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Authors

Spector, Matthew E
Sacco, Assuntina G
Bellile, Emily
et al.

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E6 and E7 antibody levels are potential biomarkers of recurrence in patients with advanced stage human papillomavirus positive oropharyngeal squamous cell carcinoma

Matthew E. Spector[*,1], **Assuntina G. Sacco**[*,2], **Emily Bellile**[1], **Jeremy M. G. Taylor**[1], **Tamara Jones**[1], **Kan Sun**[1], **William C. Brown**[3], **Andrew C. Birkeland**[1], **Carol R. Bradford**[1], **Gregory T. Wolf**[1], **Mark E. Prince**[1], **Jeffrey S. Moyer**[1], **Kelly Malloy**[1], **Paul Swiecicki**[1], **Avraham Eisbruch**[1], **Jonathan B McHugh**[1], **Douglas B. Chepeha**[1,4], **Laura Rozek**[1], and **Francis P. Worden**[1]

[1] University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan

[2] University of California at San Diego, Moores Cancer Center, La Jolla, California

[3] University of Michigan High-throughput Protein Lab, Center for Structural Biology, Life Sciences Institute, Ann Arbor, Michigan

[4] University of Toronto, Toronto, Ontario Canada

Abstract

Background—There is a paucity of biomarkers to predict failure in human papilloma-virus positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC) following curative therapy. E6/E7 viral oncoproteins are constitutively expressed in HPV+ tumors and highly immunogenic, resulting in readily detected serum antibodies. The purpose of this study is to determine if serum E6 and E7 antibody levels can potentially serve as a biomarker of recurrence in patients with HPV +OPSCC.

Methods—We evaluated E6/E7 antibody levels in patients with previously untreated, advanced stage (III, IVa-b), HPV+OPSCC receiving definitive chemoradiation under a uniform protocol from 2003-2010. Baseline and longitudinal serum samples were obtained from our archived repository. E6/E7 serum levels were measured using a glutathione-*S*-transferase capture ELISA and quantified by approximating the area under the dilution curve, and were analyzed using ANOVA and linear mixed model for longitudinal analysis.

Results—We compared 22 HPV+OPSCC patients who developed recurrence to 30 patients who remained disease-free. There were no differences in T classification, N classification, disease subsite or smoking status between the groups. In a longitudinal analysis, recurrent patients had significantly higher E6 and E7 serum antibody levels than the non-recurrent patients over the

Corresponding author: Matthew E. Spector, MD, Taubman Center 1904, 1500 E. Medical Center Dr., SPC 5312, Ann Arbor, Michigan 48109-5312, (734) 936-3172 Phone, (734) 936-9625 Fax, mspector@med.umich.edu.

*Co-first author

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follow-up period (p=0.02 and p=0.002, respectively). Patients who recurred had a lower clearance of E7 antibody than patients who remained disease free (p=0.0016).

Conclusion—Patients with HPV+OPSCC whose disease recurs have a lower clearance of E6 and E7 antibodies than patients who do not recur. The ratio of E7 antibody at disease recurrence compared to baseline is potentially a clinically significant measurement of disease status in HPV +OPSCC.

Keywords

E6 E7 Antibodies; Predictive Biomarkers; Recurrence; HPV-Positive; Oropharyngeal Squamous Cell Carcinoma

Introduction

Over the past three decades, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) has steadily increased in the United States. [1, 2] With decline in tobacco consumption, this increase in OPSCC is largely attributable to an increase in high-risk human papillomavirus (HPV) infection. [3, 4] Patients with HPV-positive OPSCC typically have a more favorable prognosis than patients with HPV-negative OPSCC, with distinct oncologic mechanisms and epidemiologic profiles associated with this subtype of cancer. [5-8]

Despite the better prognosis associated with HPV-positive OPSCC, approximately 15-20% of patients still experience treatment failure and succumb to their disease. [9, 10] Many groups have published on clinical risk factors related to disease recurrence in HPV-positive OPSCC, including a combination of advanced T and N classification [11], active smoking status [12], and matted cervical lymph nodes. [13, 14] However, there is a lack of predictive biomarkers that can identify patients who will fail standard therapy.

Previous studies have demonstrated that levels of pre-treatment serum antibodies against the HPV-related E6 and E7 oncoproteins predict disease-free survival in HPV+ OPSCC, suggesting that a highly immunogenic response to these proteins before treatment can be readily detected and may provide insight to a patient's immune status. [15] However, the detection of E6 and E7 antibodies in a post-treatment setting with longitudinal measurements of E6/E7 antibody status is needed to understand the dynamics of serum antibodies and survival in these patients. Thus, the purpose of our study was to determine if serum E6 and E7 antibody levels can potentially serve as a biomarker of recurrence in patients with HPV+ OPSCC. We hypothesized that patients who develop recurrence will have higher pretreatment serum antibody levels, and that antibody levels will be persistently elevated after treatment when compared with non-recurrent patients.

Methods

Patient Eligibility

All patients were treated under a uniform clinical protocol (University of Michigan Comprehensive Cancer Center (UMCC) protocol 2002-021), designed to evaluate the

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toxicity and efficacy of weekly concomitant carboplatin and paclitaxel with intensity-modulated radiation therapy (IMRT) for advanced stage (III, IV) OPSCC from 2003 to 2010. This study was approved by the University of Michigan Institutional Review Board. Patients were enrolled and informed consent was obtained for all patients. Patients were eligible if they had previously untreated, advanced-stage (III, IVa-b), pathologically confirmed OPSCC, were HPV positive, and had serum samples available in the University of Michigan Head and Neck Cancer Program biorepository.

Tumor staging was routinely performed by clinical examination and direct laryngoscopy in the operating room along with pre-treatment staging computed tomography (CT) or CT/positron emission tomography (PET) correlation within 4 weeks prior to starting treatment. All patients were staged based on the 2002 American Joint Committee on Cancer staging system. Patients were excluded if they had previous surgery or radiation therapy to the upper aerodigestive tract or neck.

Patient Population

There were 171 patients with advanced HPV-positive OPSCC who were treated uniformly under this paradigm. Thirty patients developed recurrence in this cohort, and 22/30 patients had baseline serum available for analysis. A control group of 30 patients was selected from patients who were treated under the same clinical protocol who did not recur after at least 2.5 years of follow-up and had available serum. When compared with the recurrent cohort, non-recurrent patients who were selected for the analysis were similar in age, T classification, N classification, and tobacco status. (Table 1) Baseline samples ranged from 26 days prior - 63 days post diagnosis and were collected prior to starting treatment. Longitudinal samples were obtained at 3 month intervals for 24 months from date of diagnosis. The mean time to recurrence (measured as time from end of treatment) within the recurrent group was 13 months. Smoking status and other epidemiologic variables were determined by health history questionnaire administered at the time of diagnosis.

Treatment Protocol

Radiation therapy was delivered using daily fractionated IMRT, 5 days per week over 35 fractions. The doses were 70 Gy at 2.0 Gy per fraction to gross disease and then 59 to 63 Gy at 1.7 to 1.8 Gy per fraction to low-risk and high-risk subclinical regions, respectively. This was done concomitantly according to published methods. [16, 17] Chemotherapy consisted of weekly carboplatin (area under the curve 1) by intravenous infusion over 30 minutes and paclitaxel (30mg/m2) by intravenous infusion over 1 hour.

HPV Status Determination

Isolation of DNA from formalin-fixed, paraffin-embedded tissue samples was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA). DNA concentration and purity were confirmed via NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). HPV status was determined by an ultrasensitive method using real-time competitive polymerase chain reaction (RT-PCR) and matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy with separation of products on a matrix-loaded silicon chip

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array, as previously described. [18] In addition, p16 staining was available for 49/52 patients and was positive in all 49 patients.

E6/E7 Antibody

Detection of E6 and E7 serum antibodies was performed using a modified version of a glutathione-*S*-transferase (GST) capture enzyme-linked immunosorbent assay (ELISA), as previously described and validated. [19] Blood drawn from patients was centrifuged at 1500 × g for 15 minutes. The resulting serum component was aspirated and stored in 0.5mL aliquots at −80°C. For the ELISA assay, wells were incubated at 4°C for 1 hour with 100μL of purified GST-E6-Tag, GST-E7-Tag, or GST-Tag recombinant antigens. The recombinant antigens had been previously generated utilizing high-quality genomic DNA isolated from the commercially available HPV16 positive Ca Ski cervical cancer cell line, which was submitted to the University of Michigan Center for Structural Biology where full length coding sequences for HPV 16-related E6 and E7 oncoproteins were generated by polymerase chain reaction (PCR) followed by large-scale purification. Simultaneously, human sera samples were serially diluted in assay diluent containing 10ng/μL of GST-Tag recombinant antigen and incubated in conical tubes for 1 hour at 4°C to eliminate background reactivity of serum components. Antigen-coated wells were then incubated at room temperature for 1 hour with 100μL of each of the diluted and pre-incubated human sera samples in duplicate. Wells were washed with PBS-T followed by 100μL of a goat anti-human IgG HRP-conjugate antibody (SC2453, Santa Cruz Biotechnology), diluted 1:20, 000 in assay diluent to remove any unbound serum components and recombinant GST-Tag. 100μL of Supersensitive Tetramethylbenzidine Liquid Substrate for ELISA (T4444, Sigma-Aldrich) was added to each well and allowed to incubate in the dark for 5 minutes at room temperature. Absorbance readings for each well were measured at 450nm. Absorbance readings for wells coated with the GST-Tag recombinant peptide represented background reactivity and were subtracted from the absorbance readings for specific reactivity with the GST-E6-Tag and GST-E7-Tag for each serum sample dilution. For antigen detection during optimization of purified GST-E6-Tag, GST-E7-Tag, and GST-Tag plate coating, a mouse monoclonal anti-SV40 KT3 Tag primary antibody (SC58664, Santa Cruz Biotechnology) was used at a 1:1000 dilution in conjunction with a goat anti-mouse IgG HRP-conjugate secondary detection antibody (SC2005, Santa Cruz) at a 1:20000 dilution.

Statistical Analysis

This study was designed to test differences in E6 and/or E7 antibody levels between recurrent and non-recurrent patients with advanced stage, HPV+ OPSCC. Both overall level of antibody and change over time between groups were explored. When designing the study from the availability of samples (30 nonrecurrent and 22 recurrent), we calculated the power to detect a mean difference in E6/E7 of at least 1 standard deviation to be >90% for a two-sample t-test, with that power being slightly less because we use the nonparametric Wilcoxon test. Other clinical characteristics of interest were age, T classification, N classification and smoking status at diagnosis. We also explored the type of recurrence (locoregional versus distant). Smoking status was defined categorically as never, prior (quit >6 months before diagnosis), or current use of cigarettes.

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Serial dilutions of each serum sample were summarized for E6 and E7 antibody levels separately by approximating the area under the dilution curve (AUDC). We performed standard bivariate comparisons between E6 and E7 at baseline, at treatment completion, and at the latest time point with clinical covariates using Wilcoxon tests (categories: e.g. smoking, Stage III vs IV, T classification, N classification, recurrence status) and Spearman correlation coefficients (continuous measures: age).

Linear mixed effects models were utilized to analyze patterns of longitudinal E6 and E7 antibody levels over time. Each model included fixed effects for time and recurrence status. Correlated measurements within subject were assumed to have a compound symmetric correlation structure. The interaction between time and recurrence was explored as well as T classification, N classification, and current smoking status in multivariable models. Separate models were analyzed for E6 antibody and E7 antibody. In addition, we derived a measure of change from baseline level to last clinically relevant time point for comparison of antibody clearance across recurrence status groups by a Wilcoxon rank-sum test. We defined this clinically relevant time point as the sample nearest recurrence (within 3 months) for the recurrent group and last available sample from follow-up for the non-recurrent group. Importantly, the samples taken from the non-recurrent patients for the clinically relevant time point were drawn within a mean difference of 36.8 days from the recurrent samples as to not have time bias as the cause of the decrease in antibody level. The extent of antibody clearance since baseline was defined as the ratio of the latest/pre-treatment antibody level.

Results

E6/E7 Baseline and 3 month post CRT Antibody Measurements

Serum from a total of 52 patients was measured at baseline and at 3 months post-CRT (22 recurrent and 30 non-recurrent). Lower levels of baseline E7 antibody were seen in current smokers (p=0.05, Table 2). Larger tumors had lower baseline E7 antibody levels (T3/4 vs T1/2, p=0.016, Table 2) and there was a trend for higher baseline E7 antibody levels by N classification (N0 vs N1/2 vs N3, p=0.09, Table 2). There was no association with pre-treatment antibody levels and recurrence (E6, p=0.61, E7, p=0.17). There was no association between the type of recurrence (locoregional versus distant) and pretreatment E6 and E7 antibody levels.

We next characterized the association between E6 and E7 antibody levels in the immediate post-treatment (within 3 months of completing treatment) and the development of recurrence. There was no association between 3 month post- treatment antibody levels and recurrence (E6, p=0.23, E7, p=0.27, Table 2), nor evidence of significantly different clearance (ratio) between the recurrent and non-recurrent patients (E6, p=0.54, E7, p=0.38, Table 3). In addition, there was no difference between the 3 month post-treatment absolute antibody levels or clearance (ratio) when stratifying by type of recurrence (locoregional versus distant).

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Longitudinal analysis of serum E6/E7 antibody Levels

The change in E6/E7 antibody levels was analyzed over time in a linear mixed model. Both recurrent and non-recurrent patients had decreases in antibody levels over time (p=0.01 and p=0.005 for E6 and E7, respectively). In addition, patients who developed recurrence had significantly higher E6 and E7 antibodies than non-recurrent patients (p=0.02 and p=0.002, respectively, Table 4). When controlling for T classification, N classification and smoking status in a multivariable linear mixed model, patients who developed recurrence continued to have significantly higher E6 and E7 antibodies than non-recurrent patients (p=0.03 and p=0.001, respectively).

The finding that patients who develop recurrence have higher E6 and E7 antibody levels in the linear mixed model was then expanded to look for time points that were particularly predictive of recurrence. This would allow a clinician to determine if there is a significant time point or change in antibody level that could be used to determine if a patient is at risk for recurrence. Figure 1 shows E6 and E7 antibody levels with Lowess curves fit to the recurrent and non-recurrent groups separately. The patterns observed support the results of the linear mixed model that both E6 and E7 decline over time and that the recurrent group has measurably higher E6 and E7 in serial measurements. A vertical line at 13 months (day 390) in each plot was added and represents the mean recurrence time among the recurrent patients. Particularly, when measuring E7 antibody ratio over time, patients who developed recurrence displayed an inflection point in their serial measurements of E7 antibody before the average recurrence time.

E6/E7 Antibody clearance from Pre-treatment to Recurrence

There were 17 patients who had serum samples pretreatment and at a time-point near their recurrence. These recurrent patients were compared to the 30 non-recurrent patients with follow-up samples. Samples used for recurrence were drawn within 3 months of clinical detection of recurrence and ranged from 1.5 months prior to 2.7 months after recurrence diagnosis. The mean (range) time to recurrence was 13 months (3-28 months). Samples taken from the non-recurrent patients for the clinically relevant time point were drawn within a mean difference of 36.8 days from the recurrent samples as to not have time bias as the cause of the decrease in antibody level.

A quantification of the extent of antibody clearance since baseline was defined as the ratio of the latest/pre-treatment antibody level (Table 3). Figure 2 shows E6 and E7 antibody clearance by recurrent status. Patients who did not recur cleared more E7 antibodies at the latest time point than recurrent patients (p=0.0016). The median amount of E7 clearance at last time point measured for the recurrent and non-recurrent patients was 30% and 60%, respectively. We did not observe a statistically significant difference by recurrence status in E6 antibody clearance. When stratifying by type of recurrence, patients who developed a locoregional recurrence had less E7 antibody clearance than patients who did not recur (p=0.004). We also saw this trend for patients who developed a distant recurrence (p=0.06).

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Discussion

Our study demonstrates that patients with advanced stage, HPV+ OPSCC who recur have higher levels of E6 and E7 antibodies with serial measurements. We also demonstrate that recurrent patients clear less E7 antibodies than patients who do not recur, and that the ratio of E7 antibody at the time of recurrence compared to baseline appears to be an important measure of recurrence in this patient population. The pattern of decline in E7 antibody relative to individual baseline shows promise as an important measure to consider when monitoring patients for recurrence after treatment. This is the first study to report the significance of the clearance of E6 and E7 antibodies after treatment, and these biomarkers have the potential to identify patients at risk of treatment failure.

Despite the overall superior survival and recurrence outcomes in HPV+ OPSCC, there is a distinct and nontrivial subset of patients (15-20%) who respond poorly to treatment. Although we have tools to diagnose a recurrence, we do not have predictive biomarkers to anticipate a recurrence. Matted nodes on the pretreatment imaging have been shown to confer an increased risk for distant metastasis but are not useful for monitoring for disease recurrence. [13, 14] Because recurrent disease in HPV +OPSCC often recurs at distant sites and is associated with a high mortality rate, it is important to develop predictive biomarkers to detect recurrence post definitive therapy. Ultimately, detecting recurrent disease earlier in these patients may allow for earlier and novel interventions aimed at improving survival.

We investigated the utility of HPV E6 and E7 antibody testing as a screening modality for tumor recurrence in HPV+ OPSCC patients. Our findings are suggestive of a novel biomarker (E7 antibody clearance) as a useful screening tool for recurrent disease in these patients. Additionally, given the ease of a liquid biopsy for cancer (blood draw), it may afford an opportunity for more comprehensive tumor recurrence monitoring.

Fahkry and colleagues have previously examined the role of E6 and E7 antibody levels in 60 patients with HPV+OPSCC patients. [15] In agreement with our data, their group also showed declining levels of E6 and E7 antibody level over time after the completion of treatment. In this cohort, only 6 patients recurred, limiting the power to detect an association, but showed higher baseline levels of E6 antibody in recurrent patients compared to the non-recurrent patients. They failed to show differences in antibody levels between recurrent and non-recurrent patients in a longitudinal analysis. The important difference in our study is that longitudinal analysis in this nested case control study allowed us to demonstrate associations between recurrence and pattern of E7 antibody clearance. We believe these findings could contribute to improved surveillance in the post treatment period. In addition, locoregional failures seemed to be better predicted by our assay (E7 clearance, p=0.004), which has the potential for future treatment (surgery, re-irradiation) options as salvage therapy.

Other investigations into predictive biomarkers of recurrence in HPV+ OPSCC have been limited to predictors of HPV status [20, 21], or use of single time point of pretreatment biomarkers. [22] The examination of post-treatment oral rinses by Rettig et al. [23] has specific merit, as this study showed the ability to detect HPV16 DNA in the oral rinses of 5

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patients who developed recurrence of their cancer. They showed high specificity for local disease recurrence (median time from earliest post-treatment detection to recurrence: 7 months), suggesting that this biomarker be used in subsequent surveillance protocols. One limitation is that patients who develop regional or distant disease did not have HPV16 in their oral rinses, which may be a limitation to this technique.

The biological importance of circulating antibodies against HPV E6 and E7 has scientific merit. Maintenance of elevated antibody levels may correlate with a continued source of immunogenicity (e.g. an active tumor), or defects in the immune system's ability to clear tumor. If an HPV+ tumor (and thus HPV+ E6/E7 tumor antigen) is cleared, there is no continued immunogenic driver for a host's immune system to continue to make E6/E7 antibodies. As a result, patients who have cleared their disease would have a drop in E6/E7 antibodies whereas those with persistent or recurrent disease may have continued generation of E6/E7 antibodies.

Of note, smoking status appears to have an effect on circulating E6/E7 antibody levels. Whether smokers with HPV+ OPSCC comprise a distinct immunogenic and prognostic population has been highly investigated. [6] There may be confounding factors in this population as it may be difficult to ascertain the underlying primary driver in HPV+ tumors in smokers, (survival for HPV+ smokers is between those of HPV+ non-smokers, and HPV− smokers). Further elucidation into the potential confounding effect of smoking needs to be performed.

There are a few considerations in interpreting these data. Our cohort size is limited, and although samples were collected in a prospective manner, the study design is retrospective in nature and requires prospective analysis. In addition, we only enrolled patients with Stage III/IV disease in this prospective clinical trial, so patients with T1/2 without cervical metastasis would not be included. This finding will need to be validated in early stage I/II HPV+ OPSCC. Further validation of our E6/E7 antibody detection methods should be performed in order to determine the reproducibility and large-scale applicability of this assay across blood samples of varying quality and age. Nevertheless, we believe these preliminary findings suggest a potential novel and valuable screening tool for HPV+ OPSCC cancer patients. A larger HPV+ cohorts, involving multiple institutions and accounting for potential confounders (e.g. smoking status), will be crucial in order to determine the utility of E7 antibody clearance as a predictive biomarker. Given this data, a prospective study would be possible with as few as 100 patients to detect a mean difference in E6/E7 levels of at least 1 standard deviation with a power of >90% for a two-sample t-test assuming a 15% recurrence rate in the cohort.

Conclusion

Patients with HPV+ OPSCC whose disease recurs have higher E6 and E7 antibodies in serial measurements. Recurrent patients clear less E7 antibodies than patients who have lived at least 2.5 years without a recurrence. The ratio of E7 antibody at disease recurrence compared to baseline appears to be a clinically significant measurement of disease status. These findings merit validation in a prospective clinical trial.

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Statement of Translational Relevance

Despite superior disease specific and overall survival in human papilloma-virus positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC), there is a distinct and nontrivial subset of patients who respond poorly to treatment. Biomarkers of recurrence are lacking in these patients, and would potentially allow early detection and treatment of recurrent disease. In this study, we are the first to report the significance of serum E6 and E7 antibodies that are persistent after treatment. Recurrent patients had significantly higher E6 and E7 serum antibody levels than the non-recurrent patients, and the ratio of E7 antibody at disease recurrence compared to baseline is potentially a clinically significant measurement of disease status. Regular monitoring of these antibody levels from time of diagnosis may allow for earlier detection of recurrent disease, which could improve overall survival and expand treatment options for patients who develop recurrent HPV+OPSCC.

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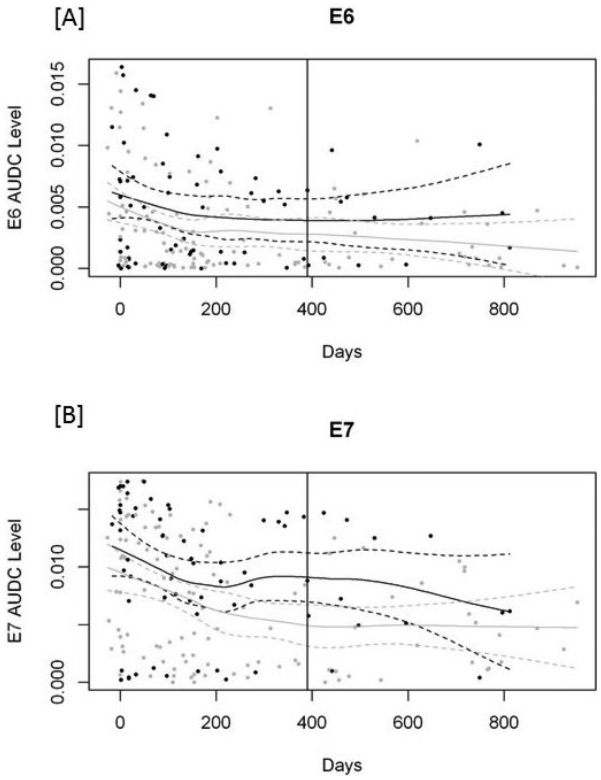

Figure 1.

E6 and E7 antibody levels with Lowess curves stratified by recurrent (red) and non-recurrent (blue) patients over time. A vertical line at 390 days represents the mean time to recurrence. The absolute levels of E6 (Panel A) and E7 (Panel B) antibodies were higher over time in recurrent compared to nonrecurrent patients. Panel B shows an inflection point of E7 antibody levels in patients who developed recurrence at approximately 200 days, which occurred before the detection of their recurrence.

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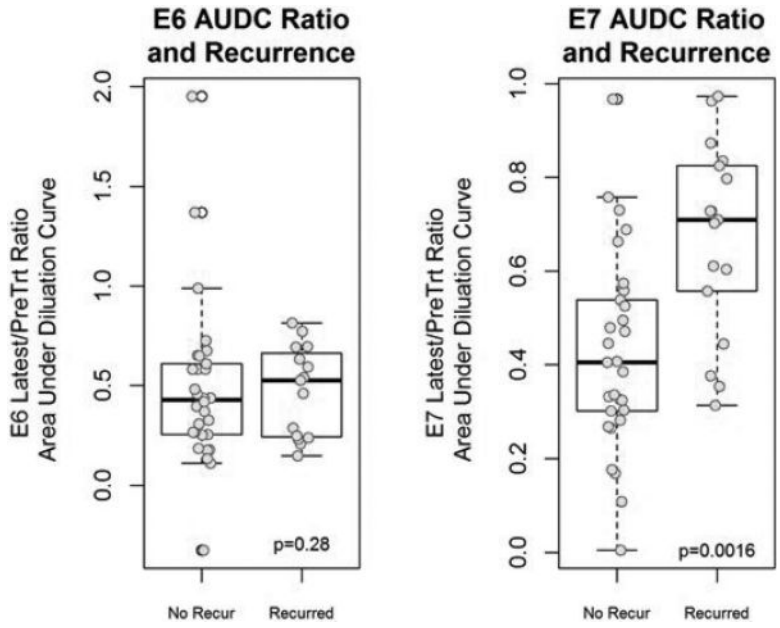

Figure 2.

Boxplots showing E6 and E7 antibody clearance by recurrent status. Antibody clearance since baseline was defined as the ratio of the latest/pre-treatment antibody level. Patients who did not recur cleared more E7 antibodies than patients who developed recurrence.

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Table 1

Clinical characteristics

Variable		Non-Recurrent n=30	Recurrent n=22	p-value
Clinical Stage	III	5	1	0.18
	IV	25	21	
T classification	T1/T2	17	8	0.15
	T3/T4	13	14	
N classification	N0	3	1	0.33
	N1	3	0	
	N2	20	16	
	N3	4	5	
Smoking	Never	7	10	0.23
	Former	8	5	
	Current	15	7	
Subsite	Tonsil	15	13	0.43
	Tongue Base	13	9	
	Other	2*	0	
Recurrence Type	Local		2	
	Local Regional		2	
	Distant		9	
	Local, Regional, Distant		2	
	Regional, Distant		2	
	Regional		5	

*Other includes the glossotonsillar sulcus(1 patient) and posterior pharyngeal wall (1 patient)

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Table 2

Median [std] of E6 and E7 antibody levels by clinical characteristics

		Pretreatment n=52					PostCRT n=45				
Variable		**n**	**E6**	**p**	**E7**	**p**	**n**	**E6**	**p**	**E7**	**p**
Clinical Stage	III	6	1.0 [4.0]	0.19	10.0 [5.7]	0.60	6	0.4 [2.6]	0.07	6.5 [4.6]	0.43
	IV	46	4.2 [5.2]		12.0 [6.1]		39	2.4 [4.3]		9.0 [5.4]	
T classification	T1/T2	25	4.0 [5.1]	0.78	13.0 [4.9]	0.02	22	2.5 [4.1]	0.46	11 [4.4]	0.01
	T3/T4	27	2.6 [5.2]		7.8 [6.4]		23	1.9 [4.3]		5.3 [5.4]	
N classification	N0	4	0.8 [4.9]	0.20	7.1 [4.7]	0.17	4	0.3 [3.3]	0.06	4.5 [3.0]	0.36
	N1	3	0.6 [2.2]		12.0 [8.3]		3	0.3 [0.7]		11 [6.5]	
	N2	36	4.8 [5.3]		12.0 [6.2]		30	3.0 [4.5]		9.0 [5.4]	
	N3	9	0.9 [4.5]		15.0 [4.7]		8	0.5 [3.1]		10 [5.2]	
Smoking	Never or Former	36	2.0 [4.3]	0.05	13.0 [5.6]	0.06	30	1.4 [3.1]	0.02	10 [5.1]	0.10
	Current	16	6.5 [6.1]		6.2 [6.2]		15	6.8 [5.0]		4.6 [5.2]	
Recur Group	Nonrecurrent	30	3.2 [5.0]	0.61	11.0 [5.7]	0.17	30	1.7 [3.6]	0.23	7.8 [5.2]	0.27
	Recurrent	22	5.1 [5.3]		14.0 [6.5]		15	2.4 [5.0]		11 [5.3]	
Recurrence Type	LocoRegional	9	5.8 [5.9]	0.45^	14.7 [5.8]	0.07^	6	6.5 [5.0]	0.05^	9.9 [5.8]	0.36^
	Any Distant	13	5.0 [5.0]	0.95^	13.7 [6.9]	0.62^	9	1.9 [4.9]	0.91^	11.0 [5.3]	0.43^

^ p-value compared to nonrecurrent group.

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Table 3

Median [std] of E6 and E7 AUDC ratios (to baseline) postCRT and latest timepoint/recurrence timepoint and clinical characteristics

			PostCRT Ratio					Latest Ratio			
Variable		**n**	**E6 Ratio**	**p**	**E7 Ratio**	**p**	**n**	**E6 Ratio**	**p**	**E7 Ratio**	**p**
Recur Group	Nonrecurrent	30	0.7 [0.3]	0.54	0.8 [14.7]	0.38	30	0.4 [0.4]	0.28	0.4 [0.8]	0.002
	Recurrent	15	0.7 [0.3]		0.7 [0.2]		17	0.5 [2.2]		0.7 [0.2]	
Recurrence Type	LocoRegional	6	0.7 [0.2]	0.95^	0.8 [0.1]	0.63^	8	0.5 [0.2]	0.44^	0.8 [0.2]	0.004^
	Any Distant	9	0.5 [0.3]	0.36^	0.7 [0.2]	0.41^	9	0.5 [3.0]	0.38^	0.6 [0.2]	0.06^

^p-value compared to nonrecurrent group.

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Table 4

Linear Mixed Model[1] Results for E6 and E7 Levels over Time

	E6		E7	
Effect	**β**	**p-value**	**β**	**p-value**
Time[2] (days)	−0.0000031	0.014	−0.0000058	0.0005
Recurrence[3] (Yes vs No)	0.0014	0.020	0.0025	0.0020

[1]Mixed effect linear model with fixed effects time (continuous) and recurrence, assuming compound symmetric variance/covariance structure for serial repeated measures.

[2]All patients in the cohort had a significant decrease in serum E6 and E7 antibodies over time as shown by a negative parameter (**β**)

[3]When comparing non-recurrent and recurrent patients, non-recurrent patients had a significant more clearance of antibody throughout the study period.