5T4-Targeted Therapy Ablates Cancer Stem Cells and Prevents Recurrence of Head and Neck Squamous Cell Carcinoma

Samuel A. Kerk1, Kelsey A. Finkel1, Alexander T. Pearson1,2,3, Kristy A. Warner1, Zhaocheng Zhang1, Felipe Nør1,4, Vivian P. Wagner4,5, Pablo A. Vargas6, Max S. Wicha2,3, Elaine M. Hurt7, Robert E. Hollingsworth7, David A. Tice7, and Jacques E. Nör1,3,8,9

Abstract

Purpose: Locoregional recurrence is a frequent treatment outcome for patients with advanced head and neck squamous cell carcinoma (HNSCC). Emerging evidence suggests that tumor recurrence is mediated by a small subpopulation of uniquely tumorigenic cells, that is, cancer stem cells (CSC), that are resistant to conventional chemotherapy, endowed with self-renewal and multipotency.

Experimental Design: Here, we evaluated the efficacy of MEDI0641, a novel antibody-drug conjugate targeted to 5T4 and carrying a DNA-damaging “payload” (pyrrolobenzodiazepine) in preclinical models of HNSCC.

Results: Analysis of a tissue microarray containing 77 HNSCC with follow-up of up to 12 years revealed that patients with 5T4high tumors displayed lower overall survival than those with 5T4low tumors (P = 0.038). 5T4 is more highly expressed in head and neck CSC (ALDHhighCD44high) than in control cells (non-CSC). Treatment with MEDI0641 caused a significant reduction in the CSC fraction in HNSCC cells (UM-SCC-11B, UM-SCC-22B) in vitro. Notably, a single intravenous dose of 1 mg/kg MEDI0641 caused long-lasting tumor regression in three patient-derived xenograft (PDX) models of HNSCC. MEDI0641 ablated CSC in the PDX-SCC-M0 model, reduced it by five-fold in the PDX-SCC-M1, and two-fold in the PDX-SCC-M11 model. Importantly, mice (n = 12) treated with neoadjuvant, single administration of MEDI0641 prior to surgical tumor removal showed no recurrence for more than 200 days, whereas the control group had 7 recurrences (in 12 mice; P = 0.0047).

Conclusions: Collectively, these findings demonstrate that an anti-5T4 antibody-drug conjugate reduces the fraction of CSCs and prevents local recurrence and suggest a novel therapeutic approach for patients with HNSCC. Clin Cancer Res; 1–12. © 2016 AACR.

Introduction

Head and neck cancer is one of the most common cancers worldwide with 46,000 new cases and 9,000 deaths in the United States each year (1). Head and neck squamous cell carcinoma (HNSCC) is the most common head and neck malignancy, frequently presenting as locally advanced disease (stage III or IVB). The standard of care for HNSCC involves a combination of surgery, radiation, and chemotherapy. Regimens with platinum-based agents such as cisplatin, 5-fluorouracil, or taxanes are frequently administered, along with targeted agents such as the monoclonal anti-EGFR antibody cetuximab (2). However, despite advances in treatment, patients with HNSCC still face a 60% risk of local recurrence and 30% risk of distant metastasis. In addition, patients with recurrent or metastatic tumors typically present enhanced morbidities and poor prognosis, with a median survival time of 10 months (3). It is rather evident that safe, more effective therapies are urgently needed for patients with HNSCC.

The cancer stem cell (CSC) hypothesis postulates that within a tumor there is a subpopulation of multipotent, tumorigenic CSCs capable of reconstituting the heterogeneity of the primary tumor by both self-renewal and differentiation. CSCs were first isolated in acute myeloid leukemia (AML) and have since been characterized in solid tumors in the breast, brain, lungs, liver, prostate, pancreas, ovaries, kidneys, and colon (4–13). In head and neck cancer, the CSC fraction shows high activity of the cytosolic enzyme aldehyde dehydrogenase (ALDH), which oxidizes retinoic acid, as well as high expression of the membrane protein CD44 (14, 15). CSCs play an integral role in treatment resistance and disease relapse. As we have shown previously, treatment with cisplatin enhances the CSC fraction in HNSCC (16). It has been proposed that the slow proliferation rate of CSCs allows them to evade treatments that target highly proliferative cells (17). Furthermore, we observed that cisplatin enhances the expression of...
Translational Relevance

Head and neck cancer is a common and deadly malignancy that is generally treated with surgical resection, platinum-based chemotherapy, and radiation. Current standard-of-care treatment typically results in high morbidity for these patients, and disease relapse is rather common. Emerging evidence suggests that the recurrence and metastases of these tumors are mediated by cancer stem cells (CSC). Here, we showed that a transmembrane glycoprotein (5T4 oncofetal antigen) is expressed primarily by head and neck squamous cell carcinoma (HNSCC) cells, particularly by CSCs, and that 5T4 expression correlates negatively with overall patient survival. We also demonstrated that MEDI0641, a novel antibody-drug conjugate targeted to 5T4, mediates the regression of patient-derived xenograft (PDX) tumors, reduces the fraction of CSCs, and prevents local recurrence. Collectively, these data suggest that patients with head and neck cancer might benefit from therapeutic ablation of 5T4-positive cells.

The antibody–drug conjugate (ADC) family of targeted therapies is a promising class of drugs that is designed to deliver cytotoxic chemotherapies specifically to cancer tissues with limited added toxicities. Indeed, when patients with HER2-positive breast cancer were treated with the ADC trastuzumab emtansine versus unconjugated lapatinib plus capecitabine, the group receiving the ADC had fewer adverse events and longer overall survival (26). The specificity of oncofetal antigen 5T4 in malignant tissue has been used to develop a novel ADC named MEDI0641. It is targeted to 5T4 and conjugated to the DNA-damaging “payload” pyrolobenzodiazepine (PBD), which binds to the minor groove of the DNA double helix, hindering its processing. The PBD dimer is released following proteolytic cleavage of the Val-Ala dipeptide, then the low pH in the lysosomal compartment results in self-immolation of the PABA spacer releasing the warhead into the cancer cell. Here, we hypothesized that head and neck CSCs can be eliminated with a 5T4-targeted ADC. Our studies demonstrate that MEDI0641 decreases the CSC fraction, mediates long-term tumor regression, and prevents tumor recurrence in PDX models of HNSCC.

Materials and Methods

Tissue microarray

Cores from paraffin-embedded tumors were prepared by a trained oral pathologist and mounted as a tissue microarray (TMA), as described previously (27). Briefly, tumor areas of the invasive front were selected and marked on a hematoxylin and eosin–stained slide using an objective marker (Nikon). The slide was then overlaid on the original paraffin block to determine the matching area to be used. Using a manual tissue arrayer (Sakura), tissue sections were exposed to rabbit anti-5T4 (Abcam), tissue was permeabilized in 0.1% Triton-x-100 (Sigma) for 20 minutes. Following blocking with Background Sniper (Biocare Medical), tissue sections were exposed to rabbit anti-5T4 (Abcam #134162) at 4°C overnight. Tissue sections were then labeled with MACH3 probe (Biocare Medical), followed by exposure to Horse-radish Peroxidase Polymer (Biocare Medical) and visualization with dianimobenzidine (DAB; Biocare Medical).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was carried out in Target Retrieval Solution (Dako). The tissue was permeabilized in 0.1% Triton-X-100 (Sigma) for 20 minutes. Following blocking with Background Sniper (Biocare Medical), tissue sections were exposed to rabbit anti-5T4 (Abcam #134162) at 4°C overnight. Tissue sections were then labeled with MACH3 probe (Biocare Medical), followed by exposure to Horse-radish Peroxidase Polymer (Biocare Medical) and visualization with dianimobenzidine (DAB; Biocare Medical).

In vivo studies

PDX tumor models of HNSCC were generated in severe combined immunodeficient (SCID) mice and characterized (28, 29).
Tumors (PDX-SCC-M0, PDX-SCC-M1, PDX-SCC-M11) were allowed to grow to 200 to 1,000 mm³ and then were treated with a single dose of 1 mg/kg MEDI0641, a weekly dose of 0.5 mg/kg MEDI0641 for 2 weeks, a weekly dose of 0.33 mg/kg MEDI0641 for 3 weeks, or nonspecific IgG1-PBD control. All mouse handling and treatments were performed in under UCICUA-approved protocols.

**Sulforhodamine B assay**

The human HNSCC cell lines UM-SCC-11B and UM-SCC-22B (generously provided by Dr. Thomas Carey) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (Invitrogen). Cells were seeded in quadruplicate wells in 96-well plates (Corning) at a density of 2,000 cells per well. Attached cells were treated with 0 to 1 µg/mL MEDI0641 or IgG1-PBD control for 24 to 96 hours. Treated cells were fixed in 50% trichloroacetic acid (Sigma), stained with 0.4% SRB (Sigma), and washed with 1% acetic acid. Bound SRB dye was solubilized in 10 mmol/L Trizma-base. Plates were read in a microplate reader at 560 nm (GENios, Tecan).

**Immunofluorescence**

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was carried out in Target Retrieval Solution (Dako). Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Following blocking with Background Sniper (Biocare Medical), tissue sections were exposed to rabbit anti-ALDH (Abcam #52492) or mouse anti-CD44 (Thermo #MS-668-R7). The tissue was then labeled with either mouse or rabbit Alexafluor 488 or 594 (Invitrogen #A10304 or #A1032) and mounted in Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories #H-1200). Alternatively, tissue sections were incubated overnight in TUNEL-binding solution (Roche #11684795910). Images were captured with a Nikon Eclipse 80i fluorescence microscope.

**Western blotting**

Whole-cell lysates were analyzed using SDS-PAGE. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (GE Healthcare Life Sciences) that was blocked in 5% non-fat dry milk and exposed to rabbit anti-5T4 (Abcam #52492) or mouse anti-CD44 (Thermo #MS-668-R7). The tissue was then labeled with either mouse or rabbit Alexafluor 488 or 594 (Invitrogen #A10304 or #A1032) and mounted in Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories #H-1200). Alternatively, tissue sections were incubated overnight in TUNEL-binding solution (Roche #11684795910). Images were captured with a Nikon Eclipse 80i fluorescence microscope.

**Flow cytometry**

Trypsinized cells were filtered through a 40-µm sterile cell strainer (Fisher) and washed with DMEM supplemented with FBS. Alternatively, tumors were harvested from mice, dissociated using the GentleMACS Dissociator Kit (Miltenyi Biotec), incubated in ACK red blood cell lysis buffer (Invitrogen), and filtered through a 40-µm sterile cell strainer. ALDH activity was quantified using the Aldefluor Kit (Stem Cell Tech.) Briefly, single-cell suspensions of 10⁶ cells were incubated in activated Aldefluor substrate (BAAA) or equivalent volume of Aldefluor inhibitor diethyl aminobenzaldehyde (DEAB). CD44 was probed with anti-CD44 (APC #559942, BD Pharmingen or V450 #561292, BD Horizon). 5T4 was probed using MEDI0641 (MedImmune) for 1 hour on ice, followed by labeling with anti-human Alexa Fluor 594 (#A11014; Invitrogen) for 30 minutes at 4°C. Human cells were distinguished by positive HLA-ABC staining (PE #560168; BD Pharmingen), and 7-aminomethylcoumarin (7AAD #00-6993-50, eBioscience) was used to isolate viable cells. For cell-cycle analysis, filtered cells were fixed in 70% ethanol for 1 hour, followed by incubation with propidium iodide (PI #P4864, Sigma) for 30 minutes at 4°C.

**Orosphere assay**

HNSCC cells were sorted for ALDHhighCD44high and ALDHlowCD44low populations by fluorescence-activated cell sorting (FACS), as we described (30). Sorted cells were cultured in DMEM-F12 (Invitrogen) and penicillin/streptomycin (Invitrogen) supplemented with N2 (Invitrogen), rhEGF (Sigma), and FGF (Sigma). Cells were seeded at 2,000 cells per well in 24-well ultra-low attachment plates (Corning). Spheres of 25 cells or more were counted (30). Orospheres were treated with 0 to 10 µg/mL of MEDI0641 (MedImmune) or IgG1-PBD control (MedImmune) for 24 to 72 hours.

**Statistical analysis**

Data were analyzed with a one-way ANOVA using Prism software (GraphPad Software), and statistical significance was defined as P < 0.05. To quantify the univariate relationship between 5T4 expression level and outcomes, we used a log-rank test. We used the Cox proportional hazards method for multivariate regression including the following covariates: age, tobacco use, alcohol use, sex, high-stage disease, chemotherapy, radiotherapy, surgical resection. The proportional hazards assumption was checked using visual inspection of the scaled Schoenfeld residuals and by testing the Schoenfeld residuals for each covariate versus scaled time. PDX tumor growth data were evaluated from the treatment start time using linear mixed models to incorporate the repeated measurements on each tumor. Model fixed effects included starting tumor size, time, treatment class, and the interaction between time and treatments and model random effects included mouse. A continuous autoregression correlation structure was employed, which assumes more correlation among temporally proximate observations. Tumor size was log-transformed to account for the exponential growth of tumors. For regression analysis with more than 2 groups, an ANOVA of the model was used to generate the P value for overall group differences. Analysis was performed using the "nlme" package in the statistical software program R version 3.1.0. For regression analysis with more than 2 groups, a Student t test of the model was used to generate the P value for differences between the 2 groups. To assess the relationship between dose and number of orospheres, we used multivariate linear regression. We included dose level, the factor- and the interaction between dose and day. Day was included in our model as a factor. Correlation between ordinal variables was tested using the Spearman rank correlation test, Association between categorical variables was tested using a χ² test.

**Results**

5T4 oncocellular antigen expression correlates with overall survival of patients with HNSCC

We first sought to understand the patterns of expression of 5T4 in HNSCC. A TMA of human HNSCC tumors (n = 77) was
evaluated for 5T4 staining by a trained oral pathologist blinded for patient outcome. We found that 5T4 is expressed primarily in the cell membrane of tumor cells, with no (or weak) observable staining in the surrounding stromal cells or in normal human oral mucosa control tissue (Fig. 1A). We also observed that high 5T4 expression correlates with shorter overall patient survival ($P = 0.038$) in patients who were followed-up for up to 12 years (Fig. 1B). Using the $\chi^2$ test, we found no significant association between 5T4 expression and gender ($P = 0.6787$), high tobacco use ($P = 1$), or high alcohol consumption ($P = 0.616$). Using Spearman correlation test, we found no association between 5T4 expression and age ($P = 0.2733$) or clinical stage ($P = 0.2525$), indicating that 5T4 might be considered an independent identifier of patient survival (Fig. 1C).

MEDI0641 induces long-term tumor regression in PDX models of HNSCC

It has been proposed that low-passage PDX models of cancer recapitulate more accurately the biology of human tumors and are valuable tools for preclinical testing of anticancer therapies (31). The PDX models used here represent clinical features observed in patients with HNSCC (28, 29). Briefly, the PDX-SCC-M0 model was generated from the local recurrence of a patient who was treated with surgery without radiation or chemotherapy. The PDX-SCC-M1 model was generated from the tumor of a previously untreated patient. The PDX-SCC-M11 model originates from the local recurrence of a tumor in the sinonasal cavity of a patient who was treated with surgery, radiation, and chemotherapy (docetaxel, carboplatin, 5-fluorouracil). These PDX models clearly express 5T4, the target of MEDI0641 (Supplementary Fig. S1). Interestingly, we also discovered via immunofluorescence that 5T4 staining colocalizes with CD44, a well-characterized marker of CSCs in HNSCC (15), in the PDX-SCC-M0 model (Supplementary Fig. S2).

A single dose of MEDI0641 resulted in complete tumor regression in the PDX-SCC-M0 model for the duration of the experiments, that is, approximately 100 to 150 days after treatment (Fig. 2A). To determine how robust were the effects of MEDI0641, we allowed the tumors to grow further and then treated 2 additional PDX models (PDX-SCC-M1, PDX-SCC-M11) with a single injection of the drug. Again, we observed significant regression in tumor volume in these 2 models. No body weight loss was observed in any of the treatments (Fig. 2A). In the PDX-SCC-M0, PDX-SCC-M1, and PDX-SCC-M11 models, autoregression correlation analysis of changes in tumor volume following treatment indicated a significant decrease ($P < 0.0001$, $P = 0.028$, $P = 0.033$, respectively) in the MEDI0641-treated groups compared with the group receiving IgG1-PBD control treatment (Fig. 2B).

We next sought to determine the impact of various dosing regimens in the PDX-SCC-M11 model, as it proved to have the least response to a single dose of MEDI0641. All treatment groups received a total dosing of 1 mg/kg MEDI0641; however, it was spread out over time in 3 distinct regimens. Mice were treated with either a single dose of 1 mg/kg MEDI0641; 1 dose every 3 weeks of 0.5 mg/kg MEDI0641 or IgG1-PBD control (total of 2 doses); or 1 dose every 3 weeks of 0.33 mg/kg MEDI0641 (total of 3 doses). Linear regression analysis showed that all 3 regimens caused a significant reduction ($P < 0.0001$) in tumor volume for at least 100 days, as compared with the IgG1-PBD control group. Notably, no significant difference in tumor volume reduction was observed among the 3 treatment regimens. Again, no body weight loss was observed during treatment (Fig. 2C).

MEDI0641 induces cell-cycle arrest and apoptosis in HNSCC cells

To begin to understand the mechanisms mediating the antitumor effect of MEDI0641, we examined the cytotoxic effects of MEDI0641 in established HNSCC cell lines. We first determined the basal 5T4 expression levels in a panel of well-characterized and genotyped HNSCC cell lines (ref. 32; Fig. 3A). Next, we examined the pharmacologic effects of MEDI0641 across a range of concentrations and time points. MEDI0641 exhibits low IC$_{50}$ in UM-SCC-11B and UM-SCC-22B cells, which express moderate to high levels of 5T4, with values of 522 and 650 ng/mL, respectively, after 72 hours of treatment (Fig. 3B). This is consistent with the values observed by Harper and colleagues in the treatment of breast, gastric, and prostate cancer cells in vitro. These doses of MEDI0641 induced a G$_2$-M cell-cycle arrest, as measured by flow cytometry with propidium iodide, with a maximum effect at 72 hours (Fig. 3C). Notably, we observed an increase in the number of apoptotic cells defined as the sub-G$_{0}$-G$_{1}$ fraction (i.e., outside the cell cycle) after treatment with MEDI0641 (Fig. 3C). Interestingly, MEDI0641 had a more modest but still significant effect in 2 HNSCC cell lines expressing lower levels of 5T4, that is, UM-SCC-14B and UM-SCC-74B (Supplementary Fig. S3). Next, we examined PDX-SCC-M11 tissue from mice that received MEDI0641 (or IgG1-PBD control) to determine whether treatment was inducing apoptosis in vivo as well. Following in situ TUNEL staining, we observed a significant increase ($P < 0.0001$) in the number of apoptotic cells in PDX tumors treated with MEDI0641 when compared with IgG1-PBD control (Fig. 3D).

MEDI0641 decreases the fraction of head and neck CSCs in vitro

We next sought to investigate the effect of MEDI0641 on the fraction of head and neck CSCs (i.e., ALDH$^{pos}$CD4$^{pos}$) in HNSCC cell lines in vitro (33). First, we examined the expression of 5T4 in CSCs in UM-SCC-11B and UM-SCC-22B cell lines by flow cytometry. We observed a general trend for increased expression of 5T4 in CSCs when compared with non-CSCs particularly in the UM-SCC-22B cell line ($P = 0.0026$; Fig. 4A). Then, we exposed UM-SCC-11B and UM-SCC-22B cells to MEDI0641 at their respective IC$_{50}$ concentrations. We observed a reduction in the CSC fraction in UM-SCC-11B from around 35% in cells treated with IgG1-PBD control to 10% in MEDI0641-treated cells ($P = 0.03$). In UM-SCC-22B cells, the CSC fraction was reduced from about 10% to 5% ($P = 0.05$) after 72 hours (Fig. 4B).

Culturing HNSCC cells in nonadherent, serum-free conditions selects for CSCs, as we have shown (30). We seeded UM-SCC-22B cells in ultra-low attachment plates and allowed orospheres to form. Once the orospheres were established, they were treated with increasing doses of MEDI0641 for 24, 48, and 72 hours. We observed a significant overall dose dependence decrease in the number of orospheres per well across all time points ($P = 0.0192$) with increasing concentrations of MEDI0641 (Fig. 4C).

MEDI0641 decreases the fraction of head neck CSCs in PDX HNSCC tumors

We next examined the effect of MEDI0641 on the fraction of CSCs in vivo. Mice harboring PDX-SCC-M0, PDX-SCC-M1, or PDX-SCC-M11 tumors were treated with a single dose of either IgG1-PBD control or MEDI0641 and euthanized after 7 days. As
Figure 1.
5T4 oncofetal antigen is expressed in HNSCC and correlates with overall survival. 

A, Immunohistochemical staining for 5T4 in human HNSCC tumor cores. Images are representative of staining patterns in 5T4low and 5T4high groups, as well as 5T4 expression in normal human oral mucosa control tissue.

B, Graph depicting overall survival over time in tumors with high and low 5T4 expression.

C, Graph depicting the breakdown of 5T4low and 5T4high tumors by disease stage at time of diagnosis.
Figure 2.
MEDI0641 induces long-term tumor regression in PDX models of HNSCC. A, Graphs depicting mean tumor volume and normalized weight over time in the PDX-SCC-M0, PDX-SCC-M1, and PDX-SCC-M11 models after treatment with a single dose of MEDI0641 or IgG1-PBD control. Mice received 0.33 mg/kg MEDI0641 or IgG1-PBD control in the experiment performed with PDX-SCC-M0 tumors or 1 mg/kg MEDI0641 or IgG1-PBD control in the experiment with PDX-SCC-M1 and PDX-SCC-M11 tumors. Black arrows indicate when MEDI0641 was administered. B, Graphs depicting autoregression analyses of data acquired in the PDX-SCC-M0, PDX-SCC-M1, and PDX-SCC-M11 models from experiments performed in A. Data for these graphs were analyzed only posttreatment. Please note the y-axes are on a log scale. C, Graphs depicting mean tumor volume, normalized weight, and autoregression analysis in the PDX-SCC-M11 model over time after receiving various dosing regimens of MEDI0641. Black arrows represent treatment initiation. Autoregression analysis was only generated for data posttreatment, as in B. Please again take note of the log scale y-axis.
Figure 3.
MEDI0641 induces G2-M cell-cycle arrest and induces apoptosis. A, Western blot analysis showing the endogenous expression of 5T4 oncofetal antigen in a panel of HNSCC cell lines. B, Graphs of SRB assays in UM-SCC-11B and UM-SCC-22B cells depicting optical density at 565 nm normalized to IgG1-PBD control over increasing concentrations of MEDI0641 at 24, 48, 72, and 96 hours. C, Modfit depictions of cell-cycle changes in UM-SCC-11B and UM-SCC-22B cells following treatment with 521 and 686 ng/mL, respectively, of either IgG1-PBD control or MEDI0641 at 72 hours. Graphs depicting triplicate analyses of the changes in cell cycle and apoptosis in UM-SCC-11Bs and UM-SCC-22Bs at 24, 48, and 72 hours. *, P < 0.05; **, P < 0.005. D, Representative images (200×) of TUNEL staining for apoptosis in PDX-SCC-M11 tissue samples treated with a single dose of 1 mg/kg MEDI0641 or control 1 mg/kg IgG1-PBD 7 days prior to euthanasia. Inserts are at 400× magnification. Pictures were taken of 5 fields per tumor in 3 tumors per group. A graph depicting TUNEL-positive cells per field in the control IgG1-PBD and MEDI0641 treatment groups.
Figure 4.
5T4 is highly expressed in CSCs and MEDI0641 targets these cells in normal and low-attachment conditions. A, Flow cytometric gating and graph depicting the expression of 5T4 in the CSC and non-CSC fractions in UM-SCC-11B and UM-SCC-22B cells. B, Flow cytometric gating and graph depicting the percentage of ALDH<sup>high</sup>CD44<sup>high</sup> cells in UM-SCC-11B and UM-SCC-22B cells following treatment with the IC<sub>50</sub> values for each line (522 or 686 ng/mL, respectively) of either IgG1-PBD control or MEDI0641. C, Graph depicting the number of UM-SCC-22B orospheres per well over time after treatment with a 2-fold concentration gradient of MEDI0641 at 0.156, 0.312, 0.625, 1.25, 2.5, 5, and 10 μg/mL. IgG1-PBD control treatments, represented by the solid black bars, were given at 10 μg/mL. Superimposed lines were generated from regression analyses of dose dependency at each time point. Representative images of orospheres after 3 days of treatment with either 10 μg/mL IgG1-PBD control or MEDI0641 at 40× and 100× magnifications.
expected, mice did not show weight loss and tumor volumes did not decrease significantly as we euthanized mice after a week to determine more accurately the effect of the drug on the fraction of CSCs (Supplementary Fig. S4). In the PDX-SCC-M0 model, FACS analysis showed complete ablation of the ALDH<sup>high</sup>CD44<sup>high</sup> fraction following treatment, and PDX-SCC-M1 and PDX-SCC-M11 models showed significant decreases (\(P = 0.0029\) and \(P = 0.0024\), respectively; Fig. 5A). Furthermore, this analysis displayed a significant decrease (\(P = 0.003\)) in CD44-positive cells independent of ALDH activity (Supplementary Fig. S5). This is perhaps not surprising, as CD44 has been shown to be a marker on its own for HNSCC CSCs (15).

In addition to flow cytometry, we also studied the effect of MEDI0641 on CSCs by immunofluorescent staining of formalin-fixed, paraffin-embedded PDX tissue. This analysis showed a significant reduction of ALDH<sup>high</sup>CD44<sup>high</sup> cells in the MEDI0641 group (\(P < 0.0001\)) as compared with the IgG1-PBD control group (Fig. 5B), confirming the FACS data.

**MEDI0641 prevents local recurrence in PDX models.**

As CSCs have been implicated in HNSCC dissemination (34), we examined the effect of MEDI0641 in preventing local recurrence in vivo. We treated the PDX-SCC-M11 model with either 1 mg/kg IgG1-PBD control or 1 mg/kg MEDI0641 7 days before surgically removing the tumors. The mice were then monitored for tumor recurrence, as defined as a tumor greater than 50 mm<sup>3</sup> in the region where the primary tumor was surgically removed. Over the course of 250 days, we observed no recurrences in 12 mice in the MEDI0641-treated group, whereas the R347 control group showed recurrences in 7 of 12 mice (\(P = 0.0047\); Fig. 6A). All recurrences were located in the same position as the resected tumor, in the subcutaneous space in the right dorsal region (Fig. 6B). No mice exhibited body weight loss (Fig. 6A). These results were confirmed when we repeated the experiment in an independent experiment (\(P = 0.011\); Supplementary Fig. S6). Histologic analysis of an original and recurrent tumor revealed similar grade, aggressiveness, and morphology as compared with the original tumor in the same mouse (Fig. 6C).

**Discussion**

The therapeutic potential of anticancer treatments with antibody–drug conjugates depends on target antigens that are highly expressed and specific to tumor cells and on the effectiveness of
the cytotoxic "payload." In this study, we examined the effect of MEDI0641 in preclinical models of HNSCC. A single dose of single-agent MEDI0641 caused regression of established PDX HNSCC tumors. Perhaps more importantly, a single dose of MEDI0641 prevented tumor recurrence when used in a neoadjuvant setting prior to surgery. Collectively, these data unveil the therapeutic potential of MEDI0641 in HNSCC, suggesting that patients with these tumors might benefit from treatment with this ADC.

Here, for the first time, we evaluated the expression and prognostic potential of 5T4 in HNSCC. We retroactively analyzed a tissue microarray of 77 patients with HNSCC for 5T4 and observed that patients harboring tumors with high 5T4 expression had lower overall survival than patients with tumors expressing
lower 5T4. This result is consistent with previous studies of 5T4 in lung, gastric, ovarian, and colorectal cancers (22–25). Interestingly, in some HNSCC tissue samples, 5T4 was found in the cytosol of the tumor cells in addition to being located in the plasma membrane. The role of 5T4 in tumor cells has not been fully characterized, and further studies regarding the differential expression of 5T4 in the membrane versus the cytosol are warranted. Of note, 5T4 staining in these patient samples was highly specific to cancer cells with very little staining in the surrounding stromal cells, validating the hypothesis that 5T4 oncofetal antigen might be a promising target for antibody–drug conjugate therapy.

We observed that MEDI0641 resulted in long-term tumor regression in PDX models of HNSCC after administering a single dose of MEDI0641 when tumors were already established. The observed tumor regression is likely due to the cytotoxic effect of MEDI0641 to both, CSCs and non-CSCs. Indeed, to challenge this therapy, we waited until the mean tumor volume reached about 1,000 mm$^3$ in the PDX-SCC-M11 model before injecting MEDI0641. Of note, we selected this model as it represents resistant disease, that is, this PDX was generated from a patient previously treated with surgery, radiation, and TPF-based chemotherapy. We observed that MEDI0641 caused long-term tumor regression in all PDX models, regardless of mean tumor volume at treatment initiation. Notably, in the PDX-SCC-M0 model, tumors were ablated completely and did not resume tumor growth for the duration of the study, almost 150 days after treatment initiation. To confirm the persistence of the tumor regression observed in the PDX-SCC-M11 model, we induced tumor growth after 80 days of treatment and monitored the outcome. Notably, tumors regressed and did not resume growth after a single injection of MEDI0641.

We next hypothesized that perhaps MEDI0641 would confer protection against local recurrence. For this analysis, we selected model PDX-SCC-M11. The patient from whom the PDX-SCC-M11 model is derived had been previously treated with chemoradiation therapy, and the tumor that was used to generate the PDX model was in fact a recurrent tumor. Indeed, we chose to analyze this model because it had already shown the potential for recurrence in a human patient and we wanted to challenge MEDI0641 with a model of resistant disease. Our hypothesis was proved to be correct, as 2 independent in vivo experiments with this model showed that treatment with MEDI0641 before surgical resection resulted in no local recurrences over a period of up to 200 days. Notably, while the PDX tumors evaluated in these recurrence studies were fairly easily resected from the mouse, HNSCC tumors are notoriously challenging to be surgically removed in humans. Further studies into the effects of MEDI0641, or other 5T4-targeted agents, on local recurrence in HNSCC with consideration for varying scopes of excision are certainly warranted.

Collectively, this work demonstrated that expression of 5T4 oncofetal antigen in the tumor correlates with survival of patients with HNSCC and showed that a single injection of a single-agent antibody–drug conjugate targeted to 5T4 is capable of causing regression and preventing recurrence of HNSCC tumors. We attribute these results to the following actions of MEDI0641: (i) Ablation of the CSC population, potentially explaining the capacity of MEDI0641 to prevent tumor regrowth (months after termination of treatment) and to prevent tumor recurrence when used in a neoadjuvant setting and (ii) Induction of apoptosis of the more differentiated tumor cells which might explain the capacity of MEDI0641 to cause tumor regression. The remarkable potency and high specificity of MEDI0641 in the work presented here warrant further studies and clinical trials. Indeed, this work suggests that anti-5T4 therapies can potentially become promising treatment options for patients with HNSCC.

Disclosure of Potential Conflicts of Interest

E.M. Hurt is a Senior Scientist at MedImmune. R.E. Hollingsworth and D.A. Tice are the Senior Directors, Oncology, at MedImmune. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S.A. Kerk, A.T. Pearson, R.E. Hollingsworth, D.A. Tice, J.E. Nör


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Finkel, K.A. Warner, V.P. Wagner, P.A. Vargas, J.E. Nör

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Kerk, A.T. Pearson, Z. Zhang, V.P. Wagner, R.E. Hollingsworth, J.E. Nör

Writing, review, and/or revision of the manuscript: S.A. Kerk, K.A. Finkel, A.T. Pearson, F. Nör, V.P. Wagner, P.A. Vargas, M.S. Wicha, J.E. Nör

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.A. Kerk, J.E. Nör

Study supervision: S.A. Kerk, Z. Zhang, R.E. Hollingsworth, D.A. Tice, J.E. Nör

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