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p16INK4a Impairs Homologous Recombination–Mediated DNA Repair in Human Papillomavirus–Positive Head and Neck Tumors

Ruveyda Dok1, Peter Kalev2,5, Evert Jan Van Limbergen1,6, Layka Abbasi Asbagh3, Iria Vázquez2,5, Esther Hauben4,7, Anna Sablina2,5, and Sandra Nuyts1,6

Abstract

The p16INK4a protein is a principal cyclin-dependent kinase inhibitor that decelerates the cell cycle. Abnormally high levels of p16INK4a are commonly observed in human papillomavirus (HPV)–positive head and neck squamous cell carcinomas (HNSCC). We and others found that p16INK4a overexpression is associated with improved therapy response and survival of patients with HNSCC treated with radiotherapy. However, the functional role of p16INK4a in HNSCC remains unexplored. Our results implicate p16INK4a in regulation of homologous recombination–mediated DNA damage response independently from its role in control of the cell cycle. We found that expression of p16INK4a dramatically affects radiation sensitivity of HNSCC cells. p16INK4a overexpression impairs the recruitment of RAD51 to the site of DNA damage in HPV-positive cells by down-regulating of cyclin D1 protein expression. Consistent with the in vitro findings, immunostaining of HNSCC patient samples revealed that high levels p16INK4a expression significantly correlated with decreased cyclin D1 expression. In summary, these findings reveal an unexpected function of p16INK4a in homologous recombination–mediated DNA repair response and imply p16INK4a status as an independent marker to predict response of patients with HNSCC to radiotherapy. Cancer Res; 74(6); 1739–51. ©2014 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and responsible for approximately half a million cases every year (1). Tobacco and alcohol consumption are two environmental risk factors associated with the development of this group of cancers. Recent epidemiologic studies have also revealed that infection with high-risk human papillomavirus (HPV) is etiologically linked to HNSCC pathogenesis (2, 3). HPV-positive HNSCCs, which are predominantly found within the oropharyngeal regions with an occurrence of 25% to 47% (4), are now widely recognized as a distinct HNSCC entity due to demographic, histologic, clinical, and molecular differences (5).

The HPV oncogene products, E6 and E7, play a key role in HPV-associated carcinogenesis by inactivating of p53 and retinoblastoma tumor suppressor functions. The functional inhibition of retinoblastoma by HPV-E7 results in high expression levels of the cyclin-dependent kinase (CDK) inhibitor p16INK4a. The p16INK4a, encoded by the CDKN2A tumor suppressor gene, is one of the core components of the cell cycle that acts by disrupting the complex between cyclin D1 and CDK4/6. The cyclin D1/CDK4/6 complex phosphorylates the retinoblastoma protein that results in the release of the transcription factor E2F and the initiation of the cell-cycle progression. Conversely, the retinoblastoma/E2F repressor complex inhibits transcription of several genes, including CDKN2A (6, 7). By inactivating retinoblastoma, HPV-E7 releases the CDKN2A gene from its transcriptional inhibition that results in p16INK4a overexpression. p16INK4a overexpression has been recently proposed to serve as a surrogate marker of HPV infection in clinical samples because of a strong correlation between HPV and p16INK4a expression status in HNSCCs (8).

Despite the differences between HPV-positive and HPV-negative HNSCC tumors, currently all locally advanced patients with HNSCC are treated uniformly with radiotherapy and concurrent chemotherapy or surgery followed by (chemotherapy) radiotherapy (9, 10). Recent clinical trials have revealed that patients with HPV-infected tumors show markedly improved treatment response to conventional therapy and have a better prognosis compared with patients with HPV-negative HNSCC (3, 8). Although recent studies showed that HPV-positive HNSCC cells are more sensitive to ionizing...
radiation (11, 12), the molecular mechanism and the function of p16INK4a in the enhanced sensitivity to ionizing radiation of these tumors remains largely unknown.

Here, we assessed the impact of p16INK4a on DNA damage repair response in HNSCC. We found that p16INK4a hampers DNA damage response independently from its ability to inhibit CDK4/6 activity or control cell-cycle progression. Decreased expression of cyclin D1 in p16INK4a-overexpressing cells impairs the recruitment of RAD51 to damaged DNA and impeded the homologous recombination–mediated DNA repair. These findings may result in novel therapeutic applications for p16INK4a-overexpressing and HPV-positive tumors.

**Material and Methods**

**Immunohistochemistry and HPV detection**

For immunostaining, routine formalin-fixed paraffin-embedded (FFPE) tumor tissues were available of 87 patients with oropharyngeal obtained before treatment with chemotherapy radiotherapy at the University Hospitals Leuven (Leuven, Belgium). The human tumor samples were acquired according protocols approved by the Ethic Board of the University Hospitals (Leuven, Belgium). Briefly, 5-μm FFPE tumor sections were deparaffinized with the DAKO PT link module and treated with primary antibodies against p16INK4a (G175–405; BD Pharmingen) or cyclin D1 (clone EP12; Dako). Immunodetection was performed with EnVision detection systems peroxidase/DAB (Dako), and sections were counterstained with hematoxylin.

DNA was extracted from FFPE tumor tissues using the QIAMP DNA FFPE Kit according to the manufacturer’s protocol. The quality of extracted DNA to use for HPV PCR was checked by control amplification of 167 basepair fragment of the *isocitrate dehydrogenase* (*IDH2*) gene using PCR. HPV positivity was determined by high-risk HPV–DNA-specific PCR reaction using GP5’/GP6’ primer set as described previously (13).

**Cell lines and reagents**

SCC154 cell line (received 2011) was purchased from the German collection of microorganisms and cell cultures (DSMZ), authentication was performed by DSMZ by short tandem repeat testing. Cells were used within 6 months of receipt after resusciation per the DSMZ recommendation. SCC090 cell line (received 2010) was a generous gift of Dr. S. Collin, the University of Pittsburgh (Pittsburgh, PA). SCC154 and SCC090 cells were cultured and maintained in minimum essential media supplemented with 10% FBS, 1% *L*-glutamine, and 1% nonessential amino acids. SQD9, SC263, and CAL27 cell lines (received 2008) were a generous gift of Dr. A. Begg, the Netherlands Cancer Institute (Amsterdam, the Netherlands) and were cultured as previously described (14). No authentication was performed in our laboratory for these cells.

Lentiviral short hairpin RNAs (shRNA) against luciferase or p16INK4a (TRCN0000255853 and TRCN0000265833) were purchased from Sigma-Aldrich. Lentiviral infections were performed as previously described (15). Infected cells were selected by treatment with 4-μg puromycin (Sigma-Aldrich) for 4 days. siRNA-targeting p16INK4a was 5’AGAACCCAGAGAGG-CUCUGATT-3’ (sip16-1) and 5’CGACCAGAUAAGGUACGUGUtt-3’ (sip16-2; Applied Biosystems). Scrambled shRNA (Silencer Negative Control; Applied Biosystems) was used as control.

Cyclin D1 plasmid (pCMV–cyclin D1) was obtained from Addgene (plasmid 19927), and pBabe HPV-E7 was a generous gift from Dr. K. Munger, Harvard University (Cambridge, MA). pLA-p16INK4a was obtained by cloning INK4a in a lentiviral pLA vector. Transient transfections were performed using lipofectamine 2000 (Life technologies) according to the manufacturer’s protocol.

DNA damage was induced by ionizing radiation using a linear accelerator (6 MV photons; Varian Medical Systems). PD-0332991 (Pfizer) was obtained from Selleckchem.

**Colony formation and cell viability assay**

Twenty-four hours upon drug (PD-0332991) treatment where after the drug is replaced by fresh full media or 72 hours after transfection with siRNAs, cells were exposed to increasing dose of ionizing radiation (2–10 Gy) and plated into 10-cm dishes. After 2 to 3 weeks cells were fixed with 2.5% glutaraldehyde in PBS and stained with 0.2% crystal violet. The colonies containing 50 cells or more were counted with ColCount colony counter (Oxford Optronix). Survival fractions were corrected for the plating efficiencies.

For cell viability assay cells were seeded with 20% confluence in 96-well plates and treated with drug or indicated doses of ionizing radiation and after 7 days a short-term survival assay (sulforhodamine B assay) was performed as previously described (14).

**Nuclear extraction, immunoblotting, and coimmunoprecipitation**

For nuclear extraction and immunoprecipitation experiments a radiation dose of 5 Gy was used as previously described (16). Whole-cell lysates were prepared in a lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Roche). Nuclear extracts were prepared in radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Roche). Protein concentrations were determined by Bradford assay (Bio-Rad) and 10 to 50 μg of proteins were subjected to SDS–PAGE. Immunoblotting was performed with antibodies recognizing β-actin (#4967; Cell Signaling Technology), RAD51 (clone 213; Abcam), cyclin D1 (clone A-12; Santa Cruz Biotechnology), p16INK4a (clone G175–405; BD Pharmingen), vinculin (clone hVIN-1; Sigma-Aldrich), and α-tubulin (clone 6-11B; Sigma-Aldrich). The soluble nuclear fraction and insoluble chromatin fraction were extracted as previously described (17, 18). The chromatin fraction was sonicated before gel electrophoresis and immunoblotting.

For coimmunoprecipitations, cells were lysed with RIPA buffer. Two micrograms of rabbit anti-cyclin D1 antibody (clone H295; Santa Cruz Biotechnology) was incubated with 1.5 mg of cells lysate overnight at 4°C, and then protein A beads were added for 1 hour at 4°C. Immunoprecipitated proteins were detected by immunoblotting using antibodies specific to RAD51 (clone ab213; Abcam) and CDK4 (clone DCS156; Cell Signaling Technology).
Immunofluorescence and light microscopy

Immunofluorescence was performed as previously described (18). Briefly, the cells were plated on μClear 96-well plates (Greiner Bio-One) and fixed with 4% paraformaldehyde. After permeabilization with methanol, primary antibodies against RAD51 (clone ab213; Abcam or clone H-29; Santa Cruz Biotechnology) were followed by a secondary antibody conjugated to fluorescein isothiocyanate (Life Technologies). The DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The immunofluorescence images were acquired using In Cell Analyzer 2000 (GE Healthcare).

For micronuclei analysis cells were irradiated with 6 Gy and fixed with 4% paraformaldehyde. Cells were stained with DAPI and analyzed by In Cell Analyzer 2000 (GE Healthcare). The frequencies of micronuclei were scored by counting the percentage of micronuclei in at least 1,000 cells per experiment.

For immunohistochemical analysis a light microscope (Olympus) with ×200 and ×400 magnification was used and pictures were taken by Cell®D software (Olympus).

Cell-cycle analysis

For cell-cycle analysis cells were irradiated with doses of 4 to 6 Gy as previously described (19), cells were fixed with 70% ethanol and stained with 10 μg/mL propidium iodide containing 100 μg/mL RNase A. Bromodeoxyuridine (BrdUrd) analysis was performed with the BD Pharmingen BrdUrd Flow Cytometry Kit and the Cell Proliferation ELISA BrdUrd Kit (Roche Applied Science) according to the manufacturer’s instruction. Cell-cycle distribution was assessed by FACSCanto (Becton-Dickinson) and FlowJo (Treestar).

Statistical analysis

Survival rates were determined on the basis of Kaplan-Meier estimates using Statistica software version 11. The log-rank test was used to compare survival intervals. To examine association in categorical data the χ² test and the nonparametric-rank test were used. For continuous variables, the unpaired Student t test or the nonparametric-rank test were used. All tests were considered statistically significant for P ≤ 0.05.

Results

High levels of p16INK4a expression correlate with better therapy response in patients with HNSCC

Recent studies have demonstrated that HPV positivity in HNSCC is strongly associated with p16INK4a overexpression. To expand these observations, we performed a parallel analysis of HPV status and p16INK4a nuclear staining in a cohort of 87 patients with HNSCCs treated with radiotherapy or chemoradiotherapy at the University Hospitals of Leuven (Leuven, Belgium). We assessed p16INK4a expression by immunohistochemical analysis in 87 tumor biopsies obtained before treatment (Fig. 1A). All tumors were classified dichotomously as either p16INK4a-positive (strong, diffuse nuclear staining in more than 10% of HNSCC cells) or p16INK4a-negative (Fig. 1A). Consistent with previous reports (8, 20), 37% of the analyzed patients demonstrated high levels of nuclear p16INK4a immunostaining (Fig. 1B).

In parallel, we determined HPV status by PCR detection of high-risk HPV-DNA using GP5+/GP6+ primers in all 87 patients of the Leuven cohort. However, we were not able to detect HPV status in 33 patients due to insufficient material or poor quality of the material (see Materials and Methods). About 25% of HNSCCs were found to be HPV-positive, which is the average HPV prevalence in Europe. We observed a significant association between HPV positivity and overexpression of p16INK4a (P value < 0.001; Yates χ² test; Fig. 1B). The same tendency was observed for the available HNSCC cell lines. HPV-positive HNSCC cell lines, SCC154 and SCC090, had high levels of p16INK4a expression, whereas we were not able to detect p16INK4a expression in HPV-negative SQP9, SC263, and CAL27 cell lines (Fig. 1C).

A recent analysis of a cohort of patients treated solely with conventional radiotherapy in the Danish Head and Neck Cancer Group 5 trial revealed that high levels of p16INK4a expression are associated with better treatment response and survival rate of patients with HNSCC treated with conventional radiotherapy. The p16INK4a expression has been shown to be an even stronger independent prognostic factor than the classical clinical parameters of tumor stage and nodal status (20). Consistent with this report, analysis of the Leuven cohort confirmed that high levels of p16INK4a nuclear expression are associated with improved locoregional control of patients with HNSCC (Fig. 1D). Together, these observations indicate that p16INK4a overexpression has a major impact on treatment response and survival in patients with HNSCC treated with conventional radiotherapy.

p16INK4a sensitizes HPV-infected HNSCCs to ionizing radiation independent from its ability to regulate cell cycle

We next examined the effect of p16INK4a overexpression on cell sensitivity to ionizing radiation. A colony formation assay of irradiated cells normalized for plating efficiencies revealed that p16INK4a-positive SCC154 and SCC090 HNSCC cell lines are more sensitive to ionizing radiation compared with p16INK4a-negative SQP9 HNSCC cells (Fig. 2A and Supplementary Fig. S1A). A short-term cell survival assay further confirmed that cell lines with lower levels of p16INK4a expression were more resistant to ionizing radiation than cell lines with high levels of p16INK4a (Fig. 2B).

To assess the contribution of p16INK4a to radiosensitivity, we suppressed p16INK4a expression in p16INK4a-positive SCC154 HNSCC cell line by lentiviral shRNA against p16INK4a. We found that p16INK4a knockdown did not alter the colony formation capacity of SCC154 cells (Supplementary Fig. S1B). In contrast, suppression of p16INK4a led to a significant increase in survival of the irradiated cells (Fig. 2C and D). We also observed an increased survival of HPV-positive SCC090 cells upon ionizing radiation when we suppressed p16INK4a in these cells by the introduction of siRNA-targeting p16INK4a (Fig. 2E and Supplementary Fig. S1C).

The presence of HPV, which inhibits retinoblastoma activity, in p16INK4a-overexpressing cells makes it unlikely that p16INK4a affects cell survival by affecting the cell-cycle progression upon DNA damage. Indeed, we found that
suppression of p16INK4a did not alter the cell-cycle distribution of HPV-positive SCC154 cells either under untreated conditions or after ionizing radiation, confirming that p16INK4a does not affect the cell-cycle progression of HPV-positive cells upon DNA damage (Fig. 3A). In addition, we did not observe any difference in BrdUrd incorporation at different time points after ionizing radiation between cells that express shRNAs against shluc or shp16INK4a (Fig. 3B). These results suggest that p16INK4a affects radiation response independent of its ability to regulate cell-cycle progression.

To confirm these observations, we overexpressed p16INK4a in HPV-negative SQD9 cells. To exclude the effect of p16INK4a on DNA damage response due to its impact on cell-cycle progression, we inhibited the retinoblastoma pathway by introducing HPV-E7 to SQD9 cells. We confirmed that p16INK4a overexpression did not alter cell-cycle distribution of SQD9E7 cells after DNA damage (Fig. 3C). On the other hand, we found that overexpression of p16INK4a in SQD9E7 cells resulted in a decreased cell survival upon DNA damage (Fig. 3D and E and Supplementary Fig. S1D). Taken together, these results strongly indicate that p16INK4a overexpression contributes to radiosensitivity of HPV-positive HNSCCs. This also suggests a novel function of p16INK4a in control of DNA damage response that is independent from its ability to regulate cell-cycle progression.

**p16INK4a affects radiosensitivity independent of its ability to inhibit CDK4/6 activity**

Previous reports demonstrated that CDKs could modulate DNA damage response and affect cell survival upon DNA damage by phosphorylating DNA repair proteins (21–23). Therefore, we hypothesize that p16INK4a overexpression affects radiosensitivity due to direct inhibition of CDK4/6 activity. To test this idea, we examined whether a specific CDK4/6 inhibitor, PD-0332991 (24, 25), has the same effect on survival of HPV-positive cells upon ionizing radiation as we observed in case of p16INK4a overexpression. Consistent with previous reports (24, 26), the presence of PD-0332991 did not affect cell-cycle distribution of HPV-positive SCC154 cells with inactivated retinoblastoma (Supplementary Fig. S2A). In contrast, we observed G1–S cell-cycle arrest and decreased proliferation of HPV-negative SQD9 cells after treatment with increasing doses of PD-0332991 (Supplementary Fig. S2A and S2B). Also, a slight increase in toxicity was seen with higher doses of PD-0332991 in HPV-negative HNSCCs. These results indicate that p16INK4a overexpression affects cell-cycle distribution of HPV-positive cells upon DNA damage but does not directly inhibit CDK4/6 activity.
SQD9 cells (Supplementary Fig. S2C). These findings confirm that the used doses of PD-0332991 efficiently inhibited CDK4/6 activity. Nonetheless, we did not find any differences in cell-cycle progression after ionizing radiation between cells expressing shRNAs against either luciferase (shluc) or p16INK4a (shp16) in the presence of PD-0332991 (Fig. 4A).
Next, we performed a colony formation assay and a short-term cell survival analysis upon ionizing radiation of SCC154 cells pretreated with PD-0332991 for 24 hours. Inhibition of CDK4/6 activity with PD-0332991 did not affect radiosensitivity of p16INK4a knockdown cells (Fig. 4B and C and Supplementary Fig. S2D). Taken together, these results indicate that increased sensitivity of p16INK4a-overexpressing/HPV-positive cells to ionizing radiation is not due to the ability of p16INK4a to inhibit CDK4/6 activity.

**p16INK4a inhibits recruitment of RAD51 to the sites of DNA damage via cyclin D1 downregulation**

It is also well established that p16INK4a inhibits CDK4/6 activity by displacing cyclin D1 from cyclin D1–CDK4/6 complexes that, in turn, leads to cyclin D1 degradation (27). In fact,
we found lower levels of cyclin D1 expression in p16INK4a-overexpressing SCC154 and SCC090 cells, whereas we observed higher levels of cyclin D1 in p16INK4a-negative SQD9, SC263, and CAL27 cells (Fig. 5A).

We also tested whether there is a correlation between cyclin D1 and p16INK4a and between cyclin D1 and HPV status in HNSCC samples by performing immunohistochemistry (IHC) analysis of cyclin D1 expression in HNSCC biopsies from the...
Leuven cohort. All HNSCC tumors were classified dichotomously as either cyclin D1–positive (strong, diffuse staining in more than 25% of carcinoma cells) or cyclin D1–negative (Fig. 5B). We found that cyclin D1 overexpression correlates with HPV negativity and low p16INK4a expression (P values of < 0.001; the Yates χ² test; and the nonparametric spearman-rank test; Supplementary Fig. S3A), further confirming that HPV status and p16INK4a overexpression could affect the expression of cyclin D1 (Fig. 5C and D and Supplementary Fig. S3A).

High levels of cyclin D1 in a number of p16INK4a-positive tumors (Fig. 5D) could be explained by cyclin D1 amplification, which is commonly observed in HNSCCs (28). Previous studies revealed that elevated levels of cyclin D1 confer radiation resistance of cancer cells (29, 30) and high levels of cyclin D1 correlate with unfavorable response to radiotherapy (31, 32). Conversely, suppression of cyclin D1
sensitizes cancer cells to DNA damage (29, 33). Our results also demonstrated that the response to ionizing radiation of HNSCC cells strongly correlated with levels of cyclin D1 expression (Figs. 2A and B and 5A). Furthermore, when we overexpressed cyclin D1 in p16INK4a-positive SCC154 cells, it led to radiation resistance of these cells (Fig. 5E and Supplementary Fig. 5B). Given that p16INK4a overexpression leads to cyclin D1 down-regulation, this suggests that p16INK4a could affect radiosensitivity by modulating cyclin D1 protein expression.

Recent reports have shown that cyclin D1 facilitates homologous recombination–mediated DNA repair through direct recruitment of RAD51 to the sites of DNA damage (34, 35). In fact, we found that overexpression of cyclin D1 in SCC154 cells enhanced radiation-induced RAD51 foci formation, a marker of homologous recombination competence (Fig. 5F).

We next examined how p16INK4a affects cyclin D1–RAD51 complex formation. We pulled down cyclin D1 from SCC154 or SCC090 cells expressing either shLuc or shp16. Consistent with our prior observations, we revealed higher expression levels of cyclin D1 in p16INK4a-depleted cells compared with shLuc-expressing cells (Fig. 6A and B). Notably, suppression of p16INK4a led to accumulation of cyclin D1–RAD51 complexes (Fig. 6A and B). Inhibition of p16INK4a expression also led to the increased amount of RAD51 associated with chromatin, whereas p16INK4a depletion did not affect RAD51 protein levels in the soluble fraction (Fig. 6C and D). We also assessed the effect of p16INK4a overexpression in SQD9E7 cells on cyclin D1–Rad51 complex formation. In contrast with p16INK4a knockdown, overexpression of p16INK4a impaired the interaction between cyclin D1 and Rad51. A lower amount of RAD51 associated with chromatin was seen in cells overexpressing p16INK4a compared with cells expressing an empty vector (Fig. 6E and F). These results strongly indicate that p16INK4a suppresses RAD51 recruitment to the chromatin by downregulating cyclin D1 protein expression.

**p16INK4a impairs homologous recombination–mediated DNA repair**

We next assessed whether p16INK4a affects the homologous recombination–mediated DNA repair pathway in HPV-positive cells by analyzing RAD51 foci formation. Consistent with the observation that p16INK4a suppression blocks RAD51 recruitment to the chromatin, we found decreased RAD51 foci formation in p16INK4a-positive SCC154 and SCC090 HNSCC cells compared with p16INK4a-negative SQD9 cells (Fig. 7A). On the other hand, depletion of p16INK4a resulted in a dramatic increase of RAD51 foci formation, indicating that p16INK4a contributes to control of the error-free homologous recombination–mediated DNA repair pathway (Fig. 7B).

We hypothesized that a decrease in the homologous recombination–mediated DNA repair pathway could result in an increase in the error-prone nonhomologous end-joining pathway (NHEJ), which commonly leads to misrepair of double-strand breaks in DNA. Increased rate of NHEJ DNA repair results in formation of both dicentric chromosomes andacentric chromosome fragments, which could be detected by micronuclei analysis (36, 37). In concordance with our idea, a micronuclei analysis revealed increased frequency of micronucleated cells in p16INK4a-positive SCC154 cells upon ionizing radiation compared with p16INK4a-depleted cells (Fig. 7C). This suggests that p16INK4a overexpression in HPV-positive cells impairs the homologous recombination DNA repair but facilitates the NHEJ pathway, leading to increased ionizing radiation–mediated genomic instability and enhanced radioresensitivity of p16INK4a-positive cells.

**Discussion**

The role of the tumor suppressor p16INK4a in the regulation of cell cycle and senescence has been studied extensively during last two decades (38, 39). On the other hand, cell-cycle–independent functions of p16INK4a are much less studied. Because the HPV oncogenic proteins, E6 and E7, disrupt cell-cycle regulation and lead to p16INK4a overexpression, HPV-infected tumors serve as an ideal platform to elucidate the involvement of p16INK4a in regulation of cellular processes other than control of cell cycle. Here, we found that p16INK4a is directly involved in control of DNA damage response independently from its ability to inhibit CDK4/6 activity and regulate cell-cycle progression. Conversely, p16INK4a expression impairs error-free homologous recombination–mediated DNA repair by downregulating cyclin D1 protein expression and thereby decreasing recruitment of RAD51 to the sites of DNA damage.

HPV status is well known to be an independent prognostic factor determining tumor control and survival after radiotherapy for head and neck cancer. Despite the prognostic value of HPV-positive HNSCCs, universal methods for stratifying patients with HPV status remain elusive (40) because direct methods of HPV detection are complex to implement in a clinical setup. Recent studies propose that p16INK4a expression could serve as a surrogate marker for HPV positivity (41, 42).

Our results strongly indicate that p16INK4a actively modulates the radiation response and has a broader role than its causal relationship with HPV infections. Consistent with our analysis of the Leuven cohort of patients with HNSCC, recent analyses of clinical data (8, 42) revealed that p16INK4a expression has a major impact on treatment response and survival in patients with HNSCC and have a stronger prognostic value than HPV status.

Importantly, the prognostic value of p16INK4a expression has been shown to depend on its cellular localization. Nuclear p16INK4a gave a better correlation with prognosis (42, 43). In our study, we classified p16INK4a tumors as positive only for samples with higher than 10% nuclear staining. This cutoff is quite strict and it could explain why some of HPV-positive tumors were considered as p16INK4a-negative. We also observed the high levels of p16INK4a expression in some of HPV-negative tumors. It could be due to other mechanisms of retinoblastoma pathway inactivation. Although retinoblastoma mutations are quite rare in HNSCC, deletions of the retinoblastoma-containing region are found in 15% to 50% of the tumors (44). Moreover, **CCND1** has been shown to be amplified in about 30% of HNSCCs (31). Taken together, our data strongly imply p16INK4a is an
independent marker for prediction of radiotherapy response in patients with HNSCC. In addition, we confirmed previous reports demonstrating a direct involvement of cyclin D1 in DNA damage response (34, 35). There are conflicting studies on how cyclin D1 expression affects response to ionizing radiation in HNSCCs. Whereas some groups reported a negative correlation between high cyclin D1 expression and radiosensitivity (30), other groups observed that high cyclin D1 expression is associated with increased radiosensitivity in oral squamous cell cancer and...
Figure 7. p16INK4a impairs homologous recombination-mediated DNA repair. A, RAD51 foci formation 4 hours after 2 Gy of ionizing radiation in the indicated cell lines. Data, mean ± SEM percentage of cells containing more than 10 foci in three experiments. Representative images of RAD51 immunostaining (supra). Scale bar, 100 μm. B, RAD51 foci formation 4 hours after 2 Gy of ionizing radiation in shluc and shp16INK4a-expressing cell lines. Data, mean ± SEM percentage of cells containing more than 10 foci in three experiments. Representative images of RAD51 immunostaining (supra). Scale bar, 100 μm. C, relative number of micronucleated cells in expressing shluc or shp16INK4a after 6 Gy of ionizing radiation. Graph, mean ± SEM of three independent experiments. Representative images of micronuclei detection (left). Arrows, micronucleated cells. Images were taken with a magnification of ×60.
early-stage of laryngeal cancer (45, 46). It is possible that the contradictory observations in these reports are due to the lack of p16INK4a and HPV detection. Moreover, the strong negative correlation between HPV status and cyclin D1 expression in the Leuven cohort and the interaction between p16INK4a, cyclin D1, and RAD51 in HPV-positive cells suggests that cyclin D1 has only a prognostic value in HPV-positive HNSCCs. Therefore, cyclin D1 IHC could be used in addition to p16INK4a as a biomarker for stratification of patients with HPV-positive HNSCC. Unfortunately, the relatively low number of patients with HPV-positive HNSCC did not allow us to make conclusions with certitude.

We believe that our data have important clinical implications by highlighting the importance of p16INK4a as a marker for stratification of patients with HNSCC. Current highly intensive chemoradiation treatment schedules lead to severe short- and long-term morbidity (47, 48). Clinical trials looking at treatment deintensification and organ-preserving therapeutic procedures for HPV-positive tumors are ongoing. Our data suggest that p16INK4a status should be taken into account for further clinical trials of novel treatment therapies.

We also found that p16INK4a overexpression dramatically impairs the homologous recombination–DNA repair pathway, which suggests the potential use of PARP inhibitors, a promising class of anticancer agents particularly effective against homologous recombination–deficient tumors (49), for treatment of p16INK4a-positive HNSCCs. However, further *in vitro* and *in vivo* studies are required to confirm the utility of this approach for patients with HNSCC.

In summary, we found that p16INK4a hampers homologous recombination–mediated DNA repair machinery and sensitizes HPV-positive cells to radiation treatment, implicating p16INK4a expression as a potent marker for stratification of patients with HNSCC.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors' Contributions**

**Conception and design:** R. Dok, P. Kalev, EJ. Van Limbergen, A. Sablina, S. Nuyts

**Development of methodology:** R. Dok, P. Kalev, S. Nuyts

**Acquisition of data** (provided animals, acquired and managed patients, provided facilities, etc.): R. Dok, P. Kalev, EJ. Van Limbergen, I. Abbasi Asbagh, I. Vázquez, E. Hauben, S. Nuyts

**Analysis and interpretation of data** (e.g., statistical analysis, biostatistics, computational analysis): R. Dok, EJ. Van Limbergen, I. Hauben, S. Nuyts

**Writing, review, and/or revision of the manuscript:** R. Dok, I. Vázquez, E. Hauben, A. Sablina, S. Nuyts

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** R. Dok, P. Kalev, I. Abbasi Asbagh

**Study supervision:** A. Sablina, S. Nuyts

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