A Novel Aldehyde Dehydrogenase-3 Activator (Alda-89) Protects Submandibular Gland Function from Irradiation without Accelerating Tumor Growth

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Abstract

Purpose: To determine the effect of Alda-89 (an ALDH3 activator) on (i) the function of irradiated (radiotherapy) submandibular gland (SMG) in mice, (ii) its toxicity profile, and (iii) its effect on the growth of head and neck cancer (HNC) in vitro and in vivo.

Experimental Design: Adult mice were infused with Alda-89 or vehicle before, during, and after radiotherapy. Saliva secretion was monitored weekly. Hematology, metabolic profile, and postmortem evaluation for toxicity were examined at the time of sacrifice. Alda-89 or vehicle was applied to HNC cell lines in vitro, and severe combined immunodeficient (SCID) mice transplanted with HNC in vivo with or without radiation; HNC growth was monitored. The ALDH3A1 and ALDH3A2 protein expression was evaluated in 89 patients with HNC and correlated to freedom from relapse (FFR) and overall survival (OS).

Results: Alda-89 infusion significantly resulted in more whole saliva production and a higher percentage of preserved acini after radiotherapy compared with vehicle control. There was no difference in the complete blood count, metabolic profile, and major organ morphology between the Alda-89 and vehicle groups. Compared with vehicle control, Alda-89 treatment neither accelerated HNC cell proliferation in vitro, nor did it affect tumor growth in vivo with or without radiotherapy. Higher expression of ALDH3A1 or ALDH3A2 was not significantly associated with worse FFR or OS in either human papillomavirus (HPV)-positive or HPV-negative group.

Conclusion: Alda-89 preserves salivary function after radiotherapy without affecting HNC growth or causing measurable toxicity in mice. It is a promising candidate to mitigate radiotherapy-related xerostomia.

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**Translational Relevance**

Xerostomia or dry mouth is the most common side effect in patients with head and neck cancer (HNC) who receive radiation therapy. Because of the close proximity of the salivary glands to the cervical draining lymph nodes, they cannot be routinely spared from high-dose radiation in many patients. Recently, adult stem cells have been identified in salivary glands, and means that can protect these stem cells from radiation damage and allow them to subsequently regenerate have the promise of preserving or restoring salivary gland function. Previously our group has identified Alda-89 as a novel ALDH3 activator that could significantly enrich submandibular gland (SMG) stem cells in vivo. Here, we report that Alda-89 infusion significantly improves postradiation SMG function in vivo without causing any measurable toxicity in treated animals. Most importantly, Alda-89 treatment does not result in accelerated growth of several HNC cell lines or tumor growth in a HNC xenograft model. Finally, neither ALDH3A1 nor ALDH3A2 protein expression in human HNC significantly correlates with prognosis. Altogether, these data indicate that short-term treatment with ALDH3 agonist can mitigate radiation-induced xerostomia without affecting tumor growth.

**Materials and Methods**

**In vivo treatment with Alda-89 (ALDH3 activator)**

All animal procedures were approved by the Stanford University Administrative Panel on Laboratory Animal Care (Stanford, CA). The procedure is conducted as previously described (6). Briefly, 4 to 5 weeks old female C57BL/6 mice or 4 to 6 weeks old SCID mice (Jackson Laboratory) were used. Alda-89 (100 mg/kg/d, ~600 μmol/L) or vehicle [PEG400/dimethyl sulfoxide (DMSO), 50:50] were delivered in a sustained fashion using an ALZET osmotic pump (DURECT Corporation). The osmotic pumps contained 3.4 mol/L of Alda-89 and delivered the drug at a rate of 0.15 μL/hour continuously for up to 6 weeks. Pumps were implanted intraperitoneally into the mice under anesthesia.

**Irradiation and saliva collection**

One week after pump placement, the submandibular and upper neck region, containing the SMGs, were exposed to either a single dose of 15 Gy (one experiment) or 30 Gy in 5 consecutive daily fractions of 6 Gy (another experiment) with the rest of the body shielded. Saliva collection was conducted at basal level (before pump placement), 1 week after pump placement, then 1, 2, 3, 4, 6, and 8 weeks after radiotherapy. Of note, 2 mg/kg pilocarpine injection (s.c.) was used to stimulate saliva production at each collection time point as previously described (6). Pumps were implanted intraperitoneally into the mice under anesthesia. The osmotic pumps contained 3.4 mol/L of Alda-89 and delivered the drug at a rate of 0.15 μL/hour continuously [calculated to deliver ~100 mg/kg/d, which is equivalent to (0.1 g/162)/(1 kg/1 kg/L) = 617 μmol/L] for 6 weeks. The measured saliva secretion was normalized to the mouse body weight (at each measurement time point) and to the basal level.

**Staining and quantification of acinar cells**

At the time of sacrifice, SMGs were removed, fixed in formalin, and embedded in paraffin. Paraffin sections were deparaffined, rehydrated, and stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS; Sigma-Aldrich, INC.) following the manufacturer’s instructions. Ten randomly selected PAS-stained images were acquired at 200 magnification using a Leica DM6000 B microscope (Leica Microsystems Inc.). The percentage of intact acinar area to total measured area was quantified using ImageJ.

**In vitro proliferation of head and neck cancer cell lines**

HN5, Fadu, SQ2B20, Cal27, ICC8, and SCC1 were obtained from the American Type Culture Collection. UM22B was obtained from the University of Michigan (Ann Arbor, MI; Courtesy Dr. Carey, The Cancer Research Laboratory of the Dept. of Otolaryngology/Head and Neck Surgery, University of Michigan). SAS cells were purchased from Japanese Cell Culture Collection. SCC90 cell line was
a gift from Dr. Robert Ferris (University of Pittsburgh, Pittsburgh, PA). Cell lines were maintained in Dulbecco’s modified Eagle medium supplemented with 10% FBS. For the in vitro cell proliferation assays, cells were plated in equal cell numbers in the presence of 60 μmol/L, 600 μmol/L Alda-89 or DMSO and counted during logarithmic growth phase with a hemacytometer.

Clonogenic survival assay

SAS cells were incubated with 200 μmol/L Alda-89 or DMSO 2 hours before radiotherapy (either single fraction of 8 Gy or a fractionated regimen of 3 Gy/fraction/day for 4 consecutive days). Cells were then plated in triplicates at different densities ranging from 300 to 10,000 cells per dish in the presence of 200 μmol/L Alda-89 or DMSO. Plating efficiency was determined by harvesting untreated cells. After 7 to 10 days, the cells were fixed and stained with a solution of 0.25% crystal violet in ethanol. Surviving fraction was determined by counting the number of colonies with more than 50 cells. Surviving fraction was normalized by the plating efficiency. The results represent the mean of triplicate with the error bars representing ± 1 SD.

Xenograft study

Six-week-old SCID mice were purchased from the Jackson Laboratory, and 5 mice per group were used. SAS (2.5 × 10^6 cells/injection) were implanted into the flanks of each mouse. When the tumor volume reached approximately 100 mm³, Alzet pumps containing either Alda-89 or DMSO were implanted intraperitoneally in the mice and allowed to release the drug up to 6 weeks at a rate of 0.15 mg/kg/d. Tumor size was measured every 1 to 2 days. Tumor volume was calculated by the formula \( \frac{L \times W \times H}{6} \). In the xenograft irradiation study, when the tumor volume reached approximately 50 mm³, Alzet pumps containing either Alda-89 or DMSO were implanted intraperitoneally in the one week after the pump placement, when the tumor volume reached approximately 130 mm³, 12 Gy was delivered to the tumor with the rest of the body shielded.

Blood test and pathology exam

Blood from Alda-89 and vehicle-treated mice were obtained in heparinized syringes by cardiac puncture at terminal bleed. A complete blood count (CBC) and a comprehensive blood chemistry panel were analyzed by the Stanford Department of Comparative Medicine.

A complete necropsy was conducted on representative mice by senior veterinarian, Dr. Richard Luong, Department of Comparative Medicine, Stanford University. All tumors were staged using the 2002 American Joint Committee on Cancer staging system (16).

Tissue microarray staining and scoring

The tissue microarray (TMA) was constructed from formalin-fixed paraffin-embedded samples of HNSCC as previously described (17). Immunoperoxidase stains for ALDH3A1 (citrate, 1:300; Abcam) and ALDH3A2 (citrate, 1:200; Abcam) were conducted on 4 μm-thick sections of the TMA. The staining results were interpreted by a pathologist (C.S. Kong) who was blinded to the clinical data and scored on the basis of cytoplasmic staining as negative (blush or no tumor staining), weakly positive (<70%), or strongly positive (>70%). For the purpose of outcome analysis, the weakly and strongly positive groups were combined together into a single positive staining group.

Statistical analysis

Data are expressed as mean ± SEM. Statistical ANOVA and t tests were used to compare the saliva secretion, acinar cell areas, clonogenic survival, and tumor growth curve in vitro and in vivo. P ≤ 0.05 is considered to be significant. Kaplan–Meier product–limit method was conducted using the Statview (Analytical Software, Inc.) statistical software as previously described (18). Log-rank test was used to compare survival curves.

Results

Alda-89 protects SMG from radiation

ALZET osmotic pumps were used to deliver Alda-89 at 34 mg/kg/d (~200 μmol/L) or vehicle to C57BL/6 mice in a continuous manner 1 week before, during, and 1 week after radiotherapy (2 weeks total). At this dose, there was a trend for a higher saliva production with Alda-89 treatment starting at week 2 (when drug delivery ended), but the difference was not statistically significant (Supplementary Fig. S1). We hypothesize that both a higher dose and a longer treatment duration may be needed to observe a functional difference. Therefore, we proceeded to administer a higher dose of Alda-89 (100 mg/kg/d, ~600 μmol/L) 1 week before and 5 weeks after radiotherapy (Fig. 1A). At this dose, there was a significant improvement in saliva production in the Alda-89–treated group (Fig. 1B). In fact, the Alda-89–treated group did not have any reduction in saliva production compared with baseline and this was maintained the same level until sacrificed after 8 weeks, whereas the vehicle control group continued to experience decline in saliva production for more than 50%, which stabilized after 6 weeks postradiotherapy. Importantly, Alda-89 treatment did not affect the general health of the mice as reflected in the body weight, which was similar

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between the 2 groups (Fig. 1C). A body weight reduction due to pump placement occurred in both groups, reflecting the stress of the procedure; however, all the mice regained their weight, which continued to climb during the observation period. H&E and PAS staining of the SMG tissues further confirmed that intact SMG acini structures were better preserved in the Alda-89–treated compared with vehicle-treated mice (Fig. 1D). The percentage of acinar area/total area per high power field was 51.05% ± 2.64% in the Alda-89 group compared with 26.71% ± 1.24% in the vehicle group (Fig. 1E; *P < 0.05). The percentage of acinar area/total area per high power field in unirradiated SGMs typically ranged from 60% to 70%.

In addition to the above single-fraction experiment, we also conducted fractionated radiation treatment (30 Gy total in 5 consecutive days with daily fraction of 6 Gy) on a different set mice receiving either Alda-89 or vehicle. The experimental schema is shown in Fig. 2A. Consistent with our finding for the single 15 Gy radiotherapy dose, Alda-89 significantly preserved the saliva flow in fractionally irradiated mice (Fig. 2B). All mice in both groups lost weight after irradiation but regained most of it over time with no significant difference between the 2 groups (Fig. 2C).

**Alda-89 does not accelerate HNC growth in vitro or in vivo**

Aldh3A1 and Aldh3a2 proteins were expressed at different levels in the examined HNC cell lines, with high expression in the UM22B, Fadu, HN5, SCC1, and ICC8, medium in SAS and Cal27, and low in SCC90 and SQB20 (Supplementary Fig. S2). To investigate the role of Alda-89 on HNC cell line proliferation in vitro, 4 HNSCC lines with different ALDH3 expression level [3 human papillomavirus (HPV)-negative lines: HN5, SCC1, and SAS and 1 HPV-positive line: SCC90] were plated in equal cell numbers in the presence of 60 μmol/L, 600 μmol/L Alda-89 or vehicle. The growth rate was calculated by counting the cell numbers during logarithmic growth phase. In all cell lines tested,
treatment with Alda-89 did not accelerate the growth rate as compared with vehicle control. There was also no difference in the growth rate between the high and low Alda-89 doses in these cell lines (Fig. 3A and Supplementary Fig. S3).

To test the effect of Alda-89 on radiotherapy-induced cell kill, we conducted clonogenic survival assay on HNC cell lines SAS and SCC90, which were pretreated with 200 μmol/L Alda-89 or DMSO 2 hours before a single dose of 8 Gy. Cells were then plated in triplicates in the presence of 200 μmol/L Alda-89 or DMSO. After 7 to 10 days, colonies were quantified to determine the surviving fraction. Alda-89 treatment did not protect cancer cells from radiotherapy; in fact, the Alda-89–treated cells had a nonsignificantly slightly lower surviving fraction than the vehicle-treated cells either with or without radiotherapy, suggesting that Alda-89, at the concentration used, was potentially toxic to these cells (Fig. 3B).

We also conducted clonogenic survival assay on SCC1, SCC90, and SAS, which were pretreated with 200 μmol/L Alda-89 or DMSO 2 hours before fractionated radiotherapy of 3 Gy/fraction/day for 4 consecutive days. Cells were then plated in triplicate in the presence of 200 μmol/L Alda-89 or DMSO. Similar to single dose of 8 Gy, Alda-89 treatment did not protect cancer cells from fractionated radiotherapy (Fig. 3C).

To evaluate the effect of Alda-89 on HNC growth in vivo, SAS tumor-bearing SCID mice were exposed to either Alda-89 (100 mg/kg/d) or vehicle delivered via the Alzet osmotic pumps when the tumors reached approximately 100 mm³. Tumors size was measured every 1 to 2 days during Alda-89 infusion and then for another 2 weeks. The tumor growth curves of the vehicle- and the Alda-89–treated mice practically overlapped, indicating that Alda-89 did not accelerate tumor growth in vivo (Fig. 3D).

To study the effect of tumor regrowth after radiotherapy, we irradiated the transplanted tumor xenograft with a single dose of 12 Gy with the rest of the body shielded. There was no difference in the tumor regrowth after radiotherapy between the 2 groups (Fig. 3E).

**Alda-89 does not induce major organ toxicity in vivo**

Because Alda-89 was delivered through an osmotic pump placed intraperitoneally, the entire mouse body was exposed to the drug. To assess for its potential toxicity, we obtained CBC and a comprehensive chemistry panel using blood from the terminal bleed. Although both groups showed hypochromic anemia and elevated absolute lymphocyte, monocyte, and eosinophil counts, which were presumably related to the pump placement procedure, there was no significant difference in any measurement between the 2 groups (Table 1).

A complete necropsy was conducted on representative mice, showing minimal tissue autolysis and normal microscopic appearance for all examined organs. All mice showed histologic evidence of chronic reactive peritonitis, which was consistent with intraperitoneal surgery and placement of the osmotic pumps.

**ALDH3 protein expression did not correlate with prognosis in patients with HNC**

To further confirm that the expression of ALDH3 isozymes does not confer a worse outcome in patients with...
HNC, we stained a TMA containing 89 HNSCC with ALDH3A1- and ALDH3A2-specific antibodies. These patients with newly diagnosed HNSCC received their treatment at Stanford University with a median follow-up of 64 months for living patients. Table 2 shows the patient, tumor, and treatment characteristics. Because p16 and HPV status are an important independent prognostic factors in HNSCC, we also assessed the prognostic significance of ALDH isozymes by HPV status as previously determined by p16 immunohistochemistry and HPV pyrosequencing (17). Seventy-five patients of the entire group had tumor assessable for both HPV status and ALDH3A1 expression: 34 patients with HPV-positive and 41 with HPV-negative tumors. As shown in Fig. 4B, there was no statistically significant difference in FFR and OS between the ALDH3A2-positive and -negative tumors for either HPV-positive or HPV-negative group.

Discussion

There is an increasing interest in stem cell therapy to replenish salivary gland function after radiotherapy damage (5, 19, 20). One direction is to promote the survival and proliferation of the rare SSC population within the gland. Different growth factors or cytokines have been tested for this purpose, including the keratinocyte growth factor (KGF; ref. 21), also known as fibroblast growth factor 7, which is a critical growth factor, supporting embryonic SMG epithelial bud proliferation and branching (22). However, because KGF is an epithelial growth factor, there is a theoretical concern that its administration before and during treatment can promote tumor growth. We evaluated the effect of Alda-89, a KGF receptor inhibitor, on the survival and proliferation of HNSCC cells in vitro and in vivo.

Figure 3. Alda-89 does not promote HNC growth in vitro or in vivo. A, cell growth curves in culture for SAS cells treated with vehicle, 60 µmol/L or 600 µmol/L Alda-89. B, clonogenic survival assay showing that 8 Gy radiotherapy (IR) dramatically reduced the SAS and SCC90 cell survival normalized to the control group. Alda-89 treatment did not affect radiotherapy cell kill compared with vehicle control. C, clonogenic survival assay showing that fractionated radiotherapy of 3 Gy/fraction/day for 4 consecutive days dramatically reduced the SAS, SCC90, and SCC1 cell survival normalized to the control group. Alda-89 treatment did not affect radiotherapy-induced cell death compared with vehicle control. D, SAS xenograft tumor growth curves over time. Treatment with Alda-89 at 100 µmol/L/day did not accelerate the tumor growth in vivo compared with the vehicle. E, SAS xenograft tumor growth curves over time after 12 Gy radiation. Treatment with Alda-89 at 100 mg/kg/d did not accelerate the tumor growth in vivo compared with the vehicle control. IR, irradiation.
growth or decrease the effectiveness of chemoradiation. Two randomized studies in HNC have shown that short-term administration of KGF did not compromise treatment cure rate; however, the duration and the dose of KGF used in these studies did not reduce radiation-related xerostomia (23).

Instead of focusing on growth factors, our group studied genes that are differentially upregulated in SSC compared with non-SSC counterparts and identified ALDH3A1 and ALDH3A2 as 2 genes that are differentially expressed at high levels in both adult human and murine SSCs (6). Aldehyde dehydrogenases are enzymes involved in oxidizing intracellular aldehydes. The family has 19 members in human with the most abundant and well-studied member being ALDH1 (24, 25), which has been found to be highly expressed in many adult tissue stem cells or progenitor cells, including hematopoietic, neuron, muscle, hepatic, adipose stem cells, and progenitor cells (7–15). Cells with high ALDH activity as selected by the Aldefluor assay have been used to treat ischemic limbs (26), myocardial infarction (22), and liver damage in animal models (14). Despite the fact that ALDH activity is considered a marker of stem cells, little is known about the exact role each ALDH isozyme plays in stem cells; this is partly because by modifying the aldehyde groups, ALDH family members play multiple functions in cells, including cholesterol and amino acid metabolism, alcohol and drug detoxification (27). In addition, ALDH enzymes are expressed in multiple cellular compartments, including the endoplasmic reticulum, mitochondria, cytosol, and the nucleus, and there is compensation effect among the different family members, making it difficult to study the specific effect of a particular member (28).

As previously mentioned, we showed that Alda-89, a novel and specific ALDH3 activator, significantly increased the c-Kit+ /CD90+ SSC number in vivo with associated increases in number and size of salispheres in culture (6). The mechanism by which activation of ALDH3 resulted in higher SCC numbers and salisphere formation has not yet been determined, but postulated to be due to better SSC survival and/or proliferation. This translated to improved salivary function and better preservation of acinar morphology, as shown in this study.

One draw back of using total stimulated saliva production to assess SGM function is the inability to distinguished contributions from SMG and other saliva glands such as parotid and minor salivary glands. However, currently we do not have a feasible way to directly quantify SMG contribution repeatedly. Previous studies have shown a direct relationship between the number of intact acini and saliva production after radiation (29). Therefore, we used this approach to indirectly assess SMG function. We found that Alda-89-treated mice had a significantly higher number of preserved acini than vehicle-treated control animals.

Table 1. Lab test for representative mice receiving vehicle or Alda-89

<table>
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<th>C1</th>
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<th>C3</th>
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<td>35.5</td>
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<tr>
<td>Glucose</td>
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<td>Calcium</td>
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Abbreviations: C, vehicle control; A, Alda-89; N/A, not applicable.
In addition of being associated with normal tissue stem cells, elevated ALDH activity has been linked to different cancer stem cells, including head and neck squamous carcinoma (30), lung (31), liver (32), pancreas (33), colon (25), breast (34), cervical (35), and prostate cancers (36). Specifically, these studies used the Alde-fluor assay, which mainly evaluates the activity of ALDH1 (24). The relationship between ALDH3 expression or activity and cancer stem cell is less clear. ALDH3 subfamily includes ALDH3A1, which is mainly expressed in the cornea, lung, esophagus, and stomach (37), and ALDH3A2, which is mainly found in the liver, and is expressed in many other tissues including kidney, intestine, stomach, skeletal muscles, skin, lung, pancreas, placenta, heart, and brain (27). ALDH3A1 has been shown to be expressed at high level in breast cancer stem cells (38), and downregulation of this enzyme resulted in reduced non–small cell lung cancer cell growth and motility (39). In contrast, neither ALDH3A2 expression nor activity has been linked to cancer development; mutations in this gene have been identified in humans and linked to a genetic condition known as the Sjögren-Larsson syndrome, characterized by mental retardation, spasticity, and ichthyosis (thick, scaly skin; ref. 40). Function and expression of either enzyme have not been studied in HNSCC. Ours is the first study to systematically evaluate the effect of activating ALDH3 in HNC cell lines and the results indicate that activation of ALDH3 with Alda-89 did not affect the pace of HNC growth in vitro or in vivo. In addition, it did not affect cell death by radiation or tumor regrowth after radiation in xenografts. Although 55% and 79% of the evaluated human HNSCC stained positive for ALDH3A1 and ALDH3A2, respectively, expression of these enzymes did not significantly correlate with either tumor relapse or OS. However, the number of patients in each subgroup is quite small and these results will need to be validated in a larger group of homogenously treated patients.

### Conclusion

In summary, we have for the first time showed that administration of a specific ALDH3 activator, Alda-89, protected the SMG function from radiotherapy damage. The drug seems to be safe in treated mice when delivered for 6 weeks at the dose of 100 mg/kg/d, and did not enhance tumor growth or protect tumor from radiotherapy. Future studies will focus on optimizing the drug delivery route, dose, duration of treatment, and sequencing with radiotherapy. In addition, a larger study will be conducted to assess the relationship between treatment outcomes and the expression of these enzymes in human HNSCC.

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### Table 2. Patient, tumor and treatment characteristics

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<th>Parameter</th>
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<tr>
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<td>27 (30)</td>
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<td>7 (8)</td>
<td>35 (41)</td>
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<td>16 (19)</td>
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<td>37 (42)</td>
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<td>32 (38)</td>
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<td>24 (27)</td>
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<td>5 (6)</td>
<td>32 (38)</td>
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<td>3–4</td>
<td>24 (27)</td>
<td>25 (28)</td>
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<td>13 (15)</td>
<td>35 (41)</td>
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<td>10 (11)</td>
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<td>12 (14)</td>
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<tr>
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<td>45 (53)</td>
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<td>58 (68)</td>
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<td>38 (45)</td>
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<tr>
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<td>Treatment</td>
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<td>51 (60)</td>
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<td>0.004</td>
<td>2 (2)</td>
<td>16 (19)</td>
</tr>
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*Seventy-five patients had tumor assessable for both HPV status (p16, HPV ISH, and HPV pyrosequencing) and ALDH3A1 staining and 72 patients had tumor assessable for both HPV status (p16, HPV ISH, and HPV pyrosequencing) and ALDH3A2 staining.

Abbreviations: CRT, chemoradiation, RT, radiotherapy.
Disclosure of Potential Conflicts of Interest

C.S. Kong has expert testimony in Martin & Jones, PLLC. D. Mochly-Rosen has ownership interest (including patents) in ALDEA Pharmaceuticals. Q.-T. Le has ownership interest (including patents) in Aldea. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Xiao, H. Cao, C.S. Kong, R. Ali, C. Chan, D. Sirjani, E.E. Graves, Q.-T. Le

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Xiao, H. Cao, R. Ali, E.E. Graves, A.C. Koong, A.J. Giaccia, D. Mochly-Rosen, Q.-T. Le

Writing, review, and/or revision of the manuscript: N. Xiao, C.-H. Chen, C.S. Kong, E.E. Graves, A.C. Koong, Q.-T. Le

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Chan

Study supervision: Q.-T. Le

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References

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Nan Xiao, Hongbin Cao, Che-Hong Chen, et al.


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