ-H2AX, and RAD51 in Vestibular Schwannoma (VS) Cells.

[A] The regression plot shows a moderate linear association (negative) between fold change in viability and γ-H2AX nuclear foci per cell, suggesting that irradiated cells with more γ-H2AX will have lower viability. [B] There is also a strong, positive correlation between γ-H2AX and RAD51 nuclear foci per cell ($p<0.0001$), suggesting that VS cells with more γ-H2AX activate more RAD51 DNA repair. [C]. VS exposed to 18 Gy of radiation had significantly higher γ-H2AX / RAD51 foci ratios. These finding suggest that high radiation can promote cell death when γ-H2AX exceeds the levels of RAD51 activation. Boxplot = 25-50-75th percentiles. Diamonds = mean. Error bar represents minimum and maximum values. ***$p<0.0001$.

Table 1.
Patient Demographics for Vestibular Schwannoma.

Patient sex, age at the time of surgery (years), tumor volume (cm^3), extent of tumor resection, speech recognition threshold (dB), word recognition score (%), and American Academy of Otolaryngology Head and Neck Surgery (AAO-HNS) Hearing Classification Scale status are provided for the patients included in this study. AAO-HNS stratifies patients into serviceable hearing (A/B) and non-serviceable hearing (C/D). M=male, F=female.

Patient	Sex	Age at Surgery (Years)	Tumor Volume (cm^3)	Extent of Tumor Resection	Speech Recognition Threshold (dB)	Word Recognition Score (%)	AAO-HNSF Hearing Classification Scale
1	M	30	4.53	Gross Total	95	0	D
2	M	72	1.23	Gross Total	20	92	A
3	F	61	0.55	Gross Total	50	24	D
4	M	39	6.2	Gross Total	90	72	C
5	M	31	15.25	Near Total	55	76	D
6	F	28	10.97	Near Total	45	8	D
Average		**43.5**	**6.46**				