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Snail as a novel marker for regional metastasis in head and neck squamous cell carcinoma

Abie H. Mendelsohn, MD¹, Chi K. Lai, MD², I Peter Shintaku, PhD², Michael C. Fishbein, MD², Katherine Brugman, BS², David A. Elashoff, PhD³, Elliot Abemayor, MD, PhD^{1,5}, Steven M. Dubinett, MD^{4,5}, and Maie A. St. John, MD, PhD^{1,5}

¹Division of Head and Neck Surgery, Department of Surgery; Center, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

²Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

³Department of Biostatistics, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

⁴Division of Pulmonary and Critical Care Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

⁵Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

Abstract

OBJECTIVE—Previous studies have shown Snail expression integral to the epithelial-mesenchymal transition during tumor progression. However, its behavior in clinical head & neck squamous cell carcinomas (HNSCC) is yet undefined. We therefore sought to: (1) investigate clinical and histopathologic characteristics of Snail-positive HNSCC, and (2) understand the link between Snail and other commonly utilized HNSCC tumor markers.

STUDY DESIGN—Retrospective case-control study.

SETTING—Large-scale academic center.

STUDY SUBJECTS—Of 51 consecutive HNSCC, 42 surgical resections were included.

METHODS—Two separate pathologists performed standard histopathologic reviews along with immunohistochemistries (Snail, E-cadherin, p16, EGFR) and in-situ hybridization (HPV). Medical review for all cases was performed.

RESULTS—22 of 42 (52%) cases stained 4+ Snail (>75% staining). The remaining 20 cases were considered negative. Snail was strongly inversely related to E-cadherin expression ($\rho =$

To whom correspondence should be addressed: Abie Mendelsohn, MD, David Geffen School of Medicine at UCLA, 62-132 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095. Tel: (310) 825-5179; Fax: (310) 206-1393; AMendelsohn@mednet.ucla.edu.

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−0.69, $p < 0.001$), but statistically independent from HPV, p16, or EGFR expression. Snail-(+) tumors were equally represented from each anatomic subsite. Snail-(+) tumors were strongly associated with poor differentiation ($p < 0.001$) and basaloid classification ($p = 0.004$). Snail-(+) tumors were also strongly associated with lymphovascular invasion ($p = 0.02$), but not perineural invasion. Ultimately, 11/22 (50%) of Snail-(+) tumors demonstrated positive nodal metastasis and 11/14 (79%) node-positive cases were Snail-(+) ($p = 0.02$).

CONCLUSION—This pilot study provides promising evidence of Snail's role as a molecular prognostic marker for HNSCC. Snail positivity is significantly predictive of poorly differentiated, lymphovascular invasive, as well as regionally metastatic tumors. As Snail positivity appears independent of HPV, p16, and EGFR expression, Snail may prove to improve upon these markers' predictive limitations.

Introduction

Patients with head and neck squamous cell carcinoma (HNSCC) are at considerable risk of mortality, with more than 300,000 deaths attributable to the disease worldwide each year¹. Ultimately, the majority of HNSCC-related deaths are due to locoregional failures. In fact, cervical lymph node metastases reduce survival by 50%. Identification of molecular markers that can pinpoint patients with increased risk of regional spread will be of the utmost importance for effective management of HNSCC.

Inflammatory mediators are dysregulated in smokers and patients with tobacco-related malignancies such as HNSCC². A chronic increase in inflammatory mediators can lead to increased HNSCC promotion, invasion, and metastasis³. Inflammatory cytokines and mediators found in the tumor microenvironment include prostaglandin E2 (PGE₂) and interleukin-1 (IL-1). IL-1 has been shown to induce activation of signal transduction pathways that regulate several early transcription factors involved in proinflammatory cytokine genes. Its subtype, IL-1 β , is not only integral for inflammatory signaling, but has also been implicated in the progression of HNSCC. Increased secretion of IL-1 β has been shown within resistant or progressing oral tumors^{4,5}. IL-1 β is one of several cytokines known to potently up regulate COX-2 expression in a variety of cells⁶⁻⁹. Tumor COX-2 plays an important role in regulating diverse cellular functions within cancer cells¹⁰. Specifically within HNSCC, COX-2 has been closely correlated with increased Snail expression and subsequent E-cadherin suppression¹¹. Snail transcription factor appears to be a key regulator due to its ability to bind to the E-cadherin promoter and block its subsequent expression¹². Figure 1 presents a streamlined diagram of this pathway.

Loss of E-cadherin is frequently observed at sites of epithelial-mesenchymal transition (EMT) during cancer development and progression, and is closely correlated with poor prognosis¹³⁻¹⁶. While several E-cadherin transcriptional repressors have been characterized (ZEB1, Snail, E12/E47, Slug, Twist, and SIP-1), in HNSCC local recurrence following treatment have been correlated specifically with Snail up-regulation¹⁷.

Based on our previous *in-vitro* and *in-vivo* delineation of this pathway¹¹, the aim of this pilot research was to identify Snail as a clinical prognostic histopathologic marker within HNSCC. Currently, as Snail has yet to be characterized clinically in HNSCC, and its

predictive value along with its relationships to other histopathologic findings are completely unknown. Additionally, while Snail's *direct* cellular pathway has been described, we aim to define its connection with other unrelated HNSCC tumor markers. Based on initial study¹¹, we hypothesize that Snail will in fact show significant association with aggressive HNSCC features.

Methods

This study was approved by the UCLA Office of Protection of Research Subjects (IRB).

Design

Because Snail has not been studied clinically in HNSCC, an exploratory study was designed from which future power calculations could be designed. We set out to include approximately 50 patients from which adequate pilot data could be gathered. Therefore, within a 12-month period, consecutive squamous cell carcinoma cases of a senior Head & Neck pathologist (C.L.) were reviewed. Tumors involving the oral cavity, oropharynx, or laryngeal (including supraglottis, glottal, and post-cricoid) were included. This yielded 51 individual specimens. 5 cases had very little tissue remaining to perform further staining, 2 cases had no cellblock, and 2 cases had inadequate staining (n=42).

Immunohistochemistry

Tissue sections (4µm thick) were cut, deparaffinized in xylene, rehydrated in alcohols, and washed twice with water. Samples were then incubated in 0.01-mol/L citrate buffer (pH 6.0) for 25min in a steamer to unmask antigens. Following cooling and rinsing with dH₂O, samples were treated for 15min with 3% H₂O₂ diluted in methanol. Tissue sections were washed in dH₂O, then PBS, then blocked with 10% normal horse serum for 30min. Each tumor marker was stained on individual slides. For E-cadherin staining, the sections were incubated overnight at 4°C with 250µg/mL mouse anti-human E-cadherin diluted in normal horse serum (BD Transduction Biosciences). After extensive rinsing with PBS, samples were incubated for 40min with 7.5µg/mL horse anti-mouse IgG-biotin (Vector Laboratories) and rinsed with PBS. Samples were then incubated for 30min at room temperature with the Vectastain ABC- kit (Vector Laboratories), followed by PBS washing, and then incubated with an alkaline phosphatase substrate kit (Vector Laboratories). Color development was followed under the microscope for 20min. The color reaction was stopped by rinsing with dH₂O. Samples were counterstained with hematoxylin. Lung and Breast tumor specimens were used as a positive control for all immunohistochemistry staining. Negative controls included incubation with nonimmune pooled rabbit or goat IgG (rabbit IgG, Vector Laboratories; goat IgG, Zymed, Invitrogen) at the same concentration as the primary antibody. Goat anti-human Snail polyclonal IgG (1:50 dilution; Abcam) was utilized for Snail immunohistochemistry. p16 (prediluted mouse monoclonal, mtm laboratories, Westborough, MA) antigen retrieval involved heating at 95° C in 0.001M EDTA, pH 8.0 for 25min in vegetable steamer, followed by a 15min cool down and rinse in 0.05M Tris buffered saline with Tween 20 (TBST). For EFGR (1/50 dilution of mouse monoclonal H11, Biocare Medical, Concord, CA), antigen retrieval involved enzymatic digestion with Proteinase K (DAKO, Carpinteria, CA) for 7min followed by washing in TBST. Following

5min blocking with Ultra V Block (Lab Vision/ThermoScientific, Fremont, CA), primary antibodies were incubated for 30min and coupled with Ultravision Value Polymer Detection System from LabVision/ThermoScientific with diaminobenzidine as chromogen. Slides were counterstained with Hematoxylin (Harris). Immunostaining was performed on DAKO Autostainer.

All slides were reviewed by two of the investigators (MF and CL). Each slide was graded for percent cells positive for each stain, and was subsequently scaled from (1+ to 4+). We examined the relationship between E-cadherin and Snail using the ordinal immunohistochemistry results (0+, 1+, etc.) and found that they were significantly negatively correlated ($\rho = -0.69$, $p < 0.001$). Subsequently, staining was dichotomized into positive (3+,4+) and negative (1+,2+) groups. The exception was Snail staining which was dichotomized into positive (4+) and negative (1+,2+,3+) groups in order to increase test specificity.

in situ Hybridization

4 μ m sections were baked for 15 minutes at 60 C and then labeled and programmed on Ventana XT autostainer to stain for HPV High Risk (genotypes 16,18,31,33,35,39,45,51,52,56,58,66), DNA control probes using ISH IVIEW Blue Plus detection protocol. HPV staining was documented as either positive or negative.

Clinical Review

Only following the completion of histopathology, the 42 clinical charts were reviewed systematically. Pathologic TNM AJCC staging was used for all primary tumor sites. Nodal staging was documented as “best of” from pathologic, radiologic, then clinical.

Statistics

Descriptive statistics were derived for all analyzed variables. A p-value < 0.05 was required for significance. Cross-tabulation analyses were performed using Pearson Chi Squared (for tables with values > 5) and Fisher Exact (for tables with values < 5) tests. Tests of mean were performed with unpaired t-tests. (SYSTAT 11, Chicago, IL)

Results

The division between the Snail-(+) group ($n = 22$) was equivalent to the Snail-(-) group ($n = 20$). Table 1 analyzes variables stratified between Snail-(+) and Snail-(-) tumors. Both groups demonstrated similar clinical characteristics including equivalent distribution of age, gender, number of patients with history of smoking and alcohol use, immunosuppressed patients, and patients with family histories of cancer. Additionally, Snail staining groups consisted of a statistically similar combination of HNSCC subsites. Unlike other HNSCC tumor markers (i.e. HPV), Snail was well represented in each subsite. Of the aggressive histopathologic factors analyzed, only perineural invasion was not significantly associated with Snail-(+) tumors. Lymphovascular invasion, poor tumor differentiation, and basaloid classification were all significantly associated with Snail-(+) tumors. When comparing Snail

expression to the selection of current HNSCC tumor markers (p16, HPV, EGFR), no association could be found.

Qualitatively, Snail immunohistochemistry revealed the well-differentiated HNSCC concentrated staining at the periphery of tumor lobules, with weaker to negligible staining within the center. The poorly differentiated HNSCC exhibited more diffuse staining. Intracellularly, Snail stained only within the nuclei. Appearance of cytoplasmic staining is due to background discoloration and was present within control slides. Examples of 1+ Snail staining (Figure 2) and 4+ Snail staining (Figure 3) are presented.

Subsequently, our entire cohort was divided into node-negative (N0) and node-positive (N+) groups. Predictive factors are stratified and analyzed in Table 2. Analysis by primary tumor site shows that there was a significant ($p=0.01$) increase of nodal involvement from oropharyngeal tumors. However, advanced T-stages did not display a significant incidence of nodal disease. The same aggressive histopathologic features which were associated with Snail-(+) tumors were also significantly correlated with an increased incidence of nodal metastasis, including lymphovascular invasion ($p=0.03$), poor tumor differentiation ($p=0.001$), and basaloid classification ($p=0.001$). Snail was subsequently demonstrated to be significantly ($p=0.02$) associated with nodal disease. The only other tumor marker which demonstrated a significant association was HPV ($p=0.002$); however, this finding was only seen within the small subgroup of HPV-(+) patients ($n=5$).

Due to the previously established inverse relationship between E-cadherin and Snail protein expression¹¹, a subset of these tumors were stained for E-cadherin ($n=20$). A significant association ($p=0.02$) was found between E-cadherin negative tumors and positive nodal metastasis, which is similar to the relationship between Snail and nodal metastasis. However, E-cadherin's relationship with poor tumor differentiation was not as strongly significant ($p=0.02$, compared to Snail $p<0.001$). Additionally, E-cadherin expression was completely without significant relationship with lymphovascular invasion ($p=0.33$), perineural invasion ($p=0.12$), or basaloid subtype ($p=0.24$). Therefore Snail expression more accurately predicts aggressive tumor behavior, and nodal metastasis, while remaining inversely correlated with E-cadherin expression. E-cadherin's limited clinical utility has been supported elsewhere as well, with E-cadherin having poor predictive value for tumor grade or metastasis in HNSCC^{18,19}.

Preliminary logistic regression model curves were configured using the primary outcome of positive nodal metastasis. While the Odds-Ratios for each factor varied depending on the variables included in each model, poor tumor differentiation significantly ($p = 0.01$) affected nodal outcomes when modeled with lymphovascular invasion or perineural invasion. When determining the Area-Under-the-Curve (AUC) of these regression curves, the addition of Snail demonstrated a modest increase in the predictive power (2-4%). In contrast, neither lymphovascular invasion or perineural invasion alone were able to affect the AUC levels.

Discussion

In HNSCC tumorigenesis, many distinct cellular pathways are altered. Our study focuses on a pathway which is now evolving as a critical portion of tumor progression. Initially, IL- β is increased during periods of inflammation. It then activates COX-2 which produces PGE₂. This COX-2 activation in turn increases Snail expression. Snail in turn binds to the promoter region of E-cadherin blocking its expression. This pathway is thought to be critical in EMT and subsequent aggressive HNSCC behavior¹⁷. However Snail's role as a marker for HNSCC, to this point, has been undefined. Our study therefore specifically focused on the clinical and histopathologic changes seen with Snail over-expression.

Smoking and alcohol are the most well known risk factor for development of HNSCC. While there are a number of established carcinogens specifically incorporated in cigarette smoke, the chronic inflammatory impact also provides a mechanism for tumorigenesis. Chronic inflammatory change has been linked with the up regulation of proinflammatory mediators (i.e. Interleukins, Cytokines), and has been directly observed in HNSCC cells². Treatment of HNSCC cells with IL- β has lead to a direct increase in COX-2 and Snail expression¹¹. The inverse relationship has also been described in oral squamous cells with Snail-(+) cells up-regulating cytokines and COX-2²⁰. Herein, we find that patients with Snail-(+) HNSCCs displayed a marked increase in mean duration (8.2 years) of smoking, although the this figure failed to reach significance.

Histopathologically, Snail-(+) HNSCC tumors were associated with highly aggressive features (poor tumor differentiation, basaloid classification). To understand the Snail-(+) tumor biology, it must be analyzed in context of its cellular pathway. Herein and previously¹¹, we have demonstrated the reciprocal expression of Snail and E-cadherin. In select HNSCC studies, loss of E-cadherin has led to significantly poorer differentiation²¹ and decreased survival²². However, on our preliminary E-cadherin analysis we have not seen a relationship with aggressive histopathologic features. This finding has been supported by studies of E-cadherin as a HNSCC tumor marker, which have not been able to predict tumor differentiation¹⁹. We therefore propose that Snail expression is a more accurate maker for tumor behavior than E-cadherin. We can also begin to demonstrate Snail's clinical utility in its association with aggressive, and therefore prognostic²³, histopathologic features such as poor tumor differentiation, and particularly, basaloid classification. Within poorly differentiated tumors, basaloid subtyping has distinctive aggressive histological characteristics²⁴. Compared with other subtypes of poorly differentiated HNSCC, basaloid tumors are more resistant to radiation therapy²⁵. Within our cohort, every basaloid tumor demonstrated Snail over-expression.

Lymphovascular invasion also demonstrated a strong association with Snail-(+) HNSCC tumors. With Snail overexpression, E-cadherin's normal intercellular binding function is lost. When E-cadherin is down regulated, tumors increase their capacity to invade and metastasize^{21,22}. Increased Snail activity suppresses E-cadherin expression and its subsequent cellular function. It is therefore not surprising that Snail-(+) cells are highly invasive. When tumor cells invade the lymphovascular space it is accepted that this is a poor prognostic indicator. Lymphovascular invasion increases regional metastasis²⁶, which in turn

impacts survival. We suggest that, as demonstrated in this initial study, Snail-(+) tumors may be useful in distinguishing the truly aggressive HNSCCs. This distinction may not only aid in prognostic information, but as Snail can be a surrogate for the COX-2 pathway, future COX-2 specific therapies may be uniquely dispensed to responsive tumors.

We also discovered that the incidence of nodal metastasis is increased in Snail-(+) HNSCCs. Based on the higher rate of lymphovascular invasive tumors, it should be expected that Snail-positivity should lead to regional metastasis, and in fact this was demonstrated. Within other carcinoma studies, Snail overexpression has been associated with the lymph node status and/or invasiveness of various carcinomas, as well as local recurrences²⁷⁻²⁹. Herein, we have shown for the first time that Snail-positivity is significantly associated with nodal disease in HNSCC. The only previous clinical study clinically demonstrated Snail's clinical nodal prognostic capacities did so in a much smaller study (n=21) involving differing breast carcinoma subtypes²⁸. Prognostic information on nodal status is a critical step in HNSCC treatment as locoregional failure is the main cause of mortality. Current treatment of cervical lymph nodes is founded on ill-defined percentages calculated by a tumor's primary site as well as a mixture of histopathologic findings. Improved outcomes and decreased patient morbidity can result from a tumor marker that can be utilized to achieve a targeted assessment of the metastatic potential of individual HNSCCs. In the present study, only three N+ tumors were Snail(-). As the overwhelming majority of nodal metastasis originated from Snail-(+) tumors we can strongly support the Snail's role in HNSCC metastatic changes. While the logistic regression data from this pilot study cannot conclusively establish Snail as a clinical prognosticator, it represents a strong foundation suggesting Snail can enhance our prognostic capabilities.

Herein we also report that Snail staining was independent of individual HNSCC tumor subsites. Prior to the present study, the clinical behavior and distribution of Snail overexpression was unknown. It may have been postulated that like HPV staining, which is found almost exclusively in oropharyngeal HNSCCs, Snail-(+) tumors would be limited to only one of the tumor subsites. Such limitations would likely limit Snail's clinical applicability. However, Snail-(+) tumors are found with similar frequencies along the upper aerodigestive tract. It could also be postulated that the multifactorial tumorigenesis of HNSCC could lead to multiple tumor marker over-expression. This idea was explored using three of the more commonly used HNSCC tumor markers (p16, HPV, EGFR). Snail overexpression was found to be separate and distinct from these other tumor markers. Specifically, as seen by HPV infection, which is unrelated to smoke exposure, Snail overexpression was found both in HPV-(+) and HPV(-) tumors. The freedom from both tumor subsite limitation and alternate tumor marker expression will allow for greater clinical applicability across a wide spectrum of HNSCC patients and tumors.

It is the metastatic potential of Snail-(+) HNSCC which is likely to have the largest clinical impact. Other similar investigations have searched through numerous markers as metastatic prognosticators. Specifically E-cadherin immunohistochemistry was shown to be diffusely positive and was not useful in prognosis¹⁹. While herein E-cadherin is associated with nodal metastasis it lacks association with aggressive histopathologic findings. Herein we show for the first time a significant association between Snail-(+) HNSCC and nodal metastasis.

Another important clinical implication of Snail-(+) immunohistochemistry relates to pathologic margin status. As seen in the present study and previously^{11,12}, Snail staining is found predominantly within the periphery of tumor growth. This finding may prove useful in assessing the true margins of the tumor. Prior studies have shown tumor markers to improve standard histopathologic margin status, which subsequently affects risk of tumor recurrence³⁰. Whether Snail staining can impact the diagnosis of challenging pathologic margins requires additional study.

There are limitations to performing a pilot study of this nature, namely the limited sample size. However, the study was able to reach significance on the main outcome variable of lymph node metastasis. The retrospective nature of the study contributes to the heterogeneous reporting of nodal stages. While every patient had accurate nodal staging documented, there was variability in the staging methodology. A uniform system would allow for more conclusive results. Currently, a large-scale prospective study is being initiated to correct for these shortcomings.

Conclusion

The present study is the first clinical investigation attempting to define Snail's clinical role in HNSCC. We have clearly demonstrated that Snail over-expression is significantly associated with regional metastasis and poor histopathologic findings in HNSCC. Associated histopathologic features such as lymphovascular invasion, poor tumor differentiation, and basaloid classification support Snail's role in HNSCC progression. Snail-(+) tumors also demonstrate significant association with nodal metastasis which corresponds to the aggressive histopathologic features of Snail-(+) tumors. We have also corroborated Snail's inverse correlation with E-cadherin, while demonstrating Snail's improved clinical utility over E-cadherin. The independence of Snail expression from HNSCC tumor subsites or from other common tumor markers supports a clinical role for Snail staining. Snail staining would allow head and neck surgeons to aggressively treat those patients most at risk for cervical metastasis. Additionally, as Snail is a surrogate for the COX-2 pathway, Snail-(+) HNSCCs may allow for targeted pharmacotherapy, such as COX-2-Inhibitors. Further large-scale studies are required to substantiate these encouraging findings.

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Figure 1. Proposed cellular pathway

Chronic inflammation increases interleukin-1-beta, which increases COX-2 activation. The COX-2 metabolite, prostaglandin-E2, leads to increased Snail expression, which actively blocks the promoter region of E-cadherin.

Figure 2. HNSCC Snail immunohistochemistry – 1+

This specimen exhibits islands of well-differentiated HNSCC (original magnification-100X) exhibiting 1+ (<25%) staining with more pronounced snail expression staining at the periphery of the lobules.

Figure 3. HNSCC Snail immunohistochemistry – 4+

This specimen exhibits infiltrating clusters of poorly differentiated squamous cell carcinoma (original magnification-100X) exhibiting 4+ (>75%) staining.

Table 1
Variable Analysis for Snail Positive Tumors

Clinical factors are free from snail staining associations. Histopathologic factors associate Snail-positivity with aggressive features including Basaloid subtype, Poor Tumor Differentiation, and Lymphovascular Invasion. There are no significant associations between the selected tumor markers and snail staining.

	Snail (-) n=20	Snail (+) n=22	p value
Clinical Factors			
Age – mean (SD)	64.8 (15.9)	63.0 (15.1)	0.70
Gender			0.87
Female	5	6	
Male	15	16	
Smoker	12	12	0.72
Pack Years – mean (SD)	21.4 (15.6)	29.6 (17.8)	0.25
Alcohol	5	6	0.87
Immunosuppression	3	4	0.78
Family History	6	9	0.46
Primary Site			0.82
Oral Cavity	11	11	
Oropharynx	3	5	
Laryngeal	6	6	
Histopathologic Factors			
Type of SCCA			0.004
Keratinizing	20	14	
Basaloid	0	8	
Tumor Grade of Differentiation			<0.001
Well to Moderate	20	10	
Poor	0	12	
Lymphovascular Invasion	3	11	0.02
Perineural Invasion	9	14	0.23
Tumor Markers			
p16 (+)	3	7	0.28
HPV (+)	1	4	0.35
EGFR (+)	13	15	0.83

Table 2
Variable Analysis for Nodal Metastasis

A significant association is demonstrated between oropharyngeal tumors and positive nodal disease. All aggressive histopathologic factors except for perineural invasion are significantly associated with nodal metastasis. Only Snail and HPV are significantly associated with positive nodal disease.

	N0 <i>n</i> =28	N+ <i>n</i> =14	<i>p</i> value
Clinical Factors			
Primary Site			0.01
Oral Cavity	18	4	
Oropharynx	2	6	
Laryngeal	8	4	
T-Stage			0.06
T ₁	6	0	
T ₂	6	7	
T ₃	1	2	
T ₄	15	5	
Histopathologic Factors			
Type of SCCA			0.001
Keratinizing	27	7	
Basaloid	1	7	
Tumor Grade of Differentiation			0.001
Well to Moderate	25	5	
Poor	3	9	
Lymphovascular Invasion	6	8	0.03
Perineural Invasion	13	10	0.13
Tumor Markers			
Snail (+)	11	11	0.02
p16 (+)	4	6	0.06
HPV (+)	0	5	0.002
EGFR (+)	19	9	0.82