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Perineural invasion in mucoepidermoid carcinoma

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PERINEURAL INVASION IN MUCOEPIDERMOID CARCINOMA

by

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

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ABSTRACT

The objective of this study was to retrospectively evaluate the prevalence of perineural invasion in cases of mucoepidermoid carcinoma (MEC). The study will determine if previously assessed perineural invasion by original pathology reports would be increased by re-review of the originally hematoxylin-eosin-(H&E) stained slides as well as review of slides reacted immunohistochemically with S100 to enhance visualization of nerves. The study will also assess whether perineural invasion or its absence in MEC is associated with clinical outcome. Thirty-one cases of major and minor salivary gland MEC were reviewed for perineural invasion and compared to the perineural invasion status stated on the original pathology report when available (13/31). All H&E-stained slides were reviewed as well as S100-reacted sections of each case's tissue blocks that contained tumor. Patient demographics and clinical outcome were collected from electronic medical records. Perineural invasion was identified in 23% (3/13) of tumors in the original reports, 13% (4/31) of the authors' re-review of the same slides, and 29% (9/31) when cases were reacted with S100. A positive relationship was seen between the discovery of perineural invasion on H&E-stained slides and a greater number of foci of perineural invasion. Perineural invasion and larger-diameter nerve involvement was significantly associated with death at 5-year follow-up. In conclusion, immunohistochemical enhancement improves the accuracy, ease and speed of perineural invasion determination. Perineural invasion is a significant factor in the decreased survival outcome of cases of MEC. These findings support continued inclusion of the presence or absence of perineural invasion as a grading parameter in MEC.

PUBLIC ABSTRACT

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm of salivary glands. Through numerous retrospective studies, recurrent and metastatic behavior of MEC has been closely related to histopathologic grade and tumor stage. Tumor grade has been shown to affect the prognosis and treatment modality. However, grading criteria has been debated over the years. Specifically, tumor's perineural invasion status has been controversial as to its effect on prognosis. From this study, we sought to validate whether perineural invasion is an appropriate parameter in grading MEC and to determine if the presence or absence of perineural invasion is associated with clinical outcome. Using the most objective histopathologic features with which to grade MEC, and subsequently determining prognosis or treatment modality is important for a patient's survival and quality of life.

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CHAPTER 1

INTRODUCTION

Salivary Gland Anatomy

Salivary glands are exocrine organs that function to produce and secrete saliva. The salivary glands include the three paired major glands and the minor glands. Major salivary glands are the parotid, submandibular, and sublingual glands. Minor salivary glands are numerous and widely distributed throughout the oral cavity and oropharynx. All salivary glands share a functional structure consisting of secretory acini, related ducts, and myoepithelial cells. However, the salivary glands have site-specific differences in function, secretions, and gross and microscopic features. The parotid gland is composed almost purely of serous acini while the sublingual and minor salivary glands are predominately mucous in type. The submandibular gland contains a mixture of both serous and mucous acini. It is these differences that affect the occurrence and type of neoplasms that arise within the salivary glands (1, 2).

General Considerations in Salivary Gland Neoplasia

The reported annual incidence of salivary gland tumors throughout the world varies from about 0.4 to 6.5 cases per 100,000 people (2). Although the cause of salivary gland neoplasia is not well understood, identified risk factors include previous ionizing radiation exposure, genetic predisposition, tobacco use, exposure to certain industrial chemicals such as nickel, chromium, and asbestos, as well as viral infections—most notably Epstein Barr virus (1-3). The majority, 64% to 80%, of all primary epithelial salivary gland tumors occur in the parotid glands. Seven percent to 11% occur in the submandibular glands, less than 1% occur in the sublingual glands, and 9% to

23% occur in the minor glands. Of these tumors, the majority, between 54% and 79%, are benign with the most common type being the pleomorphic adenoma (1, 4).

Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm of salivary glands—comprising 30% of all malignant tumors in major and minor salivary glands (1). The most common location for MEC is the parotid gland, followed by minor salivary glands and submandibular gland (1, 2). A summary of MEC's relative incidence by anatomic site is seen in Figure 1.

MEC is thought to arise from pluripotent reserve cells of excretory ducts (5). No chemical carcinogens or specific viruses have been associated with MEC. However, MEC has been associated with previous exposure to ionizing radiation (6-9). Another causative factor that has been found in some cases of MEC is a genetic translocation, t(11;19) (q21;p13.1), which has been suggested as a possible primary event in the pathogenesis of a large subset of MEC (7, 10-12).

MEC is a glandular epithelial tumor composed of varying proportions of mucous, epidermoid, intermediate, columnar, clear, and occasionally, oncocytic cells. Many tumors have a cystic component as well as solid cords, islands and/or sheets of tumor cells (1). Through numerous retrospective studies, recurrent and metastatic behavior of MEC has been closely related to histopathological grade and tumor stage (13-18). Staging criteria for minor and major salivary glands is seen in Table 1. Such studies have shown low-grade tumors exhibiting a 92% to 100% five year survival, high-grade tumors exhibiting a 0% to 43% five year survival, and intermediate-

grade tumors somewhere in between (19). However, the various histopathologic criteria for grading MEC has been debated over the years.

The grading of MEC has evolved from a two-tiered system to a three-tiered system (6, 20-24). However, which three-tiered system best classifies tumors, and is easily reproducible among multiple pathologists, remains controversial. The grading systems most commonly used today are those of the Armed Forced Institute of Pathology (AFIP) and the Brandwein system, both of which are point-based systems. The point distribution takes into account histopathologic features of aggression such as percentage of the tumor's cystic architecture, necrosis, perineural invasion, number of mitoses, presence of anaplasia, lymphovascular invasion, bony invasion, and overall pattern of invasion. The total sum of the points determines the score and tumor grade. A summary of AFIP and Brandwein grading criteria is seen in Table 2. Comparison studies have shown that the AFIP grading system has a tendency to down-grade tumors when compared to the Brandwein system (13, 25). A major component of both point-based systems is the presence or absence of perineural invasion. Perineural invasion with the AFIP criteria carries a lesser weight than that of the Brandwein criteria. In fact, in the Brandwein system, the presence of perineural invasion automatically classifies a tumor as intermediate-grade or above (13, 20).

Regardless of the system used, low-grade tumors usually do not metastasize and are often treated with surgical excision alone. High-grade tumors on the other hand are usually treated with adjuvant radiotherapy and/or neck dissection. Because intermediate-grade tumors lie somewhere on the aggressiveness spectrum between low-grade and high-grade tumors, proper treatments are often arguable with the prognosis and therapy given not necessarily consistent (1, 26-31).

Determining perineural invasion can be a difficult task. Interestingly, Kurtz et al. studied perineural and vascular invasion in head and neck squamous cell carcinomas and found that 82%

of all cases reacted with S100 showed perineural invasion. Whereas only 30% of those cases having identified perineural invasion in the original pathology reports based on hematoxylin-eosin-stained (H&E) slides alone and 62% of the authors' re-review of the same slides. Due to the high incidence of perineural invasion found, the authors did not find statistical significance correlating perineural invasion status to ultimate survival outcome (32).

S100 Protein Immunohistochemistry

The S100 protein family is a multigenic group of nonubiquitous cytoplasmic EF-hand Ca2+-binding proteins, sharing significant structural similarities at both genomic and protein levels (33). S100 protein was isolated originally from brain tissue and is historically known to be a good immunohistochemical marker for Schwann cells, melanocytes, glial cells and some central nervous system neurons (34). As such, S100 protein is an excellent marker to highlight peripheral nerves in assessing a tumor's perineural invasion (32, 35, 36).

Purpose

To the investigators' knowledge, none of the studies to date which have established the various MEC grading systems have utilized immunohistochemical enhancement of perineural invasion determination when classifying tumors.

The goal of this study was to determine if re-review of the original H&E slides would increase detection of perineural invasion in cases of salivary gland MEC whose perineural invasion status had been previously included in the original pathology report. Additionally, the present study

sought to determine if immunohistochemical enhancement of the slides with S100 would increase the number of cases of MEC found to have perineural invasion. From these findings, the present study sought to validate whether perineural invasion is an appropriate means of grading MEC and to determine if the presence or absence of perineural invasion is associated with clinical outcome.

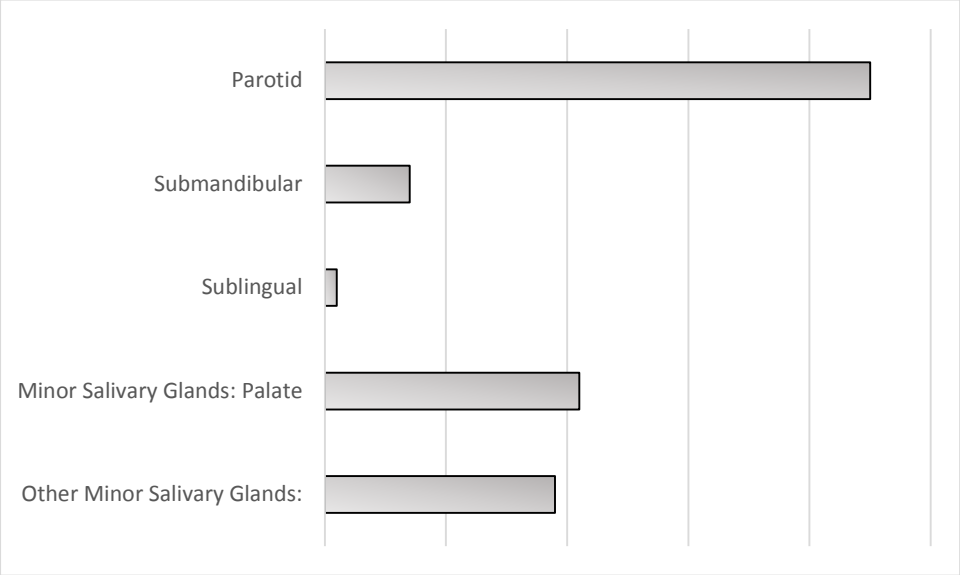
Table 1: Tumor-Node-Metastasis (TNM) staging system for salivary gland cancer

Primary Tumor (T)			
TX	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
T1	Tumor ≤2 cm in greatest dimension without extraparenchymal extension		
T2	Tumor >2 cm but ≤4 cm in greatest dimension without extraparenchymal extension		
T3	Tumor >4 cm and/or tumor having extraparenchymal extension		
T4a	Moderately advanced disease		
	Tumor invades skin, mandible, ear canal, and/or facial nerve		
T4b	Very advanced disease		
	Tumor invades skull base and/or pterygoid plates and/or encases carotid artery		
Regional Lymph Nodes (N)			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension		
N2	Metastasis in a single ipsilateral lymph node, >3 cm but ≤6 cm in greatest dimension		
	Metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension		
	Metastases in bilateral or contralateral lymph nodes, ≤6 cm in greatest dimension		
N2a	Metastasis in a single ipsilateral lymph node, >3 cm but ≤6 cm in greatest dimension		
N2b	Metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension		
N2c	Metastases in bilateral or contralateral lymph nodes, ≤6 cm in greatest dimension		
N3	Metastasis in a lymph node, > 6 cm in greatest dimension		
Distant Metastasis (M)			
M0	No distant metastasis		
M1	Distant metastasis		
Anatomic Stage/Prognostic Groups			
Stage	**T**	**N**	**M**
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	T4b	Any N	M0
	Any T	N3	M0
IVC	Any T	Any N	M1

Table 2: Comparison of the Armed Forced Institute of Pathology (AFIP) and Brandwein modified mucoepidermoid grading criteria

AFIP (1998)	**Points**	**Modified criteria, Brandwein (2001)**	**Points**
Cystic component <20%	+2	Cystic component <25%	+2
Perineural invasion	+2	Perineural invasion	+3
Necrosis	+3	Necrosis	+3
≥4 mitoses/10 HPF	+3	≥4 mitoses/10 HPF	+3
Anaplasia	+4	Anaplasia	+2
		Lymphovascular invasion	+3
		Aggressive pattern of invasion	+2
		Bony invasion	+3
Low-grade	0-4	Low-grade	0
Intermediate-grade	5-6	Intermediate-grade	2-3
High-grade	7-14	High-grade	≥4

Figure 1: Relative incidence of mucoepidermoid carcinoma by anatomic site



CHAPTER 2

MATERIALS AND METHODS

Case Selection

After obtaining Institutional Review Board approval (#201307811), the database of the department of pathology at the University of Iowa Hospitals and Clinics (UIHC) was searched for all salivary gland MEC diagnosed and operated in house between 1972 and 2007. Cases were selected by a search of the department's laboratory information system. All cases had to be previously untreated to be included in the study, and tumor recurrences were excluded. Fifty-four cases were found, and archives were searched for available microslides and tissue blocks. Only cases with available tissue blocks and complete clinical follow-up were included in the study. Twenty-one cases were not included due to missing tissue blocks. The remaining 33 cases were then reviewed by the investigators without knowledge of the subjects' outcome. Two cases were not included because the investigators agreed that the tumors were initially misclassified as MEC. One was reclassified as mammary analogue secretory carcinoma and the other as a poorly differentiated carcinoma. Of the remaining 31 cases, all patients had their surgical resections performed at this institution between 1972 and 2007. The majority of cases were followed a minimum of five years or until death. However, in two cases follow-up was available only for three years. All tumors had been examined, sectioned, and embedded according to the pathology department's protocol which is accredited in compliance with the College of American Pathologists and the Clinical Laboratory Improvement Amendments. This protocol is essentially that of a published gross room procedure manual and also follows a pathology reporting guideline (37, 38). This hospital's pathology faculty had reviewed all cases

studied, and in all cases where perineural invasion was reported, perineural invasion had been assessed with standard H&E-stained slides.

Review of Slides

The three investigators (EL, JH, RR), blinded to the status of perineural invasion according to the pathology reports, individually reviewed the original H&E-stained slides for the presence of perineural invasion. Perineural invasion was defined as tumor involvement of the perineural space with complete or partial encircling of the involved nerve. For a case to be considered positive by the investigators, only one focus of perineural invasion was required to be observed. If discrepancies were found among the investigators in regards to the perineural invasion status upon re-review of the H&E-stained slides, final perineural invasion status was determined based on the majority findings. The number of separate foci of perineural invasion was also counted in each case with the number of foci defined as the number of distinct areas of tumor invading nerves in the slides. Investigators did not count as separate foci tumor cell aggregates that were aligned in a linear pattern along a nerve because these were considered to represent tumor along the length of a single nerve. For statistical analysis, the number of separate foci used was the average among the three investigators' findings.

Immunohistochemistry

The entire collection of pathology materials was reviewed in each case to ascertain which blocks had the presence of tumor. All tumor-containing tissue blocks were recut and reacted with S100 to enhance the detection of nerves associated with tumor. Five-μm sections of formalin-fixed,

paraffin-embedded tumor tissue were deparaffinized with xylene and alcohol. Endogenous peroxide was blocked by immersing the sections in methanol/H_2O_2. No antigen retrieval was performed. Nonspecific binding was blocked using normal goat serum for 10 minutes (1:10 in phosphate-buffered saline) with 1% bovine serum albumin (in phosphate-buffered saline). The sections were then immunoreacted with monoclonal antibodies to S100 (1:500; Dako Corporation, Carpinteria, Calif) for 60 minutes at room temperature. Secondary reactions were carried out using a streptavidin-biotin complex (DAKO LINK and DAKO LSAB2; Dako) and 3,3′-diaminobenzidine tetrahydrochloride as a chromagen. Slides were rinsed and counterstained lightly with Harris hematoxylin. Internal controls of peripheral nerves were present in the tissues of interest. The same investigators evaluated the S100-reacted slides for the presence of perineural invasion. Again, if discrepancies were found among investigators in regards to perineural invasion status following S100 reaction, final status was based on majority findings. For a case to be considered positive by investigators in this study, only one focus of perineural invasion was required to be observed. The number of separate foci of perineural invasion was also counted in each case, with the number of foci defined as the number of distinct areas of tumor invading nerves in the slides. Investigators did not count as separate foci tumor cell aggregates that were aligned in a linear pattern along a nerve because these were considered to represent tumor along the length of a single nerve. For statistical analysis, the number of separate foci used was the average among the three investigators' findings. If perineural invasion was identified, the largest single nerve involved was measured in each case at a multi-headed microscope by two of the investigators (EL, JH).

Additional Features

Clinical features were obtained from electronic medical records. Subjects' clinical data were reviewed for age at time of diagnosis, sex, tumor site, tumor stage, treatment modality, tumor recurrence status, and five year survival status. Age at time of diagnosis was unavailable for one case due to inability to decipher the paper chart handwriting. In five cases, the medical records did not have tumor staging data, therefore tumors were staged using criteria provided in the original surgical pathology reports and medical records following criteria in the AJCC Cancer Staging Manual, 7th edition by the primary investigator (EL) (39). Staging criteria was unavailable for four cases. One case had no record of treatment modality or staging.

Statistical Analysis

Descriptive analyses were completed for all aspects of the study population. Statistical analysis between groups was carried out using Pearson χ^2. Differences between means were carried out using a 2-sample Student *t*-test. Kaplan-Meier estimator was used to calculate survival times. These were compared using the log-rank test (40). Significance was determined to be at $P \leqslant .05$.

CHAPTER 3

RESULTS

Patient Data

Subjects' ages at the time of diagnosis ranged from 16 to 87 years, with a mean age of 54.3 years. There were 13 men and 18 women. The number of cases originating within each subsite were as follows: parotid, eight (26%); palate, eight (26%); lip, one (3%); tongue, three (10%); tonsillar pillar, one (3%); floor of mouth, two (7%); retromolar trigone, five (16%); and buccal mucosa, three (10%). Of the tumors, 19 (61%) were originally classified as low-grade, five (16%) as intermediate-grade, and seven (23%) as high-grade. Stages of tumors were as follows: 14 were stage I, seven were stage II, one was stage III, and five were stage IV. The majority of subjects were treated with localized surgical excision only (20/30). In addition to localized surgical excision, three subjects also underwent selective neck dissection. Localized surgical excision with adjuvant radiation therapy was the treatment modality in one case. Localized surgical excision with selective neck dissection and adjuvant radiation therapy was the treatment modality in four cases. Localized surgical excision, selective neck dissection, chemotherapy and radiation therapy was the treatment modality in one case. In one case, the subject refused surgery, therefore, chemotherapy and radiation therapy were the modalities used. A summary of clinical and pathological features is seen in Table 3.

Perineural Invasion

Only 13/31 of the original pathology reports stated perineural invasion status. Of these cases, perineural invasion was reported to be present in 23% (3/13) of the cases. When original H&E-stained slides were reviewed by the investigators, 13% (4/31) of the cases were interpreted to contain perineural invasion. Examination of S100-reacted slides found 29% (9/31) of the cases to be positive for perineural invasion. A summary of perineural invasion by original report, authors' review of original slides, and review of immunohistochemical reacted slides (S100 for perineural invasion) is seen in Table 4.

Using the S100-reacted cases as the gold standard for perineural invasion status, nine cases were identified to have perineural invasion. Comparing results of the original reports with S100-reacted slides, there were no false-positive cases. There were, however, three false-negative reports of perineural invasion. A summary of original reports' perineural invasion status compared to S100-reacted slides with tumor stage break-down is seen in Table 5. The investigators identified four cases with perineural invasion on review of the H&E slides. Compared with S100-reacted slides, investigators had no false-positive cases of perineural invasion when reviewing original slides (non-immunohistochemical-reacted slides) and five false-negative cases.

Using S100-reacted slides as the standard, there were no significant differences between subjects with tumors that exhibited perineural invasion and those whose tumors did not in regard to sex, age, tumor stage, or histopathologic grade. However, statistical analysis showed that tumor location was a significant factor in perineural invasion status ($P = 0.05$) with perineural invasion being less common in the buccal mucosa, tongue, retromolar pad, and lip compared to other

locations. A summary of demographic, clinical, and pathologic features with and without perineural invasion is seen in Table 6.

Of the nine positive perineural invasion cases, the number of foci of perineural invasion ranged from one to 59 separate foci, with a mean of 14.8 foci. The largest single nerve involved in each of these cases ranged from 0.037 mm to 2.2 mm, with a mean of 0.4 mm. Of the three cases originally reported positive for perineural invasion the mean number of foci was 35 foci per case, and the mean largest single nerve size was 0.94 mm. Within the three false-negative cases from their original reports, the mean number of foci of perineural invasion was one focus per case, and the mean largest single nerve size was 0.06 mm. Of the four cases reported positive for perineural invasion on the investigators' re-review of the H&E slides, the mean number of foci of perineural invasion was 30.5 foci per case, and the mean largest single nerve size was 0.81 mm. Of the investigators' false-negative cases of perineural invasion, the mean number of foci of invasion was 2.2 per case, and the mean largest single nerve size was 0.08 mm. A summary of positive perineural invasion cases by number of foci and largest nerve size is seen in Table 7.

Of the nine positive perineural invasion cases, four cases were originally classified as low-grade, one case as intermediate-grade, and four cases as high-grade. Three of these cases were stage I tumors, two were stage II, one was stage III, and three were stage IV. Of the investigators' five false-negative cases, four cases were originally classified as low-grade and one as intermediate-grade. The largest single nerve sizes in stage I and II cases ranged from 0.037 mm to 0.125 mm. The largest single nerve sizes in stage III and IV cases ranged from 0.225 mm to 2.2 mm.

Patient Outcomes

In all but four cases, subjects were followed for at least five years or until death. In two of these cases, survival and recurrence status was available for three years, and in one case it was available for four years until the subjects were lost to follow-up. In one case, follow-up was not available. Follow-up times ranged from three to 512 months, with a mean of 124 months for all subjects. Of the 30 subjects with follow-up data, six were deceased and 24 were alive. For those deceased, time till death following initial treatment was three, 17, 19, 27, 39, and 50 months for each of these cases. Of the 24 subjects alive at the end of follow-up, one had received postoperative irradiation, and three had undergone selective neck dissection. Of the six subjects deceased at the conclusion of the study, five had also received postoperative irradiation, and four had undergone selective neck dissection. Of the deceased subjects, four had perineural invasion, and two did not. Of those alive, five had perineural invasion, and 19 did not.

Three of 30 subjects developed local recurrences. Of those cases, all had perineural invasion present. Time to recurrence from surgery dates was four months, five months, and 12 months for each of the cases.

Survival analysis was performed based on perineural invasion status alone. There was a significant effect on subject mortality based on perineural invasion status ($P = 0.002$). Median survival time for those subjects with perineural invasion was 34.7 months (25^{th}-75^{th} percentile: 27.8-50.0 months) and 129.9 months (25^{th}-75^{th} percentile: 92.7-184.7 months) for those without perineural invasion. A summary of survival distribution comparing perineural invasion status is seen in Figure 2.

Survival analysis was performed based on perineural invasion status with subjects stratified into low-stage (stages I and II) and high-stage (stages III and IV) categories. Data suggested a shorter survival time in low-stage cases with perineural invasion (median survival time of 47 vs. 126 months), but this was not significant at the 0.05 significance level ($P = 0.095$). However, for those high-stage cases, perineural invasion status did not affect survival. A summary of survival distribution comparing perineural invasion status by low-stage and high-stage cases is seen in Figures 3 and 4, respectively.

Survival analysis was also performed based on positive perineural invasion with cases stratified by small nerve size (< 0.25 mm) and large nerve size (≥ 0.25 mm). This showed that larger nerve size involvement was a significant predicator of mortality ($P = 0.002$). Median survival time for those subjects with large nerve size involvement was 19.3 months (25^{th}-75^{th} percentile: 17.7-27.8 months) compared to 48.5 months (25^{th}-75^{th} percentile: 34.7-104.4 months) for those with small nerve size involvement. A summary of survival distribution comparing perineural invasion stratified by size of the largest nerve involved per case is seen in Figure 5.

There was no significant effect on mortality in regard to number of foci of perineural invasion, sex, or tumor location. Summaries of survival distributions comparing the number of foci of perineural invasion, sex, or tumor location are seen in Figures 6, 7, and 8, respectively.

Table 3: Clinical and pathologic features of 31 subjects with salivary gland mucoepidermoid carcinoma

Case	Sex	Age (years)	Location	Tumor grade	Treatment modality	Tumor Stage	Alive at 5 years	Recurrence
1	M	71	Parotid	High	Excision, neck dissection, radiotherapy	IV	No	Yes
2	F	80	Palate	Low	Excision	I	Yes	No
3	M	39	Tongue	Low	Excision, neck dissection	I	Yes	No
4	M	80	Palate	Low	Excision	I	Yes	No
5	F	44	Parotid	Intermediate	Excision	I	Yes	No
6	F	52	Palate	Low	Excision	II	Yes^	No
7	M	16	Palate	Low	Excision	II	Yes*	No
8	F	54	FOM	Intermediate	Excision	I	Yes	No
9	F	49	Buccal mucosa	Low	Excision	II	Yes	No
10	M	78	Tongue	Intermediate	Radiotherapy, chemotherapy	IVa	No	No
11	F	64	Buccal mucosa	Low	Excision	I	Yes	No
12	M	54	Parotid	Low	Excision	I	Yes	No
13	F	23	Palate	Low	Excision	I	Yes	No
14	M	43	Tonsillar pillar	High	Excision, radiotherapy	IVa	No	Yes
15	M	54	Retromolar pad	High	Excision, neck dissection	IVa	No	No
16	M	73	Parotid	High	NA	NA	NA	NA
17	F	35	Parotid	High	Excision	II	Yes	No
18	M	83	Parotid	High	Excision, radiotherapy	IIIa	No	No
19	F	NA	Retromolar pad	Low	Excision	NA	Yes	No
20	F	20	Parotid	Low	Excision	NA	Yes	No
21	M	45	Retromolar pad	Low	Excision	NA	Yes	No
22	F	39	Palate	Low	Excision	I	Yes	No
23	F	69	Palate	Low	Excision, radiotherapy	II	Yes	No
24	M	46	Buccal mucosa	Low	Excision	I	Yes	No
25	F	69	Parotid	Low	Excision	I	Yes	No
26	F	28	Palate	Low	Excision	I	Yes*	No
27	F	80	FOM	High	Excision, neck dissection, radiotherapy, chemotherapy	IVb	No	Yes
28	F	51	Retromolar pad	Intermediate	Excision	II	Yes	No
29	F	27	Lip	Intermediate	Excision	I	Yes	No
30	M	87	Retromolar pad	Low	Excision	I	Yes	No
31	F	76	Tongue	Low	Excision, neck dissection, radiotherapy	II	Yes	No

Abbreviations: M = male; F = female; FOM = floor of mouth; NA = not available.

*Follow-up available for 3 years post-treatment

^Follow-up available for 4 years post-treatment

Table 4: Cases of mucoepidermoid carcinoma positive for perineural invasion by original report, authors' review of original slides, and review of immunohistochemical-reacted slides (S100 for perineural invasion)

	Original report using H&E slides, % (Proportion)	**Authors' review using H&E slides, % (Proportion)**	**Immunohistochemical-reacted slides (S100), % (Proportion)**
Perineural Invasion	23% (3/13)	13% (4/31)	29% (9/31)

Table 5: Comparison of cases' perineural invasion status by original report and investigators' review of immunohistochemical-reacted slides (S100 for perineural invasion)

PNI Status (original report)	S100	
	Yes	**No**
Yes (n=3)	3 (100%)	0 (0%)
	(stage1=1)	
	(stage4=2)	
No (n=10)	3 (30%)	7 (70%)
	(stage1=2)	(stage1=4)
	(stage2=1)	(stage2=2)
		(stage4=1)
Not stated (n=17)	3 (18%)	14 (82%)
	(stage2=1)	(stage1=7)
	(stage3=1)	(stage2=3)
	(stage4=1)	(stage4=1)
		(stage NA=3)

Abbreviations: PNI = perineural invasion

Table 6: Demographic, clinical, and pathologic features of 30 subjects with mucoepidermoid carcinoma with perineural invasion (S100=Yes) and without perineural invasion (S100=No)

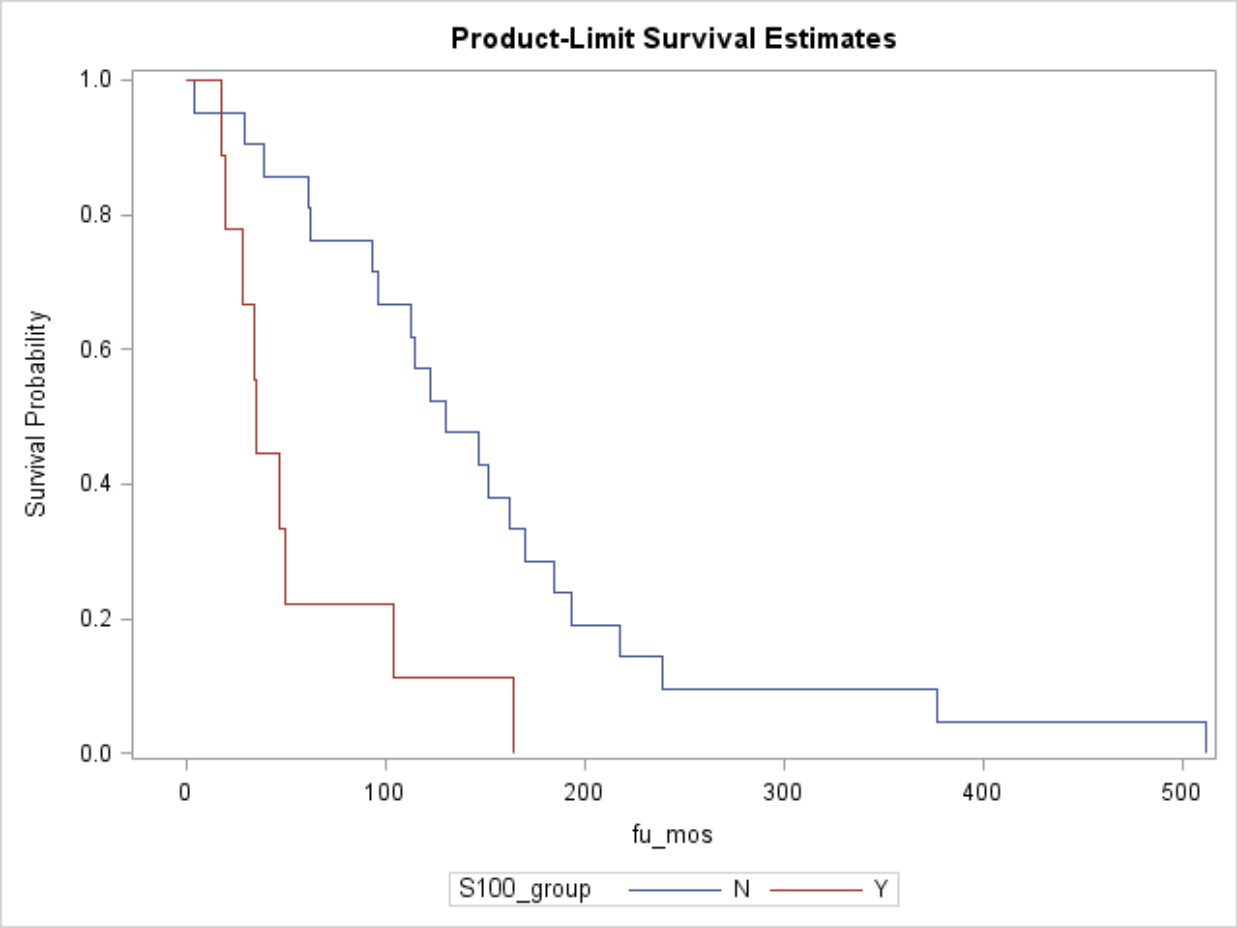
Variable	S100=Yes (n=9)	S100=No (n=21)	p-value
Sex (Male)	5 (56%)	7 (33%)	0.255 ($\chi^{2)}$
Age			0.649 (*t*-test)
Mean(SD)	56.3 (24.2)	52.4 (19.6)	
Range	16-83	20-87	
Stage		(n=18)	0.097 ($\chi^{2)}$
I	3 (33%)	11 (61%)	
II	2 (22%)	5 (28%)	
III	1 (11%)	0 (0%)	
IV	3 (33%)	2 (11%)	
Grade			0.086 ($\chi^{2)}$
High	4 (44%)	2 (10%)	
Intermediate	1 (11%)	4 (19%)	
Low	4 (44%)	15 (71%)	
Location			0.05 ($\chi^{2)}$
Parotid	2 (22%)	5 (24%)	
Palate	4 (44%)	4 (19%)	
Floor of mouth	2 (22%)	0 (0%)	
Tonsillar pillar	1 (11%)	0 (0%)	
Buccal mucosa	0 (0%)	3 (14%)	
Tongue	0 (0%)	3 (14%)	
Retromolar pad	0 (0%)	5 (24%)	
Lip	0 (0%)	1 (5%)	

Table 7: Summary of positive perineural invasion cases by number of foci and largest nerve size (mm)

Case	Reported PNI in original report	Largest Nerve Size (mm)	HE-group	S100-group	PNI foci #
1	not stated	0.25	Yes	Yes	2
4	Yes	0.075	No	Yes	3
6	No	0.037	No	Yes	1
7	not stated	0.125	No	Yes	2
8	No	0.05	No	Yes	4
14	Yes	2.2	Yes	Yes	42
18	not stated	0.225	Yes	Yes	19
26	No	0.1	No	Yes	1
27	Yes	0.55	Yes	Yes	59

Abbreviations: PNI = perineural invasion; HE = hematoxylin and eosin

Figure 2: Kaplan-Meier survival curve comparing the perineural invasion status

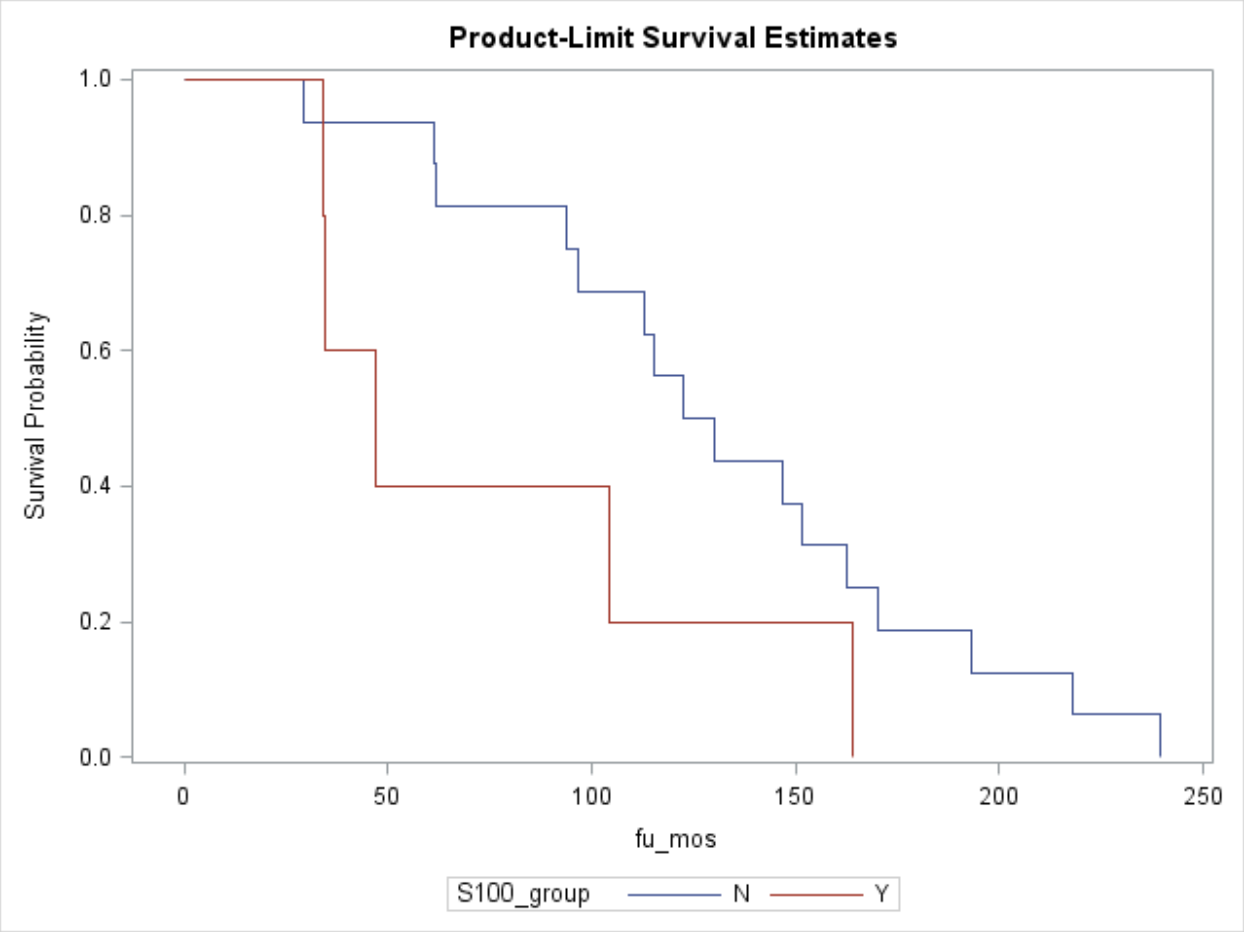


Perineural invasion = yes (S100_group Y): median survival time=34.7 months (25^{th}-75^{th} percentile: 27.8-50.0 months)

Perineural invasion = no (S100_group N): median survival time=129.9 months (25^{th}-75^{th} percentile: 92.7-184.7 months)

Log-rank test p=0.002

Figure 3: Kaplan-Meier survival curve for stages I/II: comparing cases with (S100 yes) and without (S100 no) perineural invasion

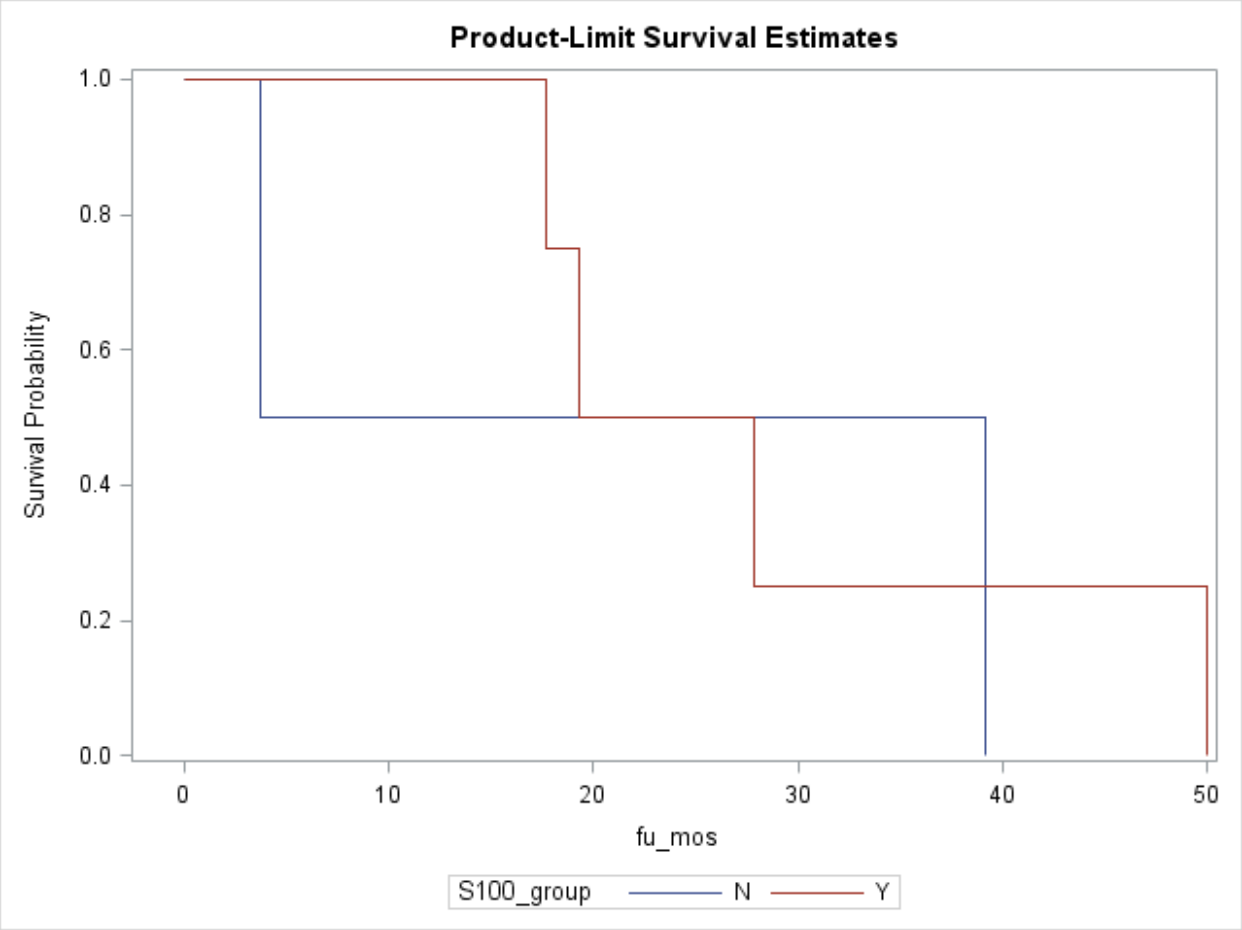


S100(yes, n=5): median survival time=47.1 months (25^{th}-75^{th} percentile: 34.7-104.4 months)

S100(no, n=16): median survival time=126.1 months (25^{th}-75^{th} percentile: 95.0-166.4 months)

Log-rank test p=0.095

Figure 4: Kaplan-Meier survival curve for stages III/IV: comparing cases with (S100 yes) and without (S100 no) perineural invasion

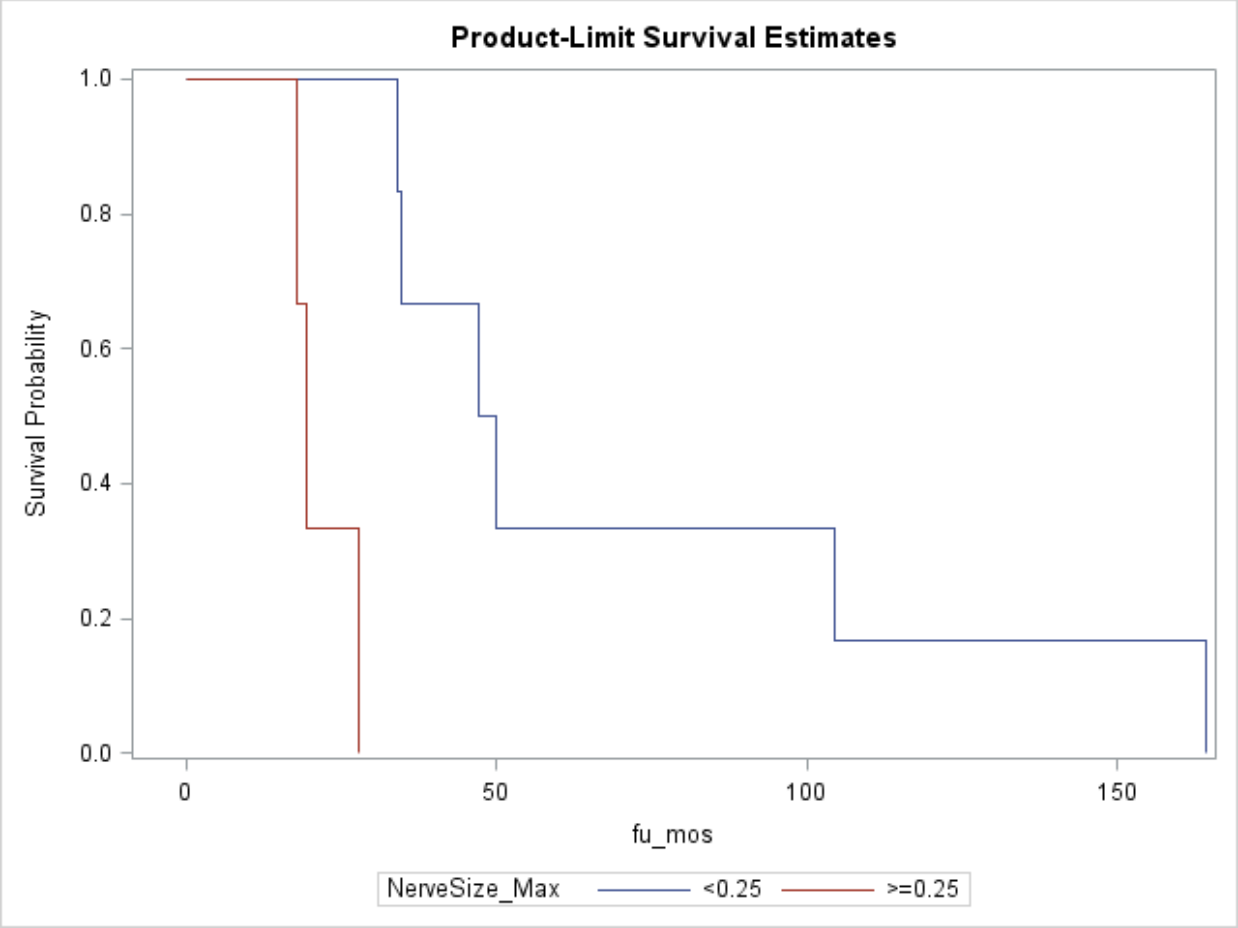


S100(yes, n=4): median survival time=23.6 months (25^{th}-75^{th} percentile: 18.5-38.9 months)

S100(no, n=2): median survival time=21.4 months (25^{th}-75^{th} percentile: 3.7-39.1 months)

Log-rank test p=0.7

Figure 5: Kaplan-Meier survival curve for comparing largest involved nerve size diameter: <0.25 vs ≥0.25 mm

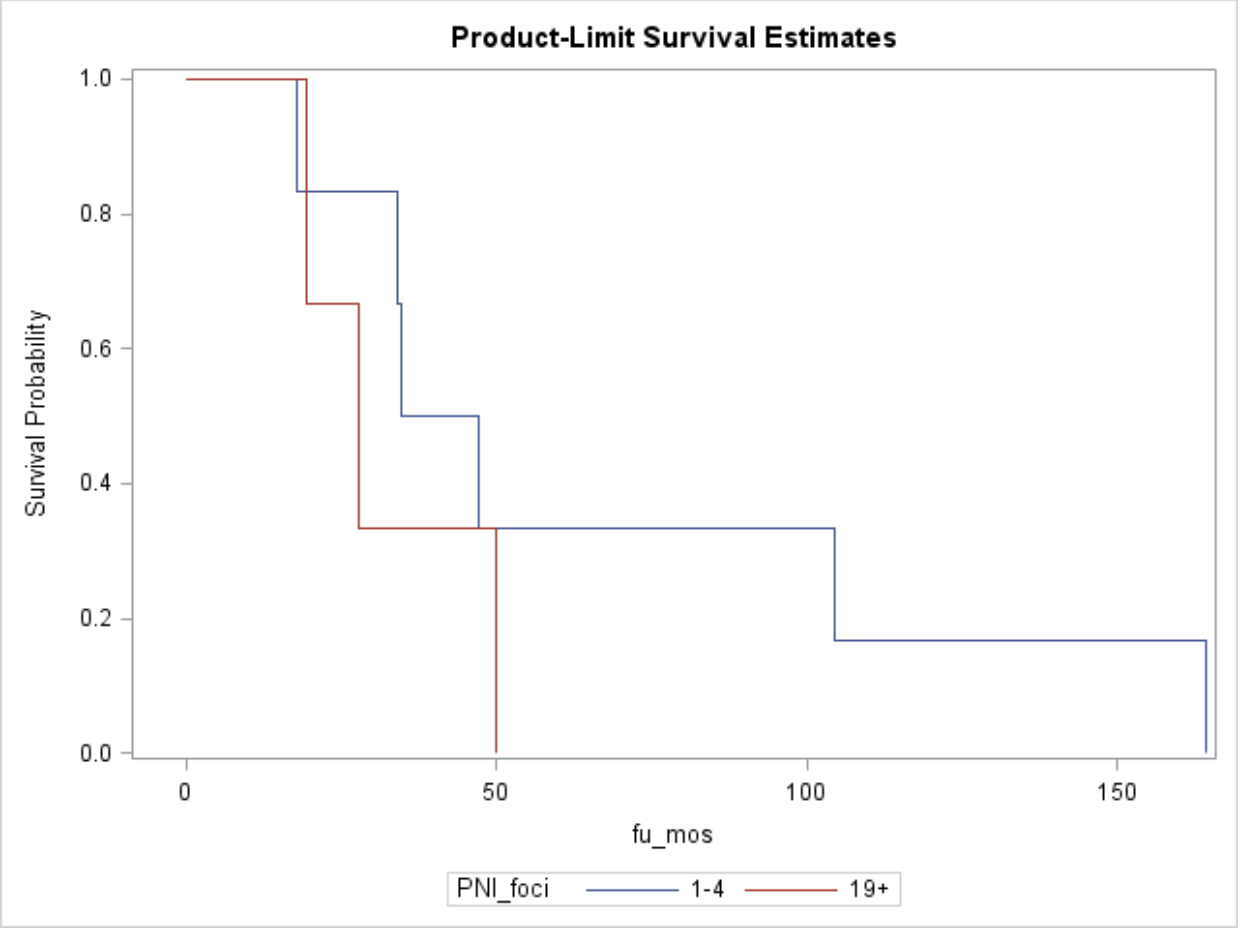


Nerve size (≥0.25 mm, n=3): median survival time=19.3 months (25^{th}-75^{th} percentile: 17.7-27.8 months)

Nerve size (<0.25 mm, n=6): median survival time=48.5 months (25^{th}-75^{th} percentile: 34.7-104.4 months)

Log-rank test p=0.002

Figure 6: Kaplan-Meier survival curve for comparing the number of perineural invasion foci: 1-4 vs ≥19

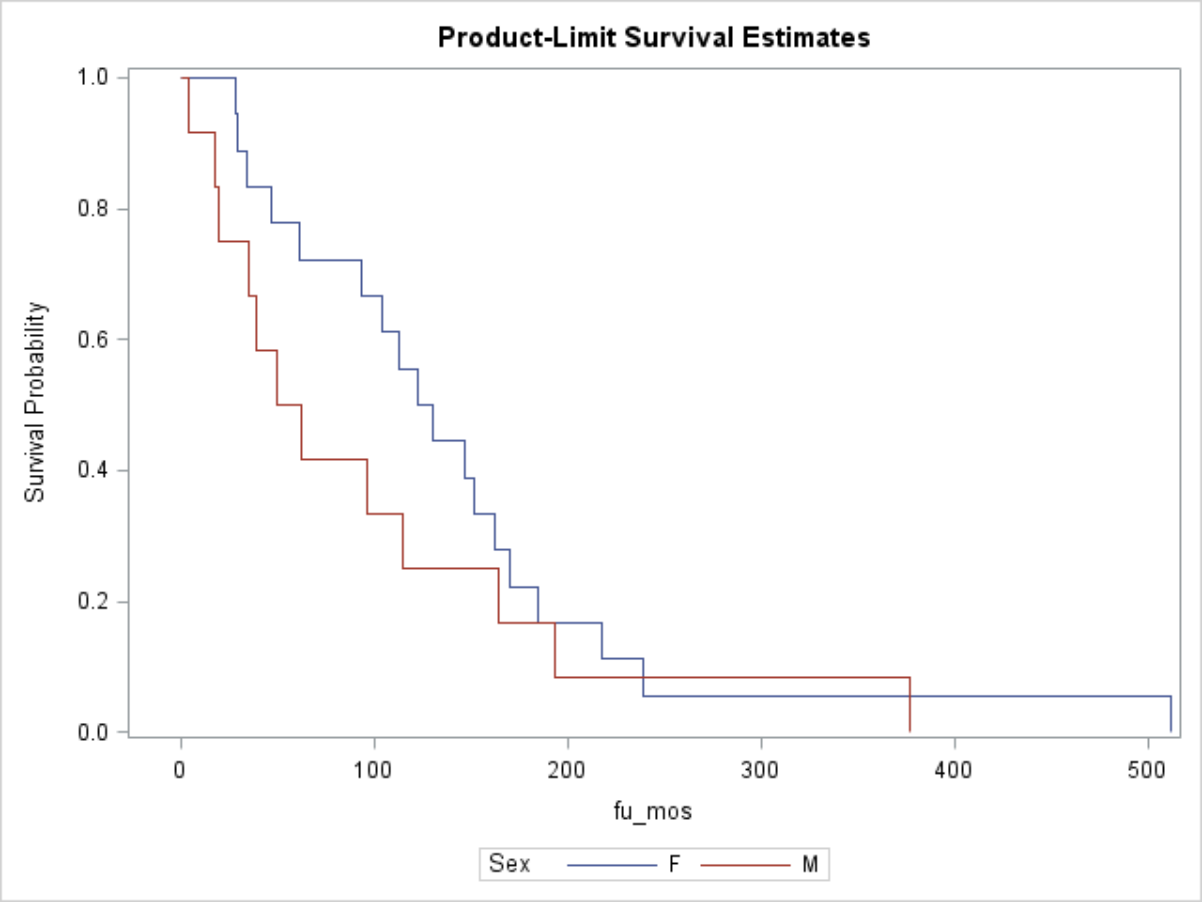


Foci number (≥19, n=3): median survival time=27.8 months (25^{th}-75^{th} percentile: 19.3-50.0 months)

Foci number (1-4, n=6): median survival time=40.9 months (25^{th}-75^{th} percentile: 33.9-104.4 months)

Log-rank test p=0.367

Figure 7: Kaplan-Meier survival curve comparing sex: female (F) vs. male (M)

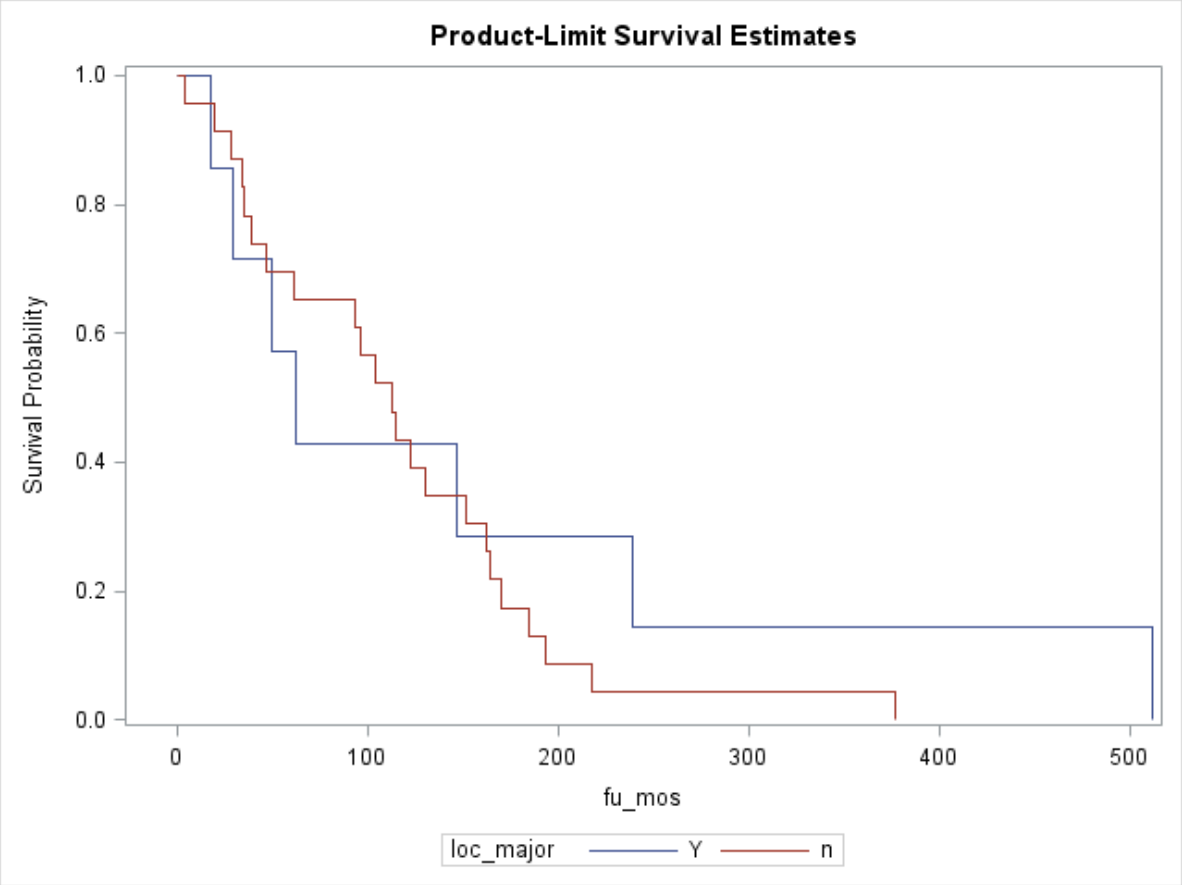


Female (n=18): median survival time=126.1 months (25^{th}-75^{th} percentile: 61.2-170.2 months)

Male (n=12): median survival time=55.9 months (25^{th}-75^{th} percentile: 27.0-139.6 months)

Log-rank test p=0.300

Figure 8: Kaplan-Meier survival curve comparing location: major salivary glands vs. minor salivary glands



Major salivary glands (n=7): median survival time=61.8 months (25^{th}-75^{th} percentile: 29.1-239.4 months)

Minor salivary glands (n=23): median survival time=112.7 months (25^{th}-75^{th} percentile: 39.1-164.2 months)

Log-rank test p=0.460

CHAPTER 4

DISCUSSION

Mucoepidermoid carcinoma is the most frequently diagnosed malignancy of salivary glands (1). Consistent with other reports that MEC has a peak prevalence in the sixth decade of life, our study showed a mean age of 54.3 years at time of diagnosis (4, 21, 41). Our study also was consistent with other reports in that the most common locations for MEC were in parotid salivary glands and minor salivary glands (1). Similar to other series that have shown a slight female preponderance, female subjects were affected 1.4 times more often than male subjects in our series (16, 21, 42).

To our knowledge, none of the studies to date which have established the various MEC grading systems have utilized immunohistochemical enhancement of perineural invasion determination when classifying tumors. However, the two grading systems most commonly used today, that of AFIP and the Brandwein grading system, both consider presence or absence of perineural invasion a major component in determining histopathologic grade of MEC cases (13, 20). These grading systems often influence the prognosis and treatment rendered. This is especially true in regard to intermediate grade MEC (1, 25-29, 43-45). It is noteworthy, however, that several studies have proposed that perineural invasion specifically should not be considered as a grading parameter in MEC (19, 26, 45). In a recent study (2014), Katabi et al., assessed 72 cases of MEC to identify those objective histopathologic features that were predictive of outcome. Interestingly, these authors did not find a significant effect of the presence of perineural invasion on overall survival, disease specific survival, or recurrence free survival (19). This is in contrast to our findings where there was a significant effect on subject mortality based on perineural invasion status ($P = 0.002$). Median survival time for those subjects with perineural invasion

was 34.7 months compared to 129.9 months for those without perineural invasion. Thus, our findings support the continued inclusion of perineural invasion as a grading parameter in MEC, regardless of which system is used. This is also supported by a 2012 study by McHugh et al. which found that high histopathological grade, advanced stage, perineural invasion, positive surgical margins, and submandibular location were all associated with poor clinical outcomes (30). This could have implications in support of using the Brandwein grading system over other systems because it allocates more points to perineural invasion.

Interestingly, our data suggested a shorter survival time in low TNM-stage cases with perineural invasion than in low TNM-stage cases without perineural invasion (median survival time of 47 vs. 126 months). Although not statistically significant ($P = 0.095$), the possibility that a larger sample size may yield significance is possible. However, we cannot determine with certainty that this finding is directly related to perineural invasion status or due to other comorbidities unrelated to the tumor itself.

Using data derived from examination of the immunohistochemically reacted slides, we found a significant correlation between larger nerve size involvement and decreased survival ($P = 0.002$). Median survival time for those subjects with large nerve size involvement was 19.3 months compared to 48.5 months for those with small nerve size involvement. This finding suggests that perineural invasion of a few small nerves embedded within the tumor is not as biologically significant as perineural invasion of large diameter nerves.

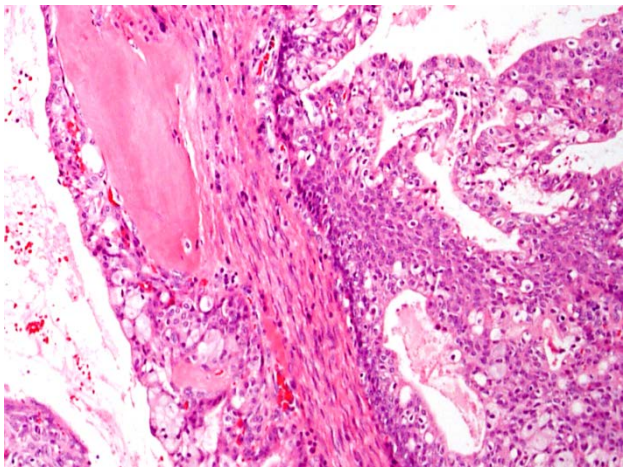
Although only a small number of cases were found to be positive for perineural invasion (9/31) in our series, using S100 reactivity as the gold standard to highlight peripheral nerves gave a 100% (9/9) detection rate as opposed to a detection rate of 44% (4/9) upon the investigators' review of the H&E-stained slides. Our data demonstrated that use of immunohistochemistry to

highlight nerves can further improve detection of perineural invasion when compared to attentive review of H&E-stained slides. We found that S100-reacted slides were especially useful for finding nerves with small diameters entrenched within the tumor and for differentiating nerves from deceivingly similar-appearing desmoplastic stroma. Use of immunohistochemical enhancement also considerably reduced the time investigators needed to search for perineural invasion. The main reason for improved speed was the distinct contrast between S100-reacted nerves and the hematoxylin counter-stain, allowing low-power detection of perineural invasion. Examples of foci of perineural invasion which were originally missed on H&E-stained slides by the investigators and later found by immunohistochemical enhancement with S100 protein are seen in figures 9 and 10. Also, not surprisingly, it appears from our study that it is more likely for pathologists to diagnose perineural invasion if the number of foci is higher with the four cases reported positive for perineural invasion on the investigators' re-review of the H&E having a mean number of foci of 30.5 foci per case compared to the investigators' false-negative cases of perineural invasion having a mean number of foci of only 2.2 per case.

Using the most objective histopathologic features with which to grade MEC and subsequently more accurately determining prognosis or treatment modality is important. This is especially true with intermediate-grade tumors since they lie somewhere in the spectrum between low-grade and high-grade tumors, and their proper treatment is often debatable (1, 26-29, 46).

Figure 9: A, Hematoxylin-eosin–stained (H&E) section of a salivary gland mucoepidermoid carcinoma (original magnification ×200). B, Corresponding section reacted with S100. The arrow demonstrates a focus of perineural invasion which was missed on H&E by the investigators (original magnification ×200).

A



B

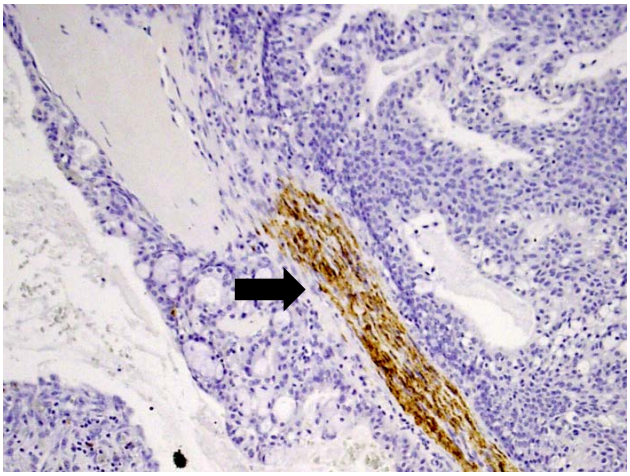
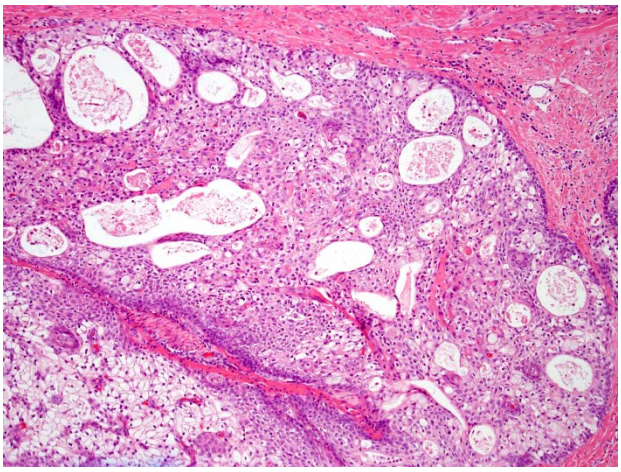
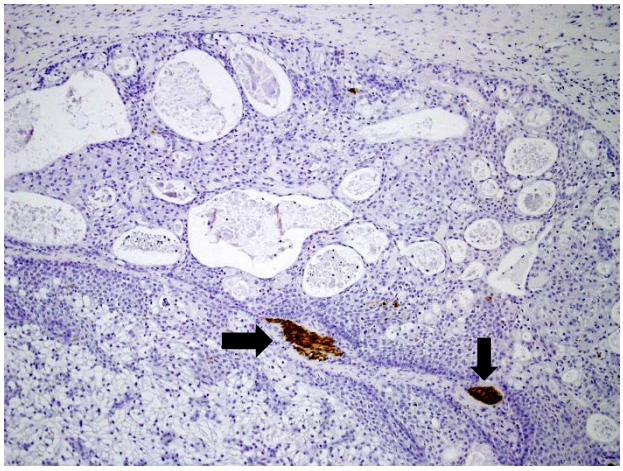


Figure 10: A, Hematoxylin-eosin–stained (H&E) section of a salivary gland mucoepidermoid carcinoma (original magnification ×200). B, Corresponding section reacted with S100. The two arrows demonstrate foci of perineural invasion which were missed on H&E by the investigators (original magnification ×200).

A 

B 

CHAPTER 5

CONCLUSION

Immunohistochemical enhancement improves the accuracy, ease, and speed of perineural invasion determination. Perineural invasion is a significant factor in survival outcome of cases of MEC. These findings support continued inclusion of perineural invasion as a grading parameter in MEC.

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