Ankara Üniv. Vet. Fak. Derg.
43: 281-285, 1996

COMPARISON OF AgNORs STAINING AND PCNA IMMUNOSTAINING METHODS, AND MITOTIC INDEX SCORES IN INTRACUTANEOUS CORNIFYING EPITHELIOMA AND SQUAMOUS CELL CARCINOMA

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Yassı hücreli kanser ve intrakutan kornifiye epitelyoma'da AgNORs ile PCNA boyama metodları ve mitotik indeks sonuçlarının karşılaştırılması

Özet: *Deri tümörleri arasında bulunan intrakutan kornifiye epitelyoma ve iyi differensiye yassı hücreli kanser birbirine benzer tümörlerdir. Bu çalışmanın amacı, prolifere hücre nüklear antijen (PCNA), klon PC10 immunhistokimyasal boyama ve argirofil nükleolar organize bölgeler (AgNORs) boyama metodlarını ve mitotik indeksi bu iki tümörde karşılaştırmaktır. Çalışma için köpeklerden alınan 5 adet intrakutan kornifiye epitelyoma ve çeşitli hayvan türlerinden temin edilen 20 adet iyi differensiye yassı hücreli kanser seçildi. Bütün örnekler formalinde fikze edilip parafinde bloklandı. Ortalama AgNOR sayısı her lezyon için 50 bazal ve 50 suprabazal hücrede immersiyon ile 1000 büyütmede saptandı. PCNA işaretli kısımlar 10 değişik büyütme alanında hesaplandı. PCNA indeksi, PCNA pozitif boyanan tümör nukleuslarının, seçilen alanlardaki toplam hücre adedine bölünüp yüzde cinsinden ifadesi ile hesaplandı. Tümörlerdeki mitotik indeks 10 değişik büyütme alanında tespit edildi. İntrakutan kornifiye epitelyoma ve iyi differensiye yassı hücreli kanser bu üç yöntemle karşılaştırıldığında Mann-Whitney U testinde AgNOR sayısı önemli ($p < 0,01$), fakat PCNA indeksi ve mitotik indeks önemli bulunmadı.*

Anahtar Kelimeler: *AgNOR boyama, intrakutan kornifiye epitelyoma, mitotik indeks, PCNA, yassı hücreli kanser*

Summary: *Among the skin tumors, intracutaneous cornifying epithelioma and well-differentiated squamous cell carcinoma are similar. The purpose of this study was to compare the immunostaining for proliferating cell nuclear antigen (PCNA) clone PC10, interphase nucleolar organizer regions staining (AgNORs) methods and mitotic index in these tumors. Five intracutaneous cornifying epitheliomas in the dogs and 20 well-differentiated squamous cell carcinomas in various species were chosen. All samples were fixed in formalin and embedded in paraffin. The mean number of AgNOR was determined in 50 basal and 50 suprabasal cells of each tumor under an oil immersion lens at a magnification of 1000. PCNA-labeled fractions and mitotic index were estimated in the same areas of these tumors in 10 high power f ields. AgNOR count ($p < 0,01$) was significant, but PCNA index and mitotic index were not significant in Mann-Whitney U test to compare intracutaneous cornifying epithelioma and well-differentiated squamous cell carcinoma.*

Key words: *AgNOR staining, intracutaneous cornifying epithelioma, mitotic index, PCNA, squamous cell carcinoma*

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Introduction

Nucleolar organizer regions (NORs) are specific nucleolar components. They represent the sites for ribosomal RNA transcription. NOR-associated proteins bind silver (5).

The scoring of interphase NOR numbers using the argyrophil technique has been shown to be of use in assessing the degree of malignancy in neoplasms (4, 6, 10).

The proliferating cell nuclear antigen (PCNA), is a 36-kD nonhistone nuclear protein which is directly involved in DNA synthesis (16). PCNA levels increase rapidly in mid-G1, remain elevated throughout S-phase and begin to decrease from G2/M to G1-phase of cell cycle (1). Several recent papers suggest that anti-PCNA antibodies could be employed to assess the proliferative activity of tumors (13, 22, 25).

Intracutaneous cornifying epithelioma is a distinct neoplasm observed only in the dog (17). Intracutaneous cornifying epithelioma and keratoacanthoma in humans are similar in many respect, but they are not identical entities (21, 26). Keratoacanthoma in humans is a skin tumor, often demonstrates a clinical and histologic resemblance to differentiated-squamous cell carcinoma. Although a variety of histological criteria have been applied to differentiate keratoacanthoma from squamous cell carcinoma in humans (12, 14, 15), some of them have proved entirely reliable.

Although there is no doubt of the diagnosis of these tumors in animals, we want to compare nucleolar organizer region (NOR) counts, PCNA and mitotic index in well-differentiated squamous cell carcinoma and intracutaneous cornifying epithelioma.

There are very few reports in the veterinary oncology on these techniques.The PCNA immunostaining method and AgNORs staining method applied in this study was used first time in veterinary oncology in Turkey.

Materials and Methods

Four μm thick sections from biopsy specimens were included in this study. After routine staining with hematoxylin-eosin of the first sections, the specimens were identified as being either intracutaneous cornifying epithelioma or well-differentiated squamous cell carcinoma.

Paraffin sections from 20 cases of histologically typical differentiated squamous cell carcinoma and five cases of intracutaneous cornifying epithelioma were stained by a modification of the silver NOR technique and immunostaining for PCNA, clone PC10.

The second sections (four μm) from the same blocks were stained by a modification of the silver NOR technique described by Ploton et al. (19).We prefer this modified technique because overstaining produce stain deposit. The sections were taken to water via xylene and graded alcohols. The sections were submitted to the AgNOR procedure at 37°C for 75 minutes. The reaction mixture comprised 2 % gelatin in 1 % aqueous formic acid. This was mixed in a proportion of 1:2 volumes with 25% aqueous silver nitrate. The reaction was stopped by washing in ultrapure water for 2x5 minutes and placed in 5% thiosulfate solution for 2x5 minutes. Tissues were washed again with distilled water. No counterstain was used. All counts were performed by direct observation. Sections were examined under an oil immersion lens at a magnification of 1000 and 100 nuclei were studied. A variation in NOR counts was apparent in different areas of the tumors, therefore fields were randomly selected 50 basal and 50 suprabasal nucleus for AgNOR counting in each tumor.

All AgNOR dots, either in nucleolus or scattered in the nucleus were counted according to Crocker et al.'s method (3). The mean number of AgNORs per nucleus was calculated for each specimen.

The third sections (four μm) from the same blocks were stained by immunostaining for PCNA, clone PC10. The sections were dried overnight at room temperature and immunostained with PC10. The tissues were deparaffinized in two changes of xylene for 10 minutes each, rehydrate in 100 %, 95% and 70% alcohol and placed in distilled water for 10 minutes. Endogenous peroxidase was blocked with 3% H_2O_2 for five minutes, then the slides were washed with tris-buffer. Sections were then slides were washed with tris-buffer. Sections were then incubated with Enhanced Polymer One-Step Staining (EPOS) anti-proliferating cell nuclear antigen/HRP (DAKO, clone PC10, Glostrup, Denmark) for 60 minutes at room temperature, after a wash in tris-buffer, the sections then were incubated with diaminobenzidine/H_2O_2 for five minutes. The slides were washed in tris-buffer and counterstained with Mayer's haematoxylin for 30 seconds. The sections were washed in tap water, taken through alcohol to xylene and mounted in synthetic medium. Negative control sections were incubated in buffer instead of anti-PCNA antibody dilution. All immunostained sections were examined under an oil immersion lens at a magnification of 1000. Totally 10 high power fields were chosen. The value of PC10 index was defined as the number of positive tumor nuclei divided by

he total number of cells expressed as a ercentage were considered.

Mitotic figures were counted in anti-PCNA tained sections in a total of 1000 cells presents in he same tumor area where PCNA indexes were stimated.Mann-Whitney U test was used to tatistical analysis.

Results

The mean values of the kinetic parameters, the AgNOR counts, PCNA and mitotic indexes for all the cases counted, are summarized in table-1.

able 1. Mean values (± Standart deviations), minimum and maximum values of the AgNOR counts, PCNA index and mitotic index in tracutaneous cornifying epithelioma and squamous cell carcinomas.

Types of turnors	Type of test	Mean	Standart Dev.	Min.	Max.
Intracutaneous Cornifying Epithelioma	AgNOR	4.65	0.99	3.49	5.46
	PCNA	4.92	2.16	2.13	7.16
	Mitosis	9.72	4.36	2.83	13.29
Squamous Cell Carcinoma	AgNOR	13.02	4.81	4.23	25.84
	PCNA	6.85	1.60	2.88	9.86
	Mitosis	10.15	6.72	1.62	28.29

In sections, AgNORs were easily dentified as brown or black dots (fig.1.A, B). he mean for the number of AgNORs per ucleus was 13,02 and the range 4,23-25,84 in ifferentiated squamous cell carcinomas. ntracutaneous cornifying epithelioma had a ean number of 4,65 AgNORs with range of ,49-5,46 (fig. 2).

The nucleus of tumor cells positive for CNA stained light to dark brown (fig.1.C, D) nd PCNA reactivity was expressed by diffuse uclear stai staining. Both strongly labeled uclei and less intensely labeled nuclei were ounted. Negative nuclei were light blue. The alue of this activity, for PCNA ranged between ,88% and 9,86% in differentiated squamous ell carcinoma and 2,13% and 7,16% in ntracutaneous cornifying epithelioma (fig.3).

The number of mitotic index ranged etween 1,62 and 28,29/1000 cells in ifferentiated squamous cell carcinoma and 2,83 nd 13,29/1000 cells in intracutaneous ornifying epithelioma (fig. 4).

Statistic analysis of AgNOR counts (p< ,01) was found significant but PCNA and itotic indexes were not statistically significant ol for these tumors in our study.

igure 1. A. AgNOR dots in intracutaneous cornifying epithelioma lls (arrows). A few dots are visible in each nucleus. Silver colloid, counterstain, x1000 magnification.

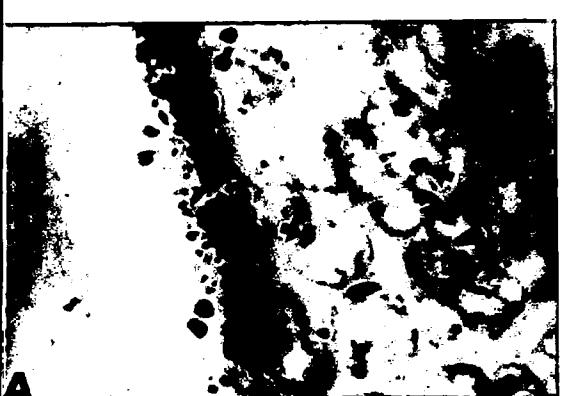


Figure 1. B. AgNOR dots in squamous cell carcinoma cells (arrows). In contrast to intracutaneous cornifying epithelioma cells in Fig 1A, these nuclei contain multiple dots. Silver colloid, no counterstain, x1000 magnification.

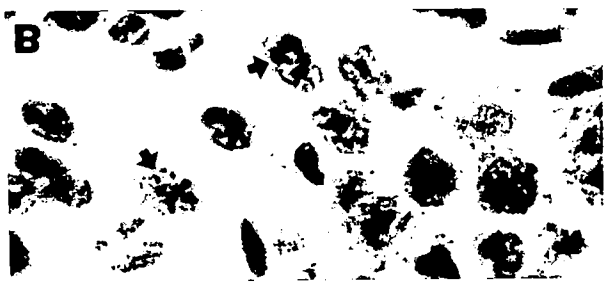


Figure 1. C. PCNA-positive nuclei in intracutaneous cornifying epithelioma (arrows). A few PCNA-positive nuclei are seen. Immunoperoxidase staining, Mayer's hematoxylin counterstain, x350 magnification.

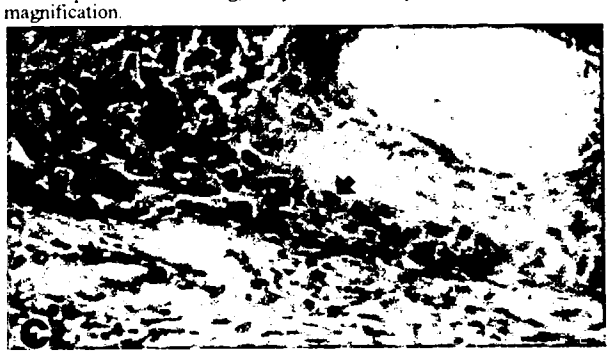


Figure 1. D. PCNA-positive nuclei in squamous cell carcinoma (arrows). A few PCNA-positive nuclei are seen. There is a little difference from Fig 1C. Immunoperoxidase staining, Mayer's hematoxylin counterstain, x1000 magnification.

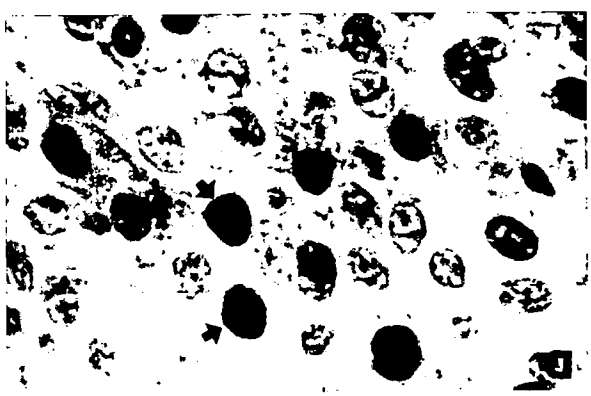


Figure 2. Box-plots showing the mean number of AgNORs per nucleus for specimens examined. As seen in diagram, squamous cell carcinomas (SCC) can easily distinguish from intracutaneous cornifying epithelioma (ICE).

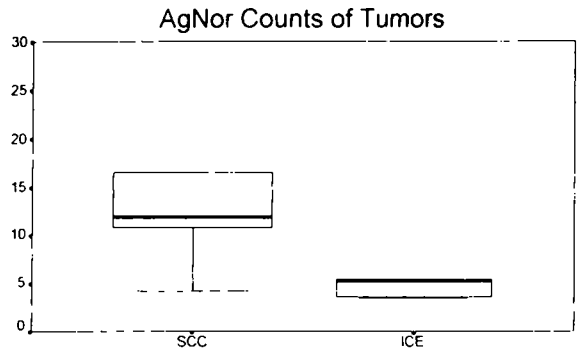


Figure 3. Distribution of PCNA indexes of the tumors in box-plots. There is not a significant difference between squamous cell carcinomas (SCC) and intracutaneous cornifying epithelioma (ICE).

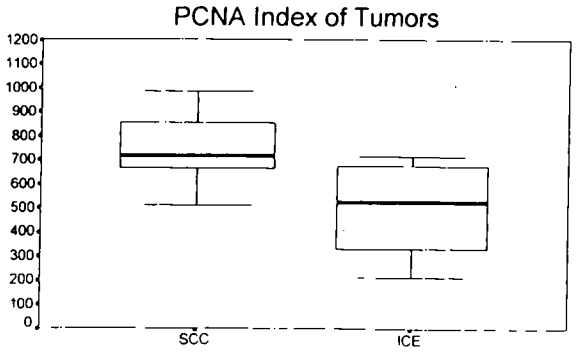
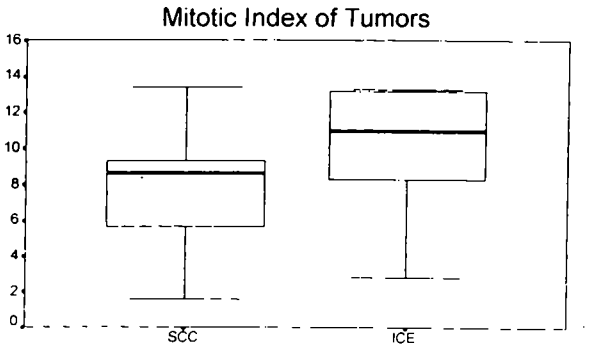


Figure 4. Distribution of mitotic indexes of the tumors. As shown in box-plots; there is no difference between squamous cell carcinomas (SCC) and intracutaneous cornifying epithelioma (ICE).



Discussion

Various methods has been reported to compare squamous cell carcinoma and keratoacanthoma in humans. It has been shown that flow cytometry measurements of DNA-index and proliferative index did not help in separating keratoacanthoma and well-differentiated squamous cell carcinoma of skin in humans (20, 27). Ho et al. (14), suggested that transforming growth factor α expression may be a marker of epithelial differentiation and may help distinguish between these two tumors. It has been reported that electron microscopy has revealed decreased numbers of tonofilaments and intercellular desmosomes in well-differentiated squamous cell carcinoma as compared to keratoacanthoma (12). However, the same authors did not believe that the subjective nature of those findings does yield reliable criteria for the individual case.

The estimation of tumor proliferative activity by means of antibodies to cell cycle related antigen is widely employed in human and animal pathology (7, 13, 23). In our study, we counted both strongly labeled and more weakly labeled nuclei which belong to different phases of cell cycle, in anti-PCNA immunostaining method. However, we could not find a correlation between intracutaneous cornifying epithelioma and well-differentiated squamous cell carcinoma and the anti-PCNA immunostaining method and also mitotic index can not be considered as a statistically significant tool in these lesions. To date no reports are available on comparison of the difference in values of these methods for the two tumors. However, Ehlers et al. (11) and Piscioli et al. (18), clearly demonstrated that keratoacanthomas are a heterogeneous group of lesions, including pseudocarcinomatous, precancerous and cancerous neoplasms by histophotometric investigation of content of nuclear DNA. They claimed that keratoacanthoma is not by definition a benign lesion in every instance, because its components' cells have a content of malignant DNA, though rarely. However, intracutaneous cornifying epithelioma is different from keratoacanthoma (21, 26), but this situation may be important also for intracutaneous cornifying epitheliomas. Although the invasion of tumor cells of intracutaneous cornifying epithelioma into the stroma from basement membran is not seen, it is thought that in this study this tumor may be a precancerous tumor according to the PCNA immunostaining method.

The counting AgNORs is used for assessing the degree of malignancy (6, 8, 9). In some cases this staining method are shown for the prognosis and treatment of the patient (2, 24, 28). In our study, only AgNOR counts were the predictor according to the other assessment of proliferative activity methods. The squamous cell carcinomas have higher AgNOR scores per nucleus than the intracutaneous cornifying epitheliomas.

AgNOR indices proved to be useful for determining the proliferative activity of squamous cell carcinoma and intracutaneous cornifying epithelioma. The results in this study confirm only the value AgNORs as the markers for proliferative activity.

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