OBJECTIVE ASSESSMENT OF PSEUDOEPITHELIOMATOUS HYERPLASIA

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ABSTRACT

Austin James Davies: Objective assessment of pseudoepitheliomatous hyperplasia
(Under the direction of Ricardo Padilla)

Introduction: Most biopsy specimens are diagnosed by interpretation of glass slides. However, some conditions like pseudoepitheliomatous hyperplasia (PEH) and squamous cell carcinoma (SCCa) may have overlapping characteristics requiring additional testing. The aims of these studies were: 1- to examine an objective method to define the incidence of PEH in granular cell tumors (GCTs), and 2- to develop a process to differentiate PEH from SCCa. **Methods:** A definition of PEH in GCTs was formulated, interobserver agreement calculated, and the incidence of PEH in GCTs determined. We histomorphometrically measured and compared the proportions of cases with positive immunohistochemical marker. **Results**: Use of our definition of PEH resulted in a high level of agreement. The proportion of melanocytes between PEH and SCCa cases was not significantly different. **Conclusion**: Our definition of PEH proved to be reliable and should be further validated. Melan-A is not capable of differentiating PEH and SCCa.

To my family and to my mentor, for shaping me into the person I am today.

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LIST OF ABBREVIATIONS

CLR	Cox conditional logistic regression
H&E	Hematoxylin and eosin
HPF	High-power field
HPV	Human Papillomavirus
IHC	Immunohistochemistry
LR	Likelihood ratio
MMP-1	Matrix metalloproteinase 1
MART-1	Melanoma antigen recognized by T cells 1
Mitf	Microphthalmia associated transcription factor
NPV	Negative predictive value
OR	Odds ratio
PEH	Pseudoepitheliomatous hyperplasia
PPV	Positive predictive value
pRB	Retinoblastoma protein
SCCa	Squamous cell carcinoma

OBJECTIVE ASSESSMENT OF PSEUDOEPITHELIOMATOUS HYERPLASIA

Introduction

In the field of oral and maxillofacial pathology, the vast majority of lesions examined by the pathologist are diagnosed by using his or her expertise to interpret the material seen on a hematoxylin and eosin-stained (H&E) glass slide. While this is accurate the majority of the time, interpreting materials subjectively leaves room for differences in opinion and potentially introduces the possibility of misdiagnosis. The main goal of diagnostic specialties is to give the correct diagnosis. Occasionally, examination of H&E stained glass slides does not result in an accurate diagnosis and the pathologist is required to perform additional special tests. At the forefront of this endeavor are molecular and cytogenetic tests that allow for highly specific and sensitive results. However, these tests are costly and can delay treatment. In addition, generation of subjective data is difficult to replicate in independent studies.

The first manuscript focuses on developing an objective operational definition of PEH in granular cell tumors (GCTs) and subsequently using it to determine its incidence in GCTs. The literature has widely varying reports of prevalence, but no standardized definition. The second manuscript aimed to objectively assess if there was a significant statistical difference between the proportions of cases that show marker expression in PEH versus SCCa, two occasionally confused but vastly different biologic entities.

INCIDENCE OF PSEUDOEPITHELIOMATOUS HYERPLASIA IN ORAL GRANULAR CELL TUMORS OF ADULTS, AN INDEPENDENT RETROSPECTIVE ANALYSIS

Introduction

Granular cell tumor (GCT), also known as Abrikossoff's tumor, granular cell myoblastoma, granular cell nerve sheath tumor, and granular cell schwannoma, is an uncommon entity of soft tissue first described by Abrikossoff in 1926.(1) Consensus has not been reached whether the GCT represents a reactive, developmental, or neoplastic process; hence its multiple designations.(2) While rare malignant forms of GCTs have been described, it typically presents as a benign process.(3) In the past, theories for the tissue of origin of this tumor have included striated muscle, histiocytic, pericytic or fibroblastic origins. Current thinking suggests that these tumors arise from stem cells with neural crest differentiation, as supported by ultrastructural and immunohistochemical studies.(4,5)

Clinically, the GCT can occur in any anatomic location. It can occur on the mucosa or epidermis, and case reports have reported lesions of the forearm**,** orbit, chest wall, gastrointestinal tract, and bronchus.(6-10) In the head and neck, the oral cavity is the most common site for GCTs, and of the intraoral ones, the tongue has the highest incidence of occurrence.(4) Tongue GCTs usually present on the dorsal surface as a painless, sessile, firm, nodule less than 1.5 cm in diameter.(3) Their color can vary, but in general, when close to the surface they appear yellowish with a normal surface texture. Other reported intraoral locations include: lateral tongue (15%), buccal mucosa (13%), palate (6%), upper lip (4%), lower lip (4%), gingiva/retromolar pad (4%), and floor of mouth (2%).(11) GCT is reported to occur at any age, but it is most commonly observed between the ages of 30 and 60 and occurs twice as commonly in women than men.(2)

Histologically, GCTs are composed of cells with granular cytoplasm arranged in a syncytial pattern, often forming nests, and ribbons separated by fibrous septa. This separation can give the appearance of invasion when the lesion occurs adjacent to or within striated muscle. As the most widely used name implies, a granular appearance of the cellular cytoplasm is always present. Ultrastructural studies have demonstrated that these granules contain lysosomes with cellular myelin.(4,12) Another occasional histological feature, and the concentration of this project, is the propensity of GCTs to induce pseudoepitheliomatous hyperplasia (PEH) of the overlying surface epithelium. (Figure 1.1) While the exact mechanism for the induction of PEH is unclear, it is seen in other head and neck lesions such as deep fungal infections with granulomatous inflammation.(13) It has also been reported that some cases of PEH have been confused with squamous cell carcinoma (SCCa) by unsuspecting or inexperienced pathologists.(14)

Observations in our institutions' oral biopsy services lead us to believe that the incidence of PEH in GCTs was closer to one third of cases, and not consistent with the literature.(4,11,15,17-19) We also noted a lack of uniformity in the definitions of PEH.(15,20,21) Therefore the goals of this study were to develop a working definition of PEH in GCTs based on published definitions, and to objectively determine the incidence of PEH in oral GCTs in our institutions by means of an independent observer study.

Material and Methods

This study was approved by the University of North Carolina at Chapel Hill's Institutional Review Board (IRB) with allowed oversight of the University of the Pacific Arthur A. Dugoni School of Dentistry's archival material (UNC IRB Study # 14-0776). All IRB procedures and regulations were followed during the entire project.

The databases of the Oral and Maxillofacial Pathology Laboratories of the University of North Carolina at Chapel Hill School of Dentistry and the University of the Pacific Arthur A. Dugoni School of Dentistry were queried for cases of GCTs. The inclusion criteria were: histopathologic diagnosis of GCT, reported anatomic location within the oral cavity, and the patient being over 21 years of age at the time of surgery for the lesion. The search yielded 70 cases from both institutions. The cases were reviewed for an accurate diagnosis of GCT. Subsequently, two board-certified Oral and Maxillofacial Pathologists were utilized as observers for this study, (R.P., D.C.). We developed a new, stringent definition to test the incidence of PEH in GCTs based on analysis of various definitions available in the current literature.(22,23) This modified working definition was:

> *Significant elongation or downgrowth of histologically benign-appearing squamous epithelium in the form of rete pegs along the surface epithelium. The individual cells are very mature, and only occasional dyskeratosis is present, usually as a keratin pearl rather than individually keratinized cells. Long, broad, intertwining rete pegs and rete ridges, when cut tangentially, can give a false, but rather strong impression of invading malignant epithelial islands of squamous cell carcinoma.*

Our PEH definition was used to calibrate the observers by having them independently review two cases that complied with that definition, and two that did not. Figure 1.2 illustrates the histological criteria for the working definition. To calculate intra-observer and inter-observer reliability, five additional blinded “reliability” cases were evaluated independently by each of the examiners before the retrospective analysis, and the same “reliability” cases were evaluated by each observer after the retrospective analysis.

After calibration, each observer was asked to apply our definition of PEH while evaluating the 70 test cases we found between the two institutions. The observers had neither demographic

background of the case or knowledge of the other observer's answers. Results were tabulated, and an unweighted Kappa (κ) statistical agreement test was applied using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

A Fischer's two way exact test was employed to test for a statistically significant difference, since in our study the majority of GCTs of the lateral tongue occurred in females.

Results

Calibration tests revealed complete intra-observer and inter-observer agreement.

In 68 out of 70 cases, both observers agreed in their responses. (κ= 0.939)(SE_κ= 0.043)(95% confidence interval: from 0.856 to 1.000) The interpretation of the Kappa agreement value was considered 'almost perfect' by the Landis and Koch's standards for the strength of agreement.(24) These results are summarized in Table 1.1. The two cases in which there was no observer consensus were further excluded from our analysis.

Of the 68 included cases, 25 had PEH (36.76%). Women constituted 79.41% of our study population. The average age of our patients was 44.9 years (Table 1.2). Anatomically, the vast majority of the lesions were from the tongue, followed by the buccal mucosa, and the lower lip. In our series, 91.4% (64/70) of our cases occurred on the tongue and buccal mucosa; and 93.33% of lateral tongue GCTs occurred in females. This last finding did not reach statistical significance (p=0.167).

When we considered exclusively PEH cases from the tongue, the dorsum was the most common location, followed by the lateral surfaces, and then the ventral surface. In our series, no GCTs were found in the gingiva, maxillary vestibule, mandibular vestibule, and upper lip.

Discussion

We strongly believe that in order to accurately diagnose and determine the incidence of PEH, a standardized, reproducible, and universally applicable definition of this entity is necessary. Such a strong definition must be supported by objectively generated data. We believe our definition of PEH complies with the parameters mentioned above. We demonstrated that our definition had agreement between the two observers as they applied the definition independently to different GCTs and agreed in 68/70 (97%) of the cases. This lead to a Kappa value of .939 or "almost perfect" agreement when compared to chance.(53) Only in two cases was agreement not attained. (Figure 3) Case one (Figure 3A) was a 51-year-old female with a GCT on the buccal mucosa. Observer one called it positive for PEH while observer two called it negative. This case had some elongation of the rete ridges and was interpreted differently by the two pathologists. Case two (Figure 3B) was a 43-year-old male with a GCT on the dorsal tongue. Observer one called it positive for PEH while observer two called it negative. This case had only minimal elongation of the epithelial rete pegs. This discrepancy in two cases highlights a frequent issue in the practice of pathology: the interpretation of subjective concepts or histological features. Ideally, all diagnostic parameters would be objective, but unfortunately, current technology is short of allowing this transition and we still often rely on the "skill" of the pathologist for subjective evaluation of some conditions and diagnoses. While the proposed definition fails to bring 100% agreement to the tissues in question, it does show that "skilled" pathologists could apply the same definition of PEH and reach a high level of agreement. While currently no definition of PEH is universal, one that gives strong intra- and inter-observer reliability, like the one offered in this paper, is worth considering for use in future projects. We encourage other investigators to test our proposed definition of PEH and its incidence in oral GCTs in order to standardize data generation.

Our study revealed a 36.76% incidence of PEH in oral GCTs as measured by two independent observers applying our proposed definition of PEH in GCTs. This incidence differs with most of the

previously published data in the English literature.(4,11, 15,17-19) All previous studies failed to disclose or apply a detailed definition of PEH and also lacked explanations of how the presence of PEH was determined. These potential flaws in most of the previous studies' methodology were the reasons that lead us to hypothesize that the incidence of PEH in GCTs has been historically overestimated. In our opinion, consensus regarding PEH incidence in GCTs cannot be reached unless a tested and standardized definition of PEH is applied. A single study from 30 years ago did report a similar incidence to the one found in our cases, but unfortunately this study also failed to provide a definition of PEH and an explanation of how they determined PEH in their cases of GCTs.(16)

The most significant misinterpretation of PEH would be a misdiagnosis of SCCa, such as reported by Lack et al. They reported a case where a misinterpretation of PEH was diagnosed as SCCa and resulted in a patient being over-treated unnecessarily with radium needle implants.(14)

Our data confirms some of the published findings regarding the distribution of GCTs. The most common area in the oral cavity was the tongue, specifically the dorsum followed by the lateral border.(11,15,17) In fact, in our study and another(15), the frequency of GCTs on the dorsal tongue was higher than in all the other intraoral sites combined. Our results also support previous reports that at least two-thirds of the patients are female (79.41% in our study). Similarly, the age range of GCTs between 30-60 years coincided with our cases average age of 44.99 years.(4,11,15-18) In our study, the majority of GCTs of the lateral tongue occurred in females (93.33%). Our data indicated a predilection for females when considering GCTs of the lateral tongue exclusively. We therefore further analyzed this discrepancy in incidence, but found it not statistically significant.

After conclusion of this project, we feel that a revised definition for determining PEH in GCTs could be:

Significant elongation or downgrowth of histologically benign-appearing squamous epithelium in the form of rete pegs below the standard depth of the oral epithelium. The individual cells appear very mature with only occasional dyskeratosis. The dyskeratosis is usually present as keratin pearls rather than individually keratinized cells.

This revised definition is more operational than our first version. We encourage future projects to test it as well, to determine the incidence of PEH in GCTs.

Conclusions

Utilization of our proposed definition of PEH in GCTs yielded 'almost perfect' Kappa inter-observer statistical reliability values. The incidence of PEH in oral GCTs in our independent retrospective analysis was 36.76%. We encourage further studies with larger case series to use the tested definition as well as the revised definition to further validate the true incidence of PEH in GCTs of the oral cavity of adults.

Tables

Table 1.1 - Results of the independent analysis to determine presence (Yes) or absence (No) of pseudoepitheliomatous hyperplasia used to calculate the unweighted Kappa Statistic (κ)

		Observer 1		
Observer 2		Yes	No	Total
	Yes	25	0	25*
	No	2	43	45
	Total	27	43*	70

* Number of observed agreements: 68/70 (97.14%)

Table 1.2 - Results of the distribution of granular cell tumors (GCTs) and pseudoepitheliomatous hyperplasia (PEH) seen in the oral cavity of adults in this study

Location	**Number of Cases**	**Presence of PEH (%)**	**Mean age***	**Percentage Females***
Dorsal Tongue	35	15 (42.86)	45.34	77.14
Lateral Tongue	15	4 (26.67)	41.4	93.33
Ventral Tongue	6	1 (16.67)	45.17	66.67
Buccal Mucosa	6	3 (50)	56.17	66.67
Lower Lip	3	1 (33.33)	26	100
Floor of Mouth	1	1 (100)	49	100
Hard Palate	1	0 (0)	70	0
Soft Palate	1	0 (0)	46	100
Total	68	25 (36.76)	44.99	79.41

* Average age and percentage female of patients with GCTs (with or without PEH)

Table 1.3 - Incidence of pseudoepitheliomatous hyperplasia (PEH) in oral mucosal granular cell tumors (GCTs) in current and past studies

Previous author and year	Incidence
Worsaae et al. (1979)	28.57%
Lack et al. (1980)	7.89%
Chaudhry et al. (1984)	"In nearly one third…"
Stewart et al. (1988)	45%
Mirchandani et al. (1989)	8.57%
Chrysomali et al. (2003)	67%
Le et al. (2004) *	100%
Vered et al. (2008)	25%
Current series. (2016)	36.76%

*8/8 + for PEH, one case had PEH as "N/A" (19)

Figures

Figure 1.1 - Pseudoepitheliomatous hyperplasia

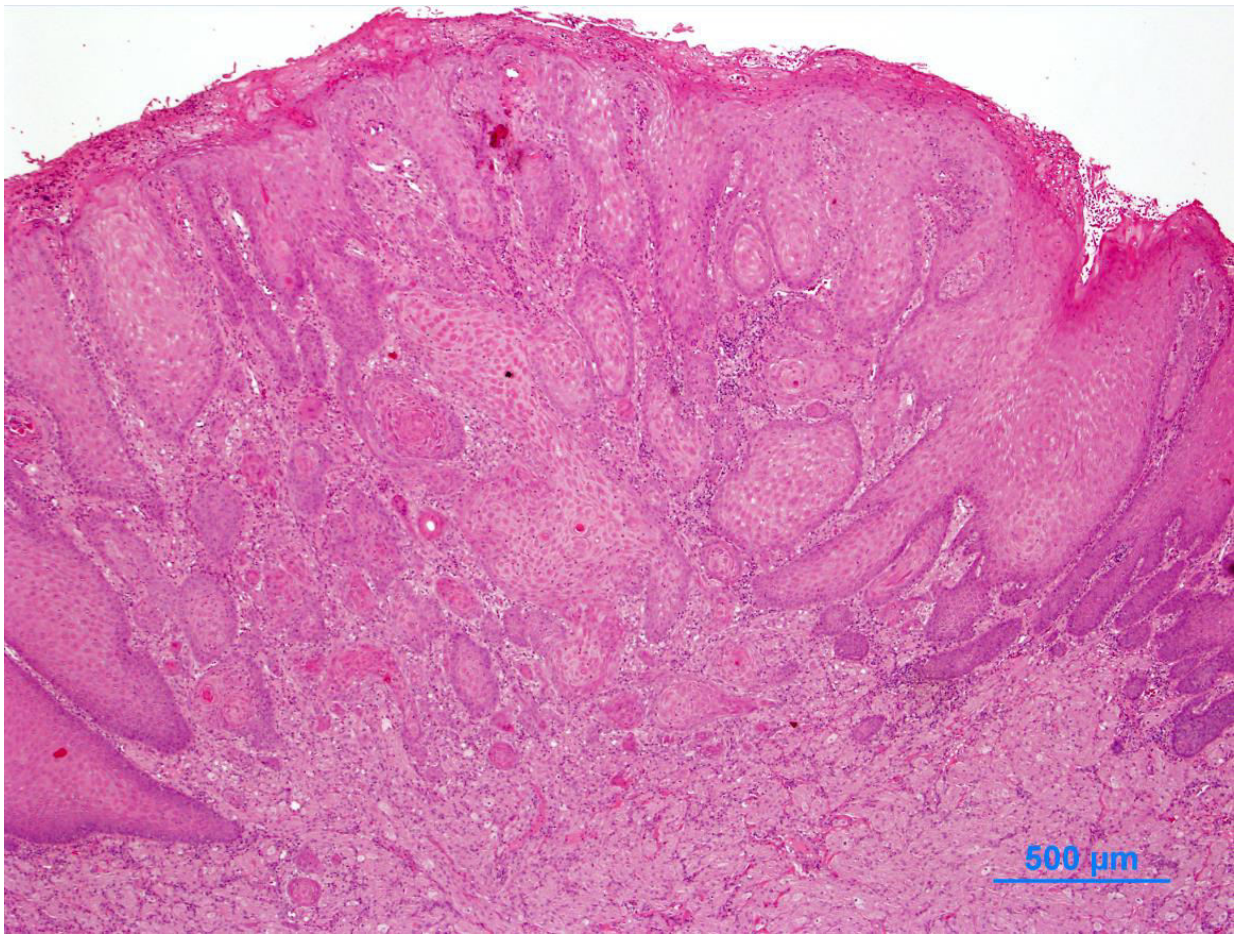

Fig 1.1 - A granular cell tumor (GCT) with overlying pseudoepitheliomatous hyperplasia (PEH). Image has been colored enhanced for brightness

Figure 1.2 - Illustrated criteria for working definition

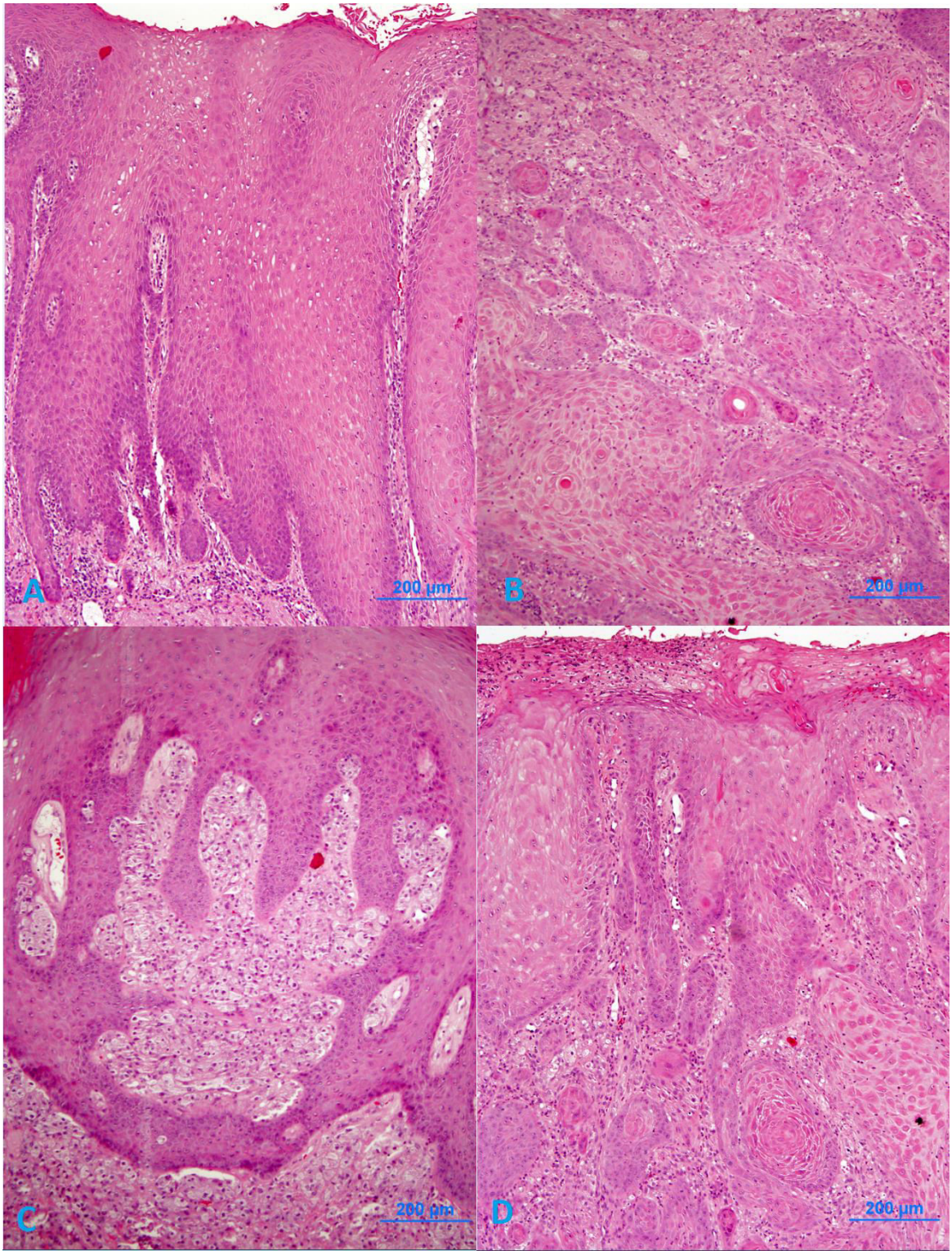

Fig 1.2 - Illustrated criteria for working definition of PEH. A. Elongation of benign appearing squamous epithelium B. Keratin pearls C. Long broad, intertwining rete pegs and rete ridges D. Long rete pegs tangentially cut giving false impression of squamous cell carcinoma

Figure 1. 3 - Cases in which agreement was not met

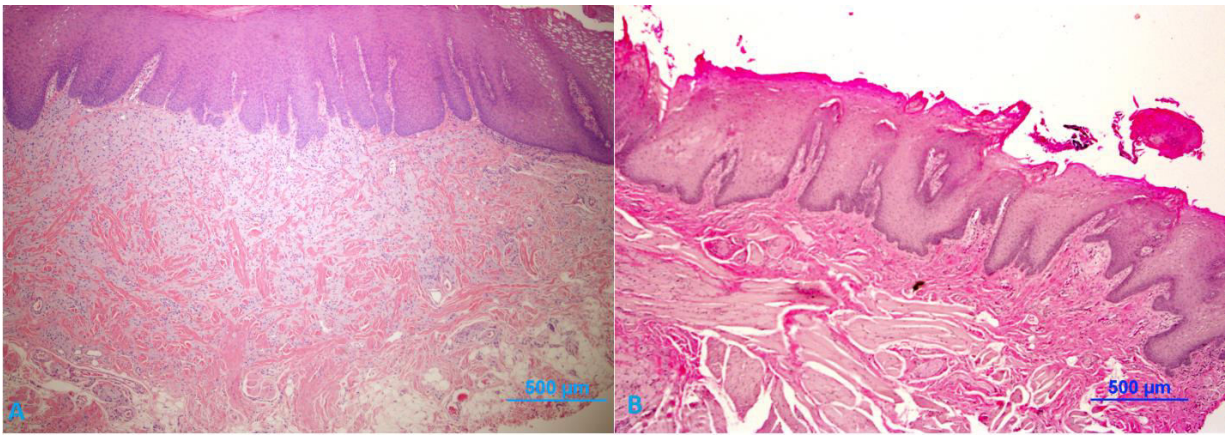

Fig 1.3 - A. and B. These two cases of granular cell tumors (GCTs) represent the only cases on which agreement between the observers were not met

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DIFFERENTIATION OF PSEUDOEPITHELIOMATOUS HYERPLASIA FROM SQUAMOUS CELL CARICNOMA OF THE ORAL MUCOSA BY IMMUNOHISTOCHEMISTRY

Introduction

Histopathological evaluation of hematoxylin and eosin stained (H&E) glass slides of tissue specimens is considered the gold standard for diagnosing numerous pathologies. In the majority of cases, this is an appropriate and sufficient diagnostic method. Occasionally, tissue artifacts and predictable histological variations are seen upon examination of H&E-stained glass slides. One such variation is pseudoepitheliomatous hyperplasia (PEH). When pathologists are aware and recognize these variations, they are able to avoid errors, and even arrive at a more accurate diagnosis. (1,2) Unfortunately, the presence of artifacts or histological variations may also occasionally lead to misdiagnosis. A common pitfall that can have dire consequences is misinterpreting PEH as squamous cell carcinoma (SCCa).

PEH is also known as “pseudocarcinomatous hyperplasia,” and is a reactive hyperplastic elongation of epithelium that occurs in association with a number of traumatic, infectious, neoplastic, and inflammatory conditions. It does not appear to arise de novo or without a causative factor.(3) Histologically PEH consists of a significant elongation or downgrowth of benign-appearing stratified squamous epithelium rete pegs.(Figure 2.1) The individual cells are very mature, appropriately differentiated, and only occasional dyskeratosis is present, usually as keratin pearls rather than individual cell keratinization. The long, broad, and intertwining rete pegs and rete ridges, when cut tangentially, can give a false, but rather strong impression of invading malignant epithelial islands of SCCa. The pathogenesis of PEH has yet to be elucidated but most investigations have revolved around

epidermal growth factor and transforming growth factor interactions with the surface epithelium.(4) Treatment of the underlying initiating factor of the PEH rather than PEH itself is recommended. This requires accurate differentiation of PEH from SCCa.

SCCa is a clonal neoplastic proliferation of keratinocytes, arising from the surface stratified squamous epithelium, that invade through the basement membrane into the supporting connective tissue stroma. The neoplastic cells invade as islands and cords of tumor. On the skin, it is one of the most common cancers in humans, with approximately 700,000 new cases each year in the United States.(5) In the United States, each year there are approximately 45,700 new cases of oral and oropharyngeal SCCa, causing about 8,600 deaths.(6) Histologically, SCCa shows cords and invasive islands of malignant keratinocytes with cytological atypia and features of malignancy, such as pleomorphic nuclei, atypical mitoses, dyskeratosis, necrosis, anaplasia, and dedifferentiation.(7). PEH may share some of the epithelial architectural features as well as some histologic features of SCCa. (Figure 2.2)(8) In cases where not all the features of SCCa are readily apparent, subtle clues can be missed and an incorrect diagnosis can be given.(9)

During the pathologic evaluation of oral mucosal lesions, one may see a number of different cells in the epithelium. Keratinocytes are the most numerous, and provide the structural foundation for the epithelium. Langerhans cells and melanocytes are also seen within the epithelium although observed with much less frequency and mostly with the aid of special stains or immunohistochemistry. Langerhans cells are found in the basal and spinous regions and function as antigen-presenting cells that intercept microbes, process them, and then present the newly formed antigen to a T-cell, thereby acting as a line of defense in the innate immune system.(10) Melanocytes are normally found in the basal layer of the epithelium and histologically appear as oval shaped cells with clear cytoplasm and dendritic processes. The function of melanocytes in the oral cavity is less clear. On the skin their role is apparent as they synthesize melanin, a pigment that protects surface

cells against damaging UV radiation. In the mouth, where UV effects are negligible, it has been hypothesized that oral melanocytes may play a role in antigen capture and processing.(11,12)

Considering that PEH is a hyperplastic condition, we believe that the stratified squamous epithelium that comprises this phenomenon will contain a diverse cellular population that mirrors that of normal epithelium, including keratinocytes, melanocytes, and Langerhans cells. Conversely, since SCCa is a neoplastic clonal proliferation, we believe that it is most likely comprised of a single lineage population of clonal keratinocytes and will not have the same cellular diversity as the normal or hyperplastic stratified squamous epithelium that we expect to see in PEH. The goal of our study was to elucidate this potential difference by means of histomorphometric analysis on the proportion of melanocytes in each condition via immunohistochemistry (IHC).

IHC relies on the concept that different cells in the body express different proteins or antigens.(13) With the basic biological concept of antigen-antibody coupling, it is possible to highlight cells or tissues that react to a specific antibody to see if a protein of interest is present. Although IHC has been commonly used to help evaluate lesions, this is the first report in the literature of Melan-A being tested as a surrogate marker for melanocytes to help differentiate PEH from SCCa.

The surrogate marker Melan-A, also known as MART-1 (melanoma antigen recognized by T cells 1), is a highly specific and sensitive IHC stain for melanocytes.(14) A well-known example of a surrogate marker is the tumor suppressor p16 protein for the detection of Human Papillomavirus (HPV) in neoplastic cells. HPV's E7 protein binds to and inactivates human retinoblastoma protein (pRB) whose function is to suppress p16 production, leading to an overproduction of p16 with subsequent surrogate marker positivity.(15) In our experiment, we suspected that the proportion of melanocytes would be higher in a cellularly diverse hyperplastic oral mucosal epithelial proliferation

(PEH), and would be diminished in a clonal, non-cellularly-diverse, keratinocyte neoplastic process (SCCa).(16)

The current study is not the first attempt to distinguish the two entities via IHC. As shown in Table 2.1, various reports by other groups using other antibodies yielded mixed results. Zarovnaya et al. showed success with immunohistochemistry for p53+, MMP-20, and E-cadherin antibodies; while multiple other groups showed negative results.(8,17-20) As stated by El-Khoury et al. in 2012 "…there is no consensus on a specific immunohistochemical panel that may aid in the differentiation between PEH and SCC and more research is needed in this field."(21) Because of the striking histologic similarity, subjective interpretation of the entities, drastically different treatment hinging on the correct diagnosis, and lack of definitive work in this area, we explored whether a specific IHC marker could help separate the two conditions. Our null hypothesis was: there is no significant statistical difference between the proportion of cases that show marker expression in PEH versus SCCa. Our alternate hypothesis was: there is a significant statistical difference between the proportion of cases that show marker expression in PEH versus SCCa.

Methods

This study was approved by the University of North Carolina at Chapel Hill's Institutional Review Board (UNC IRB Study # 14-0776).

The archives of the Oral and Maxillofacial Pathology Laboratory of the University of North Carolina at Chapel Hill School of Dentistry dating from 2005-2014 were queried for qualifying cases for our study. The search fields included "pseudoepitheliomatous hyperplasia", "pseudo-epitheliomatous hyperplasia", "pseudocarcinomatous hyperplasia", and the laboratory's retrieval code for PEH. The search yielded 80 cases of PEH. All cases were re-reviewed by a board certified oral and maxillofacial pathologist. Cases were excluded on three counts: if no additional tissue was available in our archives;

if no PEH could be detected in the last cut glass slide; or if the patient was a minor at the time of biopsy. 42 of the original eighty cases remained after the exclusion criteria were applied.

The same protocol was followed to retrieve cases of SCCa. The search fields included "squamous cell carcinoma" and the laboratory's retrieval code for SCCa. All cases were re-reviewed by a board-certified oral and maxillofacial pathologist. The same exclusion criteria were used.

For analysis, 42 cases of SCCa were matched to the 42 cases of PEH on the basis of age group, sex, and location for a total of 84. Age groups were originally divided as 18-54, 55-69, and >70 years. These age groups were adapted from a study by the Surveillance, Epidemiology, and End Results Program (SEER Program) from the National Cancer Institute.(22) For the purposes of tabulating the location of the lesions within the oral cavity, ten specific locations were defined as follows: gingiva, maxillary vestibule, mandibular vestibule, buccal mucosa, hard palate, soft palate, lateral tongue, dorsal tongue, ventral tongue, and floor of mouth.

Immunohistochemical staining was performed using the Bond Fully-Automated Slide Staining System (Leica Microsystems Inc., Norwell, MA). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in a dewax solution and hydrated in a wash solution. Antigen retrieval for Melan A antibody (Leica Microsystems) was performed using an epitope retrieval solution at pH 9.0 for 20 minutes. After pretreatment, anti-Melan A was applied for 15 minutes. Detection of Melan-A was performed using a polymer refine detection system. Stained slides were dehydrated and cover-slipped. Positive and negative controls (no primary antibody) were included. Stained slides were digitally imaged at 20 × magnification using the Aperio ScanScope XT (Aperio Technologies, Vista, CA). Digital images were stored in the University of North Carolina Translational Pathology Laboratory (TPL) eSlide Manager Database (Aperio; https://tpl-spectrum.med.unc.edu) for subsequent histomorphometric analysis.

All slides contained epithelium; a board certified pathologist was recruited to discern which areas of the slides were to be histomorphologically analyzed by the computer software. The pathologist was blinded to any knowledge of the case demographics, clinical information, diagnosis, etc. The pathologist's instructions were to place pre-drawn standard-area circles, on each of the digitalized Melan-A stained slides, on the five most concerning areas for possible SCCa. If there were less than five concerning areas, he would only place circles in the areas concerning for possible SCCa. If no questionable areas existed, then he would not place any circles on that slide. The digitally pre-drawn standard area annotation circles were 0.5mm in diameter, which corresponds to the same area as a traditional microscopic high-power field (HPF).(23)

The Tissue Studio software (version 2.4.2; Definiens Inc., Carlsbad CA) was then calibrated to record a positive finding. A positive finding was defined as any positive stain signal that surrounded a pale blue area of counter stain in any of the circled fields. This area represented the periphery of the nucleus of the melanocyte. The results for each case were reported in a binary fashion: 0 = no melanocytes present, and 1 = melanocytes present.(Figures 2.3 and 2.4)

Due to lack of a high number of specimens for each of the age-sex-location matched pairs, the groups were re-assigned into broader, though still biologically relevant, groups: 1- gingiva and hard plate; 2 - maxillary vestibule, mandibular vestibule, buccal mucosa, soft palate, dorsal tongue, ventral tongue, lateral tongue, and floor of mouth. Due to small sample size, the age categories were also reassigned as: A - 18-55 years; and B - 56 years and older.

The data was then subjected to multiple McNemar's tests, and multiple conditional logistic regression tests (CLR) using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). The level of significance was set at alpha = 0.05 for all tests.

Results

The results are shown in detail on Tables 2.2 and 2.3. When calculated by McNemar's test, the proportion of Melan-A positive melanocytes between PEH and SCCa was not significantly statistically different for all cases and subgroups. Using CLR, an odds ratio (OR) of 2.75 was obtained when comparing all groups of PEH versus SCCa, but this value did not attain statistical significance (p=0.083). None of the subgroups were significantly statistically different.

Discussion

The aim of this study was to attempt to differentiate between PEH and SCCa by measuring the proportions of melanocytes. Analysis of our data determined that there were no significant statistical differences when comparing PEH to SCCa in either statistical test of all groups. While this value did not attain statistical significance (p=0.083), an OR of 2.75 that compared all cases of PEH and SCCa was obtained using CLR, and interestingly the CLR values in Table 2.3 approached a closer statistical significance than in the McNemar's tests in Table 2.2. We believe that this difference may be in part due to the nature of the tests themselves, and also due to the incorporation of the Yates correction factor to the McNemar's test in SPSS in order to make the test more conservative.(24)

Another issue worth elaborating on is how our cases were selected and arranged. We matched our cases in 1:1 pairs. This pairings offered advantages in statistical power calculations due to low sample size, and addressed any possible cofounding variables.(25) The unquestionable disadvantage of pairing the data is that diagnostic tests calculations cannot be performed. Diagnostic tests include sensitivity, specificity, likelihood ratio (LR), positive predictive value (PPV), and negative predictive value (NPV). These calculations are the standard for how markers are compared to one another. If our experiment had showed promise, larger studies could be performed using independent samples from which diagnostic tests could be calculated.

In regards to the IHC, multiple melanocytic markers are commercially available. We selected to use Melan-A in this project. The diagnostic test values for Melan-A are: 95%, 97%, 75, 97%, and 94% in regards to sensitivity, specificity, LR, PPV, and NPV, respectively. Another choice could have been microphthalmia associated transcription factor (Mitf), which has better or equal sensitivity, specificity, LR, PPV, and NPV at: 100%, 97%, 90, 97%, and 100% respectively.(Table 2.4)(26) Besides comparing diagnostic test values, one can also look at staining properties. Melan-A staining is constrained to the cytoplasm, while Mitf is constrained to the nucleus. This is important because the melanocyte is a dendritic cell that in cross section would show multiple apparently independent positive stains. If these stains were to all be counted, it would overestimate the true number of melanocytes. Conversely because cells were often cut in cross section, a total surrounding of the nucleus by the stain was not always possible. This then could have underestimated the number of melanocytes. Choosing Mitf would have allowed for any positive signal detected to be considered positive, potentially improving the objectiveness of the results. In summary, we believe that Mitf would be a better antibody to objectively evaluate for the number of melanocytes.

A previous publication by Zaronayva and Black reported success with immunohistochemical differentiation between PEH and SCCa. Their IHC choices are shown in Table 2.1. They used independent samples and thus were able to report diagnostic utility for one of their markers, Matrix metalloproteinase-1 (MMP-1) with 81% sensitivity, 94% specificity, 93% PPV, and 84% NPV; LR was not calculated in that study.(Table 2.5)(8) In their study, the use of two observers allowed for cases of disagreement to be subjectively resolved, as opposed to ours, in which the determination of marker positivity was a completely objective and automated process. This controlled for some interobserver reliability errors, which are very common problems encountered in diagnostic pathology.

There are numerous examples of intra- and inter-observer variability in the practice of surgical pathology.(27-29) We believe that this is mainly due to the subjective diagnostic parameters used in

the interpretation of the material. IHC is also a procedure that usually needs the pathologist's interpretation, and is only occasionally interpreted in an automated fashion.(30) A new trend in pathology practice is cytogenetic and molecular testing of tissue.(31) These new tests do have more objective criteria and often yield binary data, creating an unequivocal result. As technology related to these tests advances, the ability to discern subjective difficult cases will likely improve.(32) This study attempted to take a step in that direction by trying to objectively differentiate between the proportion of melanocytes in PEH and SCCa.

Conclusion

We fail to reject our null hypothesis: there is no significant statistical difference between the proportion of cases that show marker expression in PEH versus SCCa.

Tables

Table 2.1 - Published attempts of differentiating PEH from SCCa.

Group	Year	IHC	Helpful?
Lee, YS et al.	1994	p54	No
Sarda et al.	1995	AgNOR	No
Li et al.	1996	PCNA	No
Zarovnaya et. al	2005	p53, MMP-1, E-Cad, Collagen IV	Yes: p53, MMP-1, E-Cad, No: Collagen IV
Galan et al.	2007	CD1a	No
Davies et al.	2015	Melan-A	No

Table 2.2 - Results of the McNemar's tests

Group	P value	Odds Ratio	Confidence Interval
All	0.118	2.75	0.8756-8.6365
F	0.146	3	0.8122-11.0815
M	1	*	*
Age Group A	0.453	*	*
Age Group B	0.289	*	*
Location 1	0.687	*	*
Location 2	0.18	3.5	0.7271-16.8485

* = not calculated as $\alpha > 0.2$

Table 2.3 - Results of the conditional logistic regressions

Group	P Value	Odds Ratio	Confidence Interval
All	0.083	2.75	0.876-8.636
F	0.099	3	.812-11.081
M	0.571	*	*
Age Group A	0.273	*	*
Age Group B	0.178	3	.606-14.864
Location 1	0.423	*	*
Location 2	0.118	3.5	0.727-16.848

* = not calculated as $\alpha > 0.2$

Table 2.4 - Results of the diagnostic test evaluations.

	Melan-A	Mitf Comparison
Statistic	**Value**	**Value**
Sensitivity	95%	100%
Specificity	97%	97%
Likelihood Ratio	75	90
Positive Predictive Value	97%	97%
Negative Predictive Value	94%	100%

Table 2.5 – Previous study by Zarovnaya and Black that successfully differentiated between PEH and SCCa using MMP-1

Statistic	**Value**
Sensitivity	81%
Specificity	94%
Likelihood Ratio	(*)
Positive Predictive Value	93%
Negative Predictive Value	84%

* Not reported

Figures

Figure 2.1 - Pseudoepitheliomatous hyperplasia

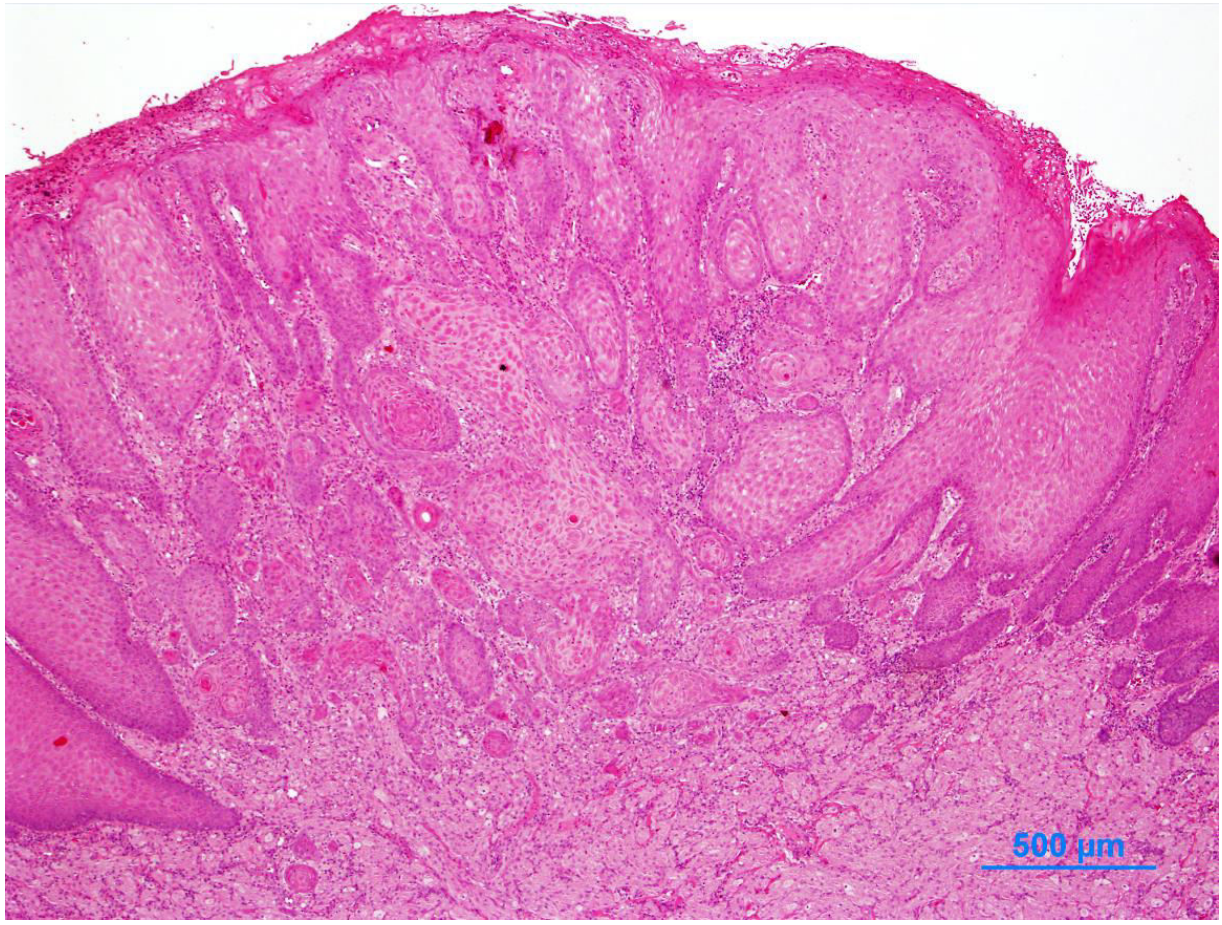

Fig 2.1 - Pseudoepitheliomatous hyperplasia (PEH) overlying a granular cell tumor (GCT). Image has been colored enhanced for brightness.

Figure 2.2 – Squamous cell carcinoma

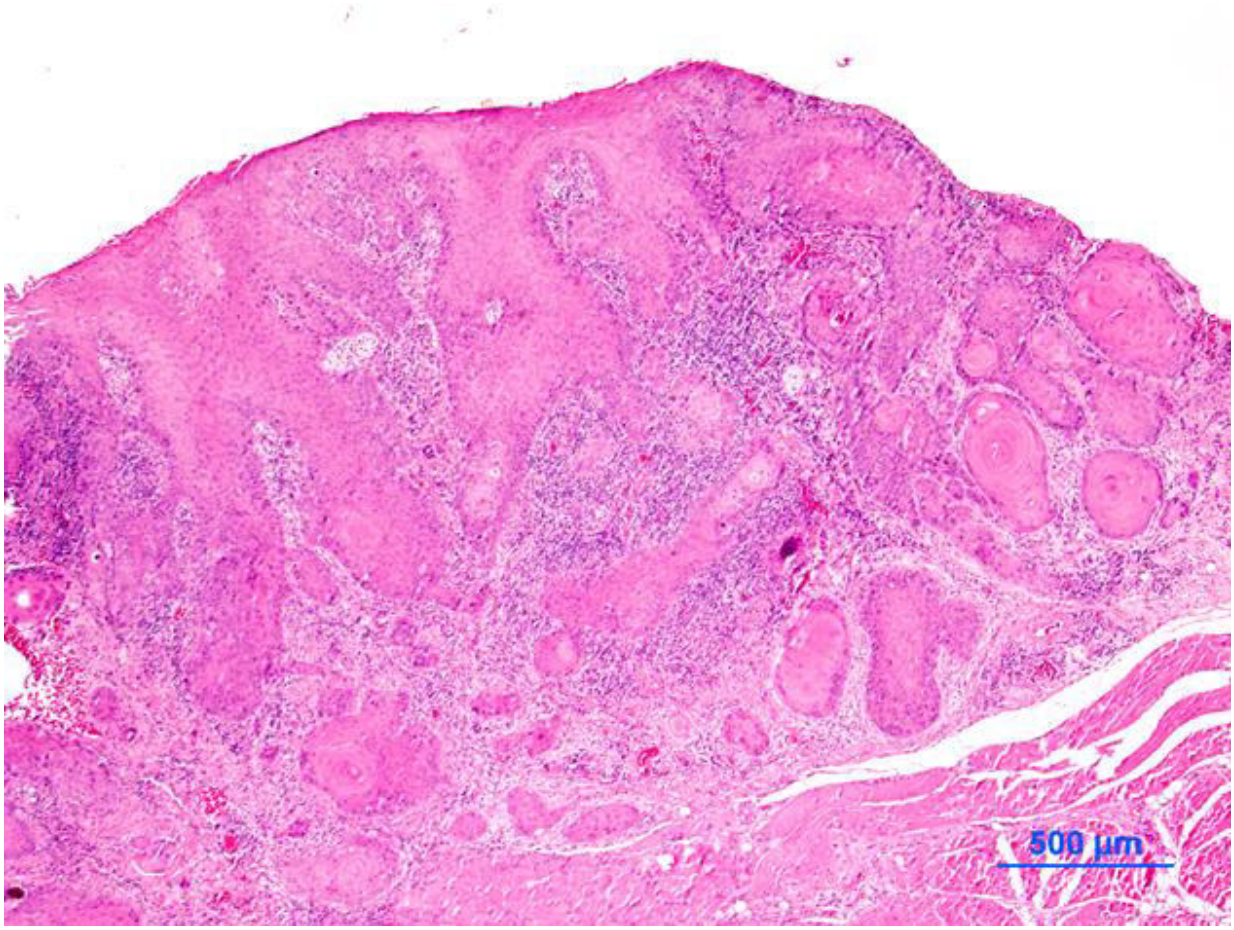

Fig 2.2 – Squamous cell carcinoma. Image has been colored enhanced for brightness

Figure 2.3 – Immunohistochemical stain and cellular quantitative analysis in a positive marker example

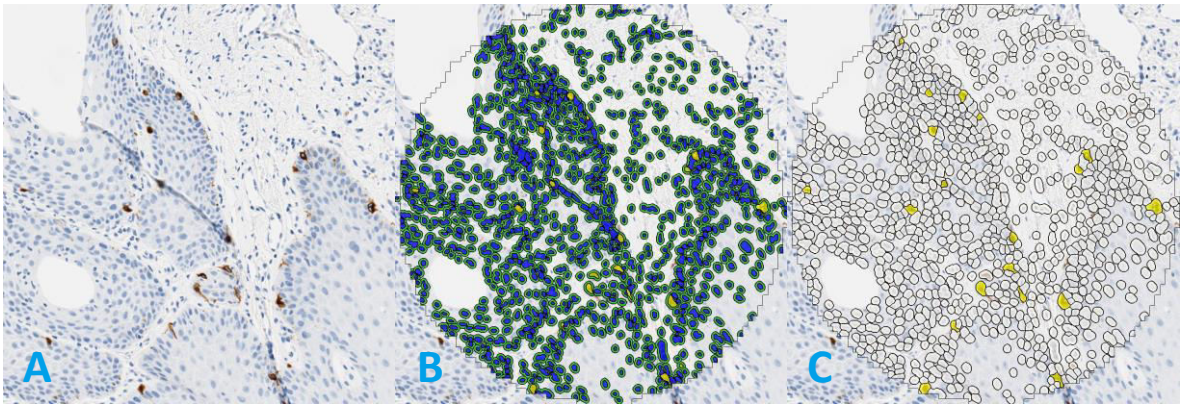

Fig 2.3- Immunohistochemical stain and cellular quantitative analysis in a positive marker example A. Immunohistochemical specimen containing melanocytes that are highlighted by the melanocytic marker Melan-A. B. Cellular analysis of all cells recognized by Tissue Studio software (version 2.4.2; Definiens Inc., Carlsbad CA). The circle indicates the pathologist's region of interest. C. Cellular analysis of suspected melanocytes recognized by Tissue Studio software (version 2.4.2; Definiens Inc., Carlsbad CA) yielding a positive marker result. The circle indicates the pathologist's region of interest.

Figure 2.4 – Immunohistochemical stain and cellular quantitative analysis in a negative marker example

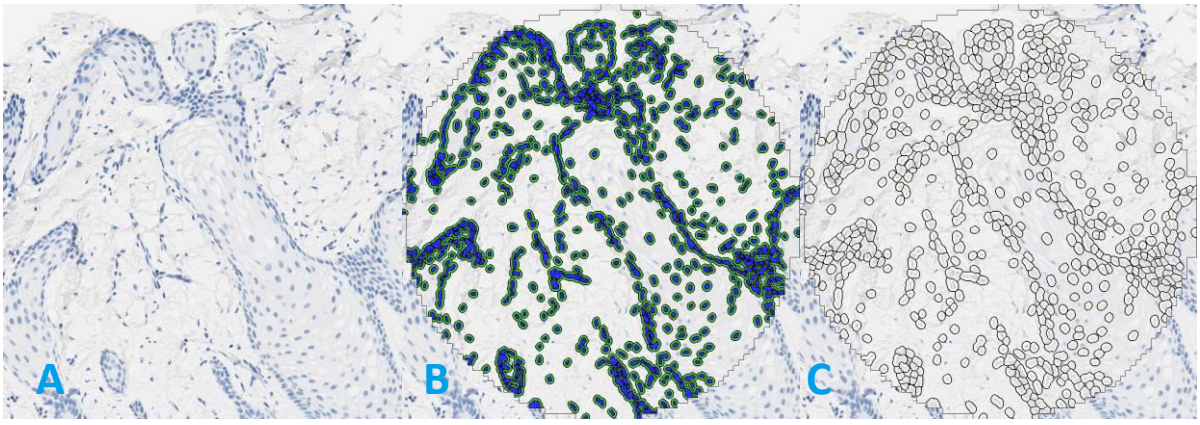

Fig 2.4- Immunohistochemical stain and cellular quantitative analysis in a negative marker example A. Immunohistochemical specimen containing no detectable melanocytes by the melanocytic marker Melan-A. B. Cellular analysis of all cells recognized by Tissue Studio software (version 2.4.2; Definiens Inc., Carlsbad CA). The circle indicates the pathologist's region of interest. C. Cellular analysis of of no melanocytes yielding a negative marker result. The circle indicates the pathologist's region of interest.

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