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p16 expression in cutaneous squamous cell carcinoma of the head and neck is not associated with integration of high risk HPV DNA or prognosis

Laveniya Satgunaseelan
Royal Prince Alfred Hospital

Noel Chia
University of New South Wales

Hyerim Suh
University of New South Wales

Sohaib Virk
University of New South Wales

Bruce Ashford
University of Wollongong, Illawarra Shoalhaven Local Health District, Chris O'Brien Lifehouse, bga095@uowmail.edu.au

See next page for additional authors

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Abstract
Head and neck cutaneous squamous cell carcinoma (HNcSCC) can present with cervical metastases without an obvious primary. Immunohistochemistry for p16 is established as a surrogate marker of human papillomavirus (HPV) in oropharyngeal cancer. p16 expression in HNcSCC needs to be elucidated to determine its utility in predicting the primary site. The aim of this study was to evaluate the rate of p16 expression in HNcSCC and its association with prognostic factors and survival. p16 immunohistochemistry was performed on 166 patients with high risk HNcSCC (2000-2013) following histopathology review. Chromogenic *in situ* hybridisation (CISH) for HPV was performed. Fifty-three (31.9%) cases showed strong, diffuse nuclear and cytoplasmic p16 expression including 14 (41%) non-metastatic and 39 (29.5%) metastatic tumours ($p = 0.21$). HPV CISH was negative in all cases. p16 expression significantly increased with poorer differentiation ($p = 0.033$), but was not associated with size ($p = 0.30$), depth of invasion ($p = 0.94$), lymphovascular invasion ($p = 0.31$), perineural invasion ($p = 0.69$), keratinisation ($p = 0.99$), number of involved nodes ($p = 0.64$), extranodal extension ($p = 0.59$) or survival. Nearly 32% of HNcSCCs, particularly poorly differentiated HNcSCCs, show p16 expression. A primary HNcSCC should be considered in p16 positive neck node metastases in regions with high prevalence of HNcSCC. p16 expression is not associated with improved survival in HNcSCC.

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Laveniya Satgunaseelan, Noel Chia, Hyerim Suh, Sohaib Virk, Bruce Ashford, Trina Lum, Marie Ranson, Jonathan Clark, and Ruta Gupta

p16 Expression in Cutaneous Squamous Cell Carcinoma of the Head and Neck is Not Associated with Integration of High Risk HPV DNA or Prognosis.

Laveniya Satgunaseelan[a], Noel Chia[b], Hyerim Suh[b], Sohaib Virk[b], Bruce Ashford[c,d,e,g], Trina Lum[a], Marie Ranson[c,d], Jonathan Clark[f,g], Ruta Gupta[a,f]

[a]Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Sydney NSW Australia,
[b]University of New South Wales, Sydney NSW Australia,
[c]University of Wollongong, Wollongong NSW Australia,
[d]Illawarra Health and Medical Research Institute, Wollongong NSW Australia,
[e]Illawarra Shoalhaven Local Health District, Wollongong NSW Australia,
[f]Central Clinical School, University of Sydney, Sydney NSW Australia,
[g]Sydney Head and Neck Cancer Institute, Chris O'Brien Lifehouse, Sydney NSW Australia.

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Corresponding Author:
A/Prof Ruta Gupta MBBS, MD, FRCPA
Staff Specialist, Department of Anatomic Pathology and Diagnostic Oncology,
Royal Prince Alfred Hospital,
Building 94, John Hopkins Drive
Camperdown, NSW 20150
Australia
P: +61295158354. F: +61295158405 E. Ruta.Gupta@sswahs.nsw.gov.au

Abstract

Background: Head and neck cutaneous squamous cell carcinoma (HNcSCC) can present with cervical metastases without an obvious primary. Immunohistochemistry for p16 is established as a surrogate marker of human papillomavirus (HPV) in oropharyngeal cancer. p16 expression in HNcSCC needs to be elucidated to determine its utility in predicting the primary site.

Objectives: To evaluate the rate of p16 expression in HNcSCC and its association with prognostic factors and survival.

Methods: p16 immunohistochemistry was performed on 166 patients with high risk HNcSCC (2000-2013) following histopathology review. Chromogenic in situ Hybridisation (CISH) for HPV was performed.

Results: 53 (31.9%) cases showed strong, diffuse nuclear and cytoplasmic p16 expression including 14 (41%) non-metastatic and 39 (29.5%) metastatic tumours (p=0.21). HPV CISH was negative in all cases. p16 expression significantly increased with poorer differentiation (p=0.033), but was not associated with size (p=0.30), depth of invasion (p=0.94), lymphovascular (p=0.31), perineural invasion (p=0.69), keratinization (p=0.99), number of involved nodes (p=0.64), extranodal extension (p=0.59) or survival.

Conclusion: Nearly 32% of HNcSCCs, particularly poorly differentiated HNcSCC, show p16 expression. A primary HNcSCC should be considered in p16 positive neck node metastases in regions with high prevalence of HNcSCC. p16 expression is not associated with improved survival in HNcSCC.

Key words: p16, high risk cutaneous squamous cell carcinoma, HPV

Introduction

The incidence of head and neck cutaneous squamous cell carcinoma (HNcSCC) is increasing with the aging population in regions with high solar ultraviolet (UV) index. HNcSCC is known to present with cervical metastases without an obvious primary site. Immunohistochemistry (IHC) for p16 is routinely performed in the setting of nodal metastases and the presence of p16 expression is frequently used as a surrogate marker for human papilloma virus (HPV) and an indicator of an oropharyngeal primary [1]. The recent American Joint Commission on Cancer (AJCC) staging system also recommends that p16 positive cervical neck node metastases be staged as per the nodal staging system for oropharyngeal carcinomas[2].

There is increasing data demonstrating that p16 expression is also driven by mechanisms unrelated to HPV, and is observed in other head and neck malignancies such as oral squamous cell carcinoma [3]. Moreover, there is evidence that UV radiation and immune suppression, both of which are associated with increased risk of cutaneous squamous cell carcinoma of the head and neck (HNcSCC) can also lead to p16 upregulation [4]. However, the incidence of p16 expression or its association with the conventional prognostic factors and survival outcomes is not well established in the context of HNcSCC. Thus, the aim of this study is to evaluate the incidence of p16 expression in high risk HNcSCC, both in the primary and the nodal metastases and analyse its association with conventional prognostic factors and survival outcomes.

Material and methods

A cohort of 166 patients with high risk HNcSCC, defined as per 7th edition of AJCC criteria [5] who had undergone primary surgical treatment with curative intent for HNcSCC from

2000-2013 were identified from a prospective clinicopathological database maintained by the Sydney Head and Neck Cancer Institute following approval from the institutional human research ethics committee (X14-0231 & HREC/14/RPAH/301). This cohort includes patients with non-metastatic high-risk HNcSCC (N=34) and patients with concurrent primary and metastatic high risk HNcSCC (N= 132). Patients without archival slides and blocks or detailed clinical follow up were excluded from the study.

Clinicopathologic data

Clinical data included the patient's age, gender, date of surgery, type of surgery and adjuvant therapy. Clinical follow-up information documenting local or regional recurrence, metastases or death was also obtained.

Histopathological analysis

Archival slides of the patients were analysed for histopathological data, which included the tumour size, depth of invasion, pattern of invasion, tumour differentiation (based on assessment of the architecture, presence of keratinisation, nuclear pleomorphism, and proliferation rate [6]), keratinisation, lymphovascular involvement, perineural invasion, number of involved lymph nodes and extranodal extension. The histopathological evaluation was performed prior to the tissue microarray construction and p16 immunohistochemistry.

Tissue microarray construction

Tissue microarrays were constructed using Beecher Manual Tissue Microarrayer (Model MTA-1, Diagnostic Technology). Two cores of 1.0mm diameter were extracted from formalin fixed paraffin embedded tissue blocks of the primary as well as the metastatic HNcSCC as applicable. Using the archival slide as a guide, areas of high cell density were

selected. Areas with hemorrhage, necrosis, excessive keratin deposition or tissue damage due to diathermy were avoided.

Immunohistochemistry to determine p16 expression

Tissue sections from the TMAs were cut at 3µm thickness onto charged SuperFrost Ultra Plus slides (Menzel-Glaser, Thermo Fisher Scientific). p16 immunohistochemical staining was performed on Leica-Bond III autostainer using pretreatment ER2 (epitope retrieval buffer) for 30 minutes. Following heat-induced epitope retrieval with an EDTA based buffer, pH 9 (Leica Microsystems, VIC, Australia), tissue sections were incubated with a ready-to-use Mouse anti-Human p16 (E6H4) primary antibody (CINtec Histology Kit, Roche Ventana, Tucson, AZ). Biotin-free Polymer Refine Detection Kit (Leica Microsystems, VIC, Australia) was used for p16 protein detection. The reaction product was visualised with 3, 3'-diaminobenzidine chromogen and enhanced with DAB enhancer before counterstaining with haematoxylin.

Whole sections of primary and metastatic cSCC were also stained with p16 in a similar manner in 51 cases to identify the heterogeneity if any of p16 expression in cSCC.

A positive p16 status was defined as diffusely strong nuclear and cytoplasmic staining for p16 in more than 70% of the tumour cells [7] (Figure 1).

HPV Chromogenic In-Situ Hybridisation (CISH)

DNA *in situ* hybridization was performed on 3-µm TMA sections using the fully automated Benchmark Ultra staining platform (Ventana Medical Systems, Tucson, AZ) using Inform ISH iView Blue Plus Detection Kit and Red Counterstain II, (Ventana, Tucson AZ) according to the manufacturer's instructions. The assay utilised the Ventana HPV III Family

16 ISH Probe cocktail, which hybridised with high risk HPV genotypes including 16, 18, 31, 33, 35, 45, 51, 52, 56, 58 and 66.

Either large, homogenous, navy blue precipitate (episomal pattern) or discrete, stippled navy blue dots (integrated pattern) were required within the nuclei of malignant cells for scoring the cases as positive.

Appropriate negative and positive controls of cervical intraepithelial neoplasia (CIN3) and tonsillar squamous cell carcinoma were included for both p16 IHC and HPV CISH.

Statistical analysis

Statistical analysis was undertaken to determine the association of p16 status in cSCC and outcome using SPSS version 22.0 (IBM, Armonk, NY). Analysis of p16 status and categorical data such as demographic and clinicopathologic factors was conducted using a chi-square test. The Mann-Whitney U test was used to analyse non parametric data. A two sided p-value of less than 0.05 was statistically significant.

Survival curves were constructed using the Kaplan-Meier product-limit method for overall survival, disease specific survival and disease free survival by p16 status. Disease free survival was calculated from the date of the surgery to the date of the first disease recurrence, death or the most recent follow up date if there was no recurrence. The overall survival was from the date of the surgery to the date of the patient's death or the most recent follow up. Survival analysis was performed using the log-rank test.

Results

The study population included a total of 166 patients, the majority of which are males (87%) with a median age of 74.1 years. 34 patients had non-metastatic high risk HNcSCC while 132 patients had metastases to cervical lymph nodes, intraparotid nodes or both. 88 patients received adjuvant radiotherapy. The median follow-up was 1.8 years (0.002 – 16.1 years). Local recurrence was observed in 11 (7%) patients, regional recurrence was noted in 13 (8%) patients, while 3 (2%) developed distant metastases. 69 (41.6%) deaths were recorded, of which 18 (10.8%) were due to HNcSCC. Table 1 summarises the relevant clinicopathological parameters.

p16 expression

Strong and diffuse nuclear and cytoplasmic immunostaining with p16 was observed in 53 (32%) of the cases, these included 14 (41%) patients with non-metastatic HNcSCC and 39 (29.5%) patients with metastatic cSCC. The rate of p16 expression in the metastatic versus non-metastatic groups was not significantly different (p=0.20). (Table 2).

Of those primary tumours with p16 expression, their corresponding metastases also showed p16 staining with IHC, thus indicating that p16 expression is retained in all metastatic tumours.

Whole sections from 51 tumours (48 primary and 3 metastatic) were stained to exclude the possibility of heterogeneity in p16 expression. A concordance of 98% was observed between the whole sections and their TMA counterparts.

p16 expression and high risk HPV integration

Integration of high risk HPV was not observed in any cases of HNcSCC in this cohort indicating that p16 expression in HNcSCC is unrelated to high risk HPV.

p16 expression and histopathologic characteristics

Patients who were p16 positive were significantly more likely to have poorly differentiated tumours (p=0.033). However p16 expression was not associated with tumour size (p=0.30), depth of invasion (p=0.94), lymphovascular (p=0.31), perineural invasion (p=0.69) or keratinization (p=0.99). Amongst the patients with metastases, p16 expression was not associated with the number of involved nodes (p=0.64) or presence of extranodal extension (p=0.59).

p16 expression and survival

p16 expression was not associated with overall (p=0.60), disease free (p=0.86) or disease specific survival (p=0.81) on univariable analysis. (Figures 2A to 2C).

p16 expression and adjuvant therapy:

Adjuvant radiotherapy was given to 88 (53%) patients in this cohort. Of these 27 (31%) showed strong diffuse p16 expression.

There was no significant difference in number of patients who received adjuvant radiotherapy between p16 positive (50.9%) and p16 negative (53.9%) groups. Also, there was no significant difference in the disease free (p=0.56), disease specific (p=0.81) and overall survival (p=0.42) amongst the p16 positive and negative patients receiving radiotherapy.

Discussion

The current study including 166 cases of HNcSCC demonstrates that 32% of HNcSCC show strong diffuse nuclear and cytoplasmic immunostaining with p16. None of the cases showed

integration of high risk HPV. p16 expression was significantly more frequent in poorly differentiated tumours, but was not associated with any other conventional prognostic factors or disease free or disease specific survival.

There are limited studies evaluating the incidence of p16 expression in HNcSCC. Beadle et al. and McDowell et al. report a similar incidence of 30% in their studies of 27 and 143 cases of HNcSCC respectively [8,9]. p16 expression was not associated with a specific demographic profile as has also been described by Kusters-Vandevelde et al. in their study of cSCC from all sites of the body [10].

p16 expression was significantly more frequent in poorly differentiated HNcSCC. Most poorly differentiated HNcSCC tend to show a basaloid appearance due to minimal cytoplasm and keratinization and closely mimic oropharyngeal cancers as has also been described by Nilsson and Burnworth [11,12]. This finding is of particular importance in patients who present with metastases of unknown primary in the neck nodes, particularly in communities with relatively high incidence of HNcSCC. The patient is often unaware of or may not recollect having a primary cutaneous lesion and histologically the metastatic carcinoma closely mimics oropharyngeal carcinomas in appearance. There is no substitute for a detailed clinical history and comprehensive clinical and radiological examination. However, our data and that of McDowell et al. indicate in-situ hybridization (ISH) testing for HPV can be a useful adjunct in this context as metastases from non-oropharyngeal sites, such as HNcSCC and oral cavity SCC do not show high risk HPV integration [8,13].

The presence of p16 expression in cSCC can be largely attributed to non-HPV factors such as UV radiation exposure analogous to the aberrant p53 expression which has been well

characterized as a 'UV-type mutation' [11,14]. It is postulated that UV radiation induces keratinocytes to upregulate p16 as an adaptive mechanism to prevent damaged cells from proliferating. This action is mediated by cyclin dependent kinase inhibitors including p16 which hypophosphorylate retinoblastoma (Rb) protein and prevent transcription of genes required for cell cycle progression. [10,15,16]. As is well known, the dysregulation of Rb, whose gene is located on long arm of chromosome 13 (13q14.2) can occur via one of two mechanisms. The HPV mechanism is well described, where the E7 oncoprotein of HPV competitively binds to Rb. The other mechanism is via chromosomal instability, in particular loss of heterozygosity at 13q14, which has been characterized in cutaneous SCC [17]. Non-HPV mechanisms leading to p16 overexpression have been described. These mechanisms include the TP53 gene, where silencing of the gene in embryonic carcinoma stem cells results in upregulation of CDKN2A/P16INK4A (the gene which encodes the p16 protein)[18]; induction of p16 mRNA by microRNA (miR-877-3p) as shown in bladder carcinoma[19]; as well as MUC4-modulated cellular senescence pathways, which have found to be p16-dependent in HNSCC[20]. Furthermore, Nindl et al. described higher frequency of mutations in CDKN2A in metastatic cSCCs as compared to primary tumours [8,15]. It appears that p16 plays critical roles in growth arrest and senescence of tumour cells. Thus, given the protective role of p16 upregulation in carcinogenesis, it is biologically reasonable to speculate that the presence of p16 expression would be more frequent in smaller, early stage tumours and loss of p16 expression would be more frequent in larger and metastatic HNcSCC. In our cohort, the rate of p16 expression was higher in the non-metastatic group (41% vs 29.5%), though this did not reach statistical significance due to the smaller number of non-metastatic high risk primaries in this study. Also, p16 expression was not associated with tumour size, depth of invasion, number of nodal metastases, extracapsular spread or survival in our cohort. McDowell et al. reported similar findings in their study of 143 cases of

HNcSCC [8]. This may be attributed to the generally favorable prognosis of locally metastatic HNcSCC or the mutational profile of HNcSCC [21] . It is well established that cSCC harbours a significantly high number of somatic mutations [12,22]. It is plausible that p16 is rendered a less reproducible marker of prognosis in this complex mutational landscape.

Radiotherapy plays a vital role in locoregional control of HNcSCC, resulting in lower rates of locoregional recurrence and improved disease-free survival rates, as compared to surgery alone [23,24]. There was no difference in the incidence of adjuvant radiotherapy amongst p16 positive and negative patients. Furthermore, there was no difference in overall survival in p16 positive and negative patients amongst those receiving radiotherapy in this cohort. Thus in contrast to oropharyngeal SCC, p16 expression in HNcSCC is unlikely to predict better response to therapy [1].

The 8th edition of AJCC recommends that p16 positive lymph node metastases of SCC should be staged as per the staging recommendations for oropharyngeal carcinoma The staging [2] system does not make recommendations regarding the possible sites of the involved nodes [2]. This would lead to under-staging nearly 32% of patients with metastatic HNcSCC. The treatment implications would be more significant in the cohort of patients who present with neck node metastases and HNcSCC of unknown primary. Testing for HPV by ISH will be critical in this context as metastatic p16 positive squamous cell carcinomas of cutaneous or oral origin do not demonstrate integration of high risk HPV.

The findings of our study emphasise the incidence of p16 expression in HNcSCC and highlight its lack of association with better prognosis. The incidence of HNcSCC is on the rise with the aging population in several countries and thus awareness of the incidence of p16

expression in HNcSCC is critical. These factors need to be considered while managing patients with metastatic squamous cell carcinoma to the neck, particularly in areas with high incidence of cSCC.

FIGURE LEGENDS

1A) Moderately differentiated HNcSCC (H&E x100)

1B) Corresponding p16 immunohistochemistry interpreted as positive

2A) Kaplan-Meier analysis of overall survival (years) and p16 expression in HNcSCC

2B) Kaplan-Meier analysis of disease free survival (years) and p16 expression in HNcSCC

2C) Kaplan-Meier analysis of disease specific survival (years) and p16 expression in HNcSCC

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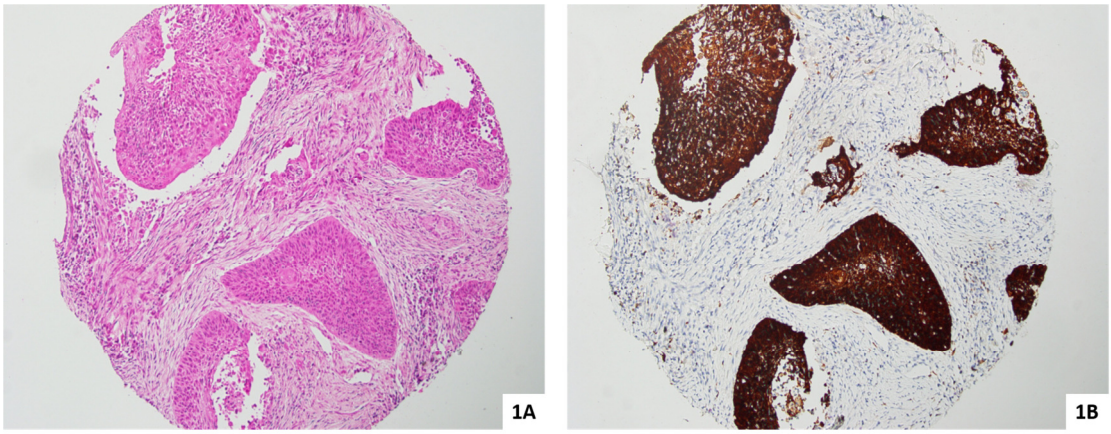

Figure 1

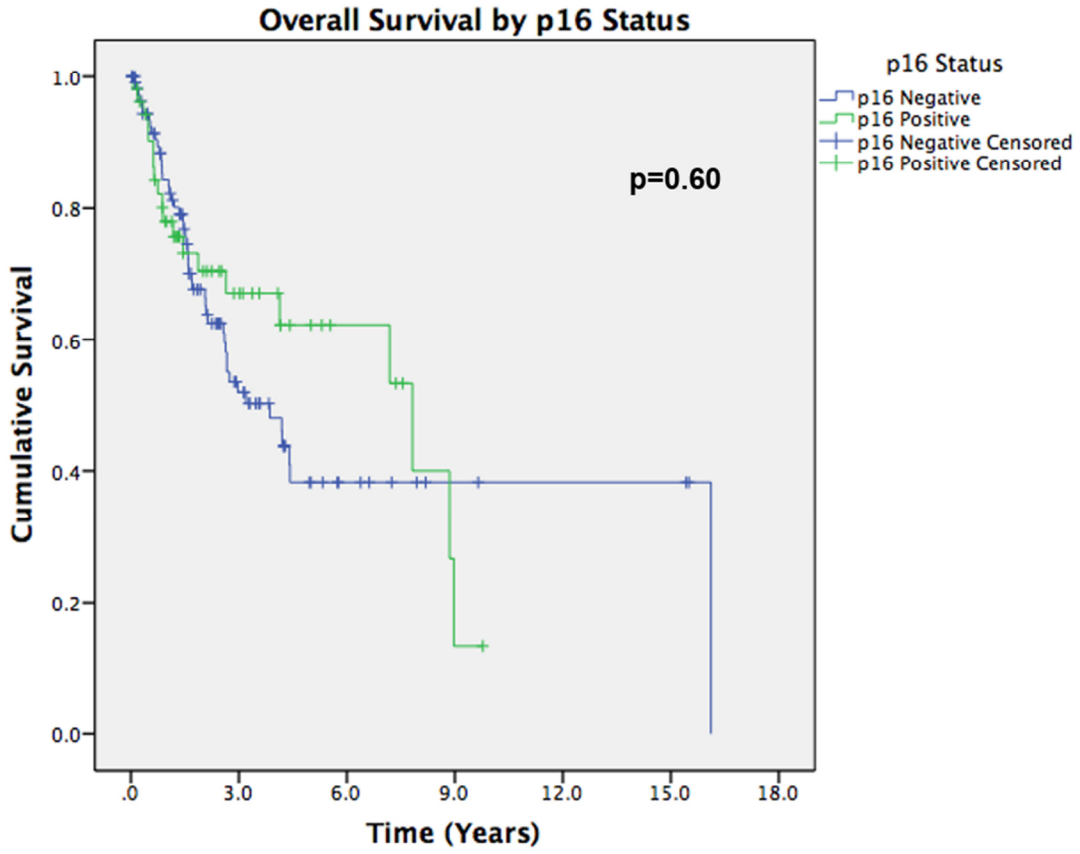

Figure 2A

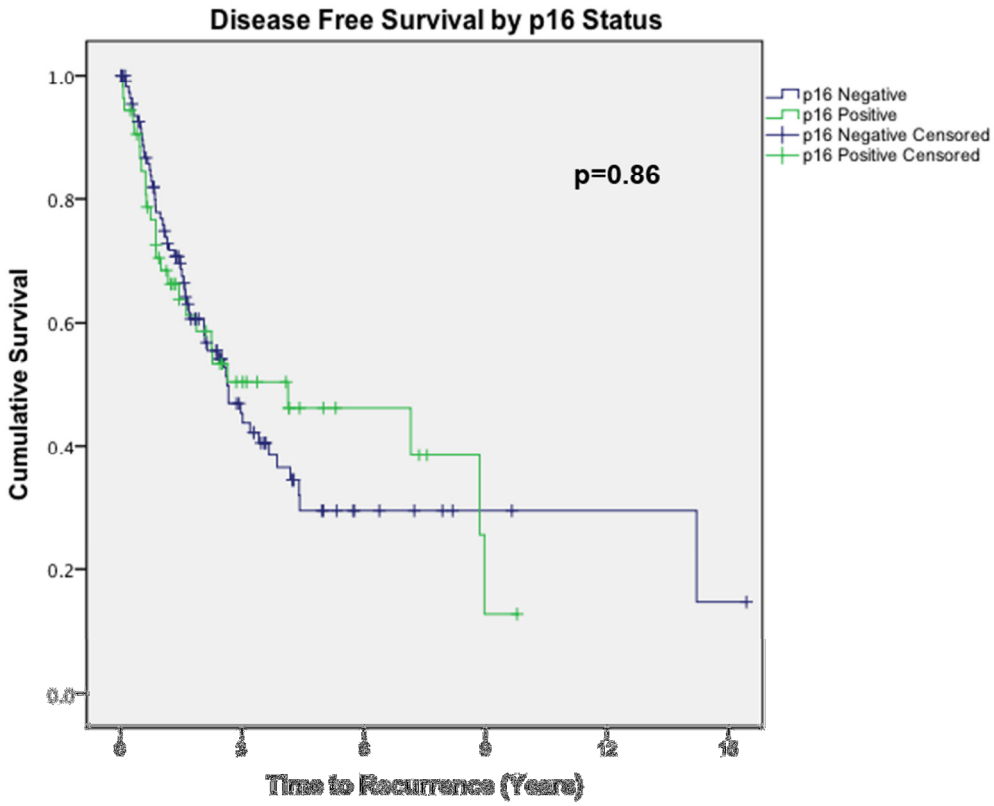

Figure 2B

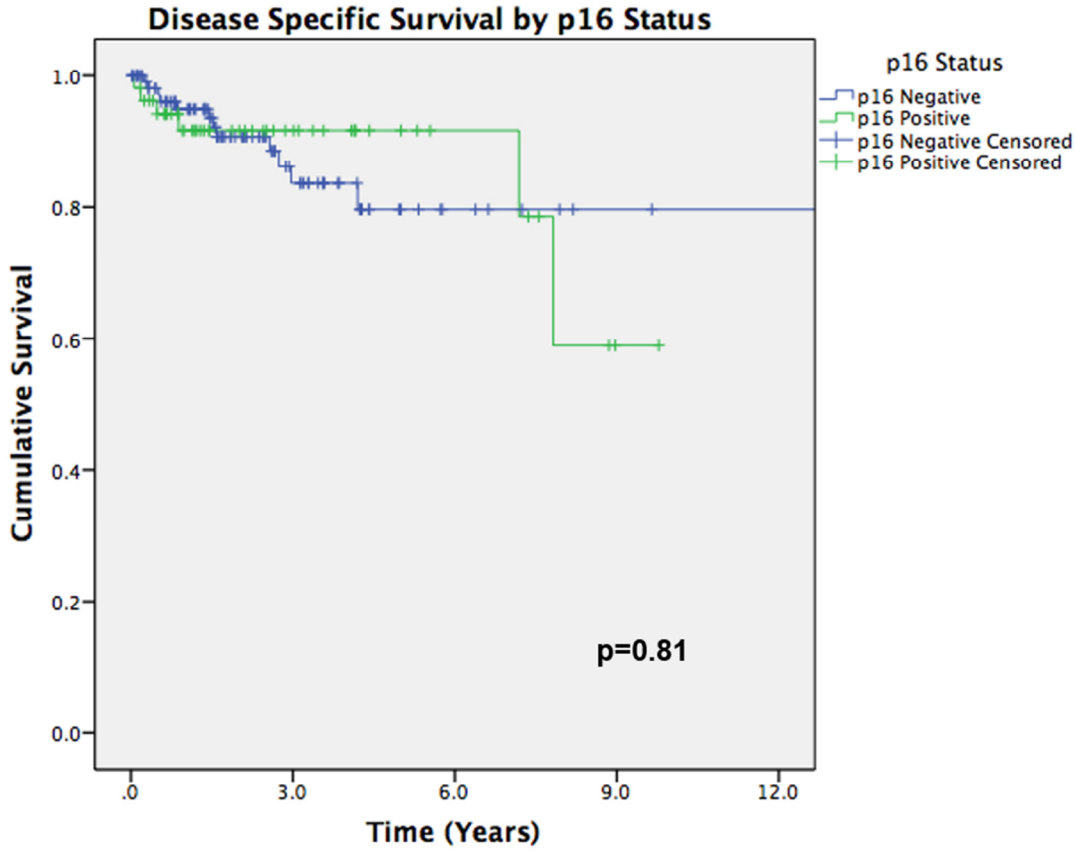

Figure 2C

Table 1 – Clinicopathological data

Variable	n (%)/Median[IQR]
Age (years)	74.1 [64.2 – 83.8]
Gender • Male • Female	 144 (87) 22 (13)
p16 • Positive • Negative	 53 (32) 113 (68)
Keratinization	44/166 (27)
Border • Pushing • Irregular • Disjointed • Tentacular	 2/71 (3) 16/71 (23) 23/71 (32) 30/71 (42)
Differentiation • Well • Moderate • Poor	 5/110 (5) 61/110 (55) 44/110 (40)
T Stage • T1 • T2 • T3 • T4	 11/82 (13) 49/82 (60) 6/82 (7) 16/82 (20)
N stage • N0 • N1 • N2 • N3	 34/161 (21) 51/161 (32) 75/161 (47) 1/161 (1)
ECS Positive	42 (25)
Lymphovascular Invasion	19 (11)
Perineural Invasion	44 (27)
Maximum Tumor Diameter (mm)	25 [15 – 49]
Tumor Thickness (mm)	11 [6 – 19]
Margin • Clear • Involved • Close	 16/96 (17) 35/96 (36) 45/96 (47)
Radiotherapy	88/166 (53)

Table 2 – Association between p16 expression and clinicopathological parameters

Variable	N/ Median[IQR]		P-value
	p16 Positive	**p16 Negative**	
Age (years)	73.7 [61.0 – 79.9]	74.1 [65.3 – 84.1]	0.28
Gender • Male • Female	 44/53 9/53	 100/113 13/113	0.33
Keratinization	30/113 (27)	14/53 (26)	0.99
Border • Pushing • Irregular • Disjointed • Tentacular	 1/27 4/27 12/27 10/27	 1/44 12/44 11/44 20/44	0.28*
Differentiation • Well • Moderate • Poor	 1/37 15/37 21/37	 4/73 46/73 23/73	0.033*
T Stage • T1 • T2 • T3 • T4	 6/30 17/30 1/30 6/30	 5/52 32/52 5/52 10/52	0.51*
N Stage • N0 • N1 • N2 • N3	 14/52 16/52 22/52 0/52	 20/109 35/109 53/109 1/109	0.64*
Nodal involvement (N+ve)	38/52	89/109	0.21
ECS Positive	12/53	30/113	0.59
Lymphovascular Invasion	8/53	11/113	0.31
Perineural Invasion	13/53	31/113	0.69
Maximum Diameter (mm)	21 [12 – 40]	25 [15 – 47]	0.30
Tumor Thickness (mm)	11 [6 – 16]	10 [5 – 20]	0.94
Margin • Clear • Involved • Close	 5/35 8/35 22/35	 11/61 27/61 23/61	0.050
Radiotherapy	27/53	61/113	0.71

* P-value is for Fisher's exact test