ORIGINAL ARTICLES

Immunohistochemical Study of Calretinin in Normal Hair Follicles and Tumors With Follicular Differentiation

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Abstract. *Background.* Selective immunostaining for calretinin labels the innermost layer of the outer root sheath of normal hair follicles, which is difficult to distinguish with hematoxylin-eosin staining.

Objective. The aim of this study was to determine whether immunohistochemistry for calretinin allows identification of cutaneous adnexal tumors with follicular differentiation towards cells of the outer root sheath.

Material and methods. We analyzed the staining pattern for calretinin by immunohistochemistry in 49 biopsies of cutaneous adnexal tumors with follicular differentiation.

Results. Fifteen biopsies corresponded to trichilemmomas/inverted follicular keratosis and had staining for calretinin in the epithelium of the most superficial areas of the lesions and in squamous eddies. Ten were trichilemmal cysts, which displayed staining of the cyst wall. Three were basal cell carcinomas with variable staining according to the type of follicular differentiation in each variant. One was a panfolliculoma that had focal staining. Two were folliculosebaceous cystic hamartomas with staining of the excretory duct of the sebaceous glands. Two pilomatricomas and 3 proliferative trichilemmal tumors had positive staining in the cellular layers close to the lumen of the cystic structures. Nine trichoblastomas/trichoepitheliomas, 2 infundibular cysts, 1 dilated pore of Winer, and 2 acanthomas of the follicular sheath were negative for calretinin.

Conclusion. Immunohistochemistry for calretinin allows identification of cutaneous adnexal tumors of the hair follicle or a component of the follicle with differentiation towards cells of the outer root sheath.

Key words: calretinin, cutaneous adnexal tumors, follicular differentiation, companion layer.

ESTUDIO INMUNOHISTOQUÍMICO DE LA CALRETININA EN EL FOLÍCULO PILOSO NORMAL Y NEOPLASIAS CON DIFERENCIACIÓN FOLICULAR

Resumen. *Introducción.* La inmunotinción selectiva con calretinina evidencia la capa más interna de la vaina radicular externa del folículo piloso normal, difícil de distinguir con la tinción de hematoxilina-eosina.

Objetivo. Conocer si calretinina nos permite identificar neoplasias anexiales con diferenciación hacia la vaina radicular externa del folículo piloso.

Material y métodos. Hemos analizado el patrón de inmunotinción para calretinina en 49 biopsias de distintas neoplasias anexiales cutáneas con diferenciación folicular.

Resultados. Quince de las 49 tinciones correspondían a tricolemomas/queratosis folicular invertida, observándose positividad con calretinina en el epitelio de las áreas más superficiales de la lesión y en los remolinos escamosos; 10 quistes tricolémicos con positividad en su pared, tres carcinomas basocelulares con positividad variable dependiendo del tipo de diferenciación folicular de cada variante, un panfoliculoma con positividad focal, dos hamartomas sebáceos infundíbulo-quísticos con positividad en el conducto excretor de las glándulas sebáceas, dos pilomatricomas y tres tumores tricolemales proliferantes con positividad en las capas celulares cercanas a la luz de las estructuras quísticas, 9 tricoblastomas/tricoepiteliomas, dos quistes infundibulares, un poro dilatado de Winer y dos acantomas de la vaina folicular resultaron negativos.

Conclusión. El estudio inmunohistoquímico de la calretinina nos permite identificar neoplasias anexiales del folículo piloso o componentes de éste, con diferenciación hacia la vaina radicular externa del folículo piloso.

Palabras clave: calretinina, neoplasias anexiales con diferenciación folicular, capa compañera.

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Introduction

Calcium is an intracellular messenger that acts mainly through a series of proteins able to bind calcium. There are 2 main calcium binding proteins: calmodulin-like proteins that belong to the EF-hand family, that is, they contain the EF-hand domain in their structure allowing them to interact with 2 calcium ions, and those—grouped under the generic name of annexins—that bind calcium and phospholipids.

Calretinin is a 29-kDa calcium binding protein that belongs to the EF-hand family, which also includes proteins such as protein S100 and parvalbumin. Calretinin has a characteristic structure containing 6 EF-hand domains. The calretinin gene displays 60% homology with calmodulin and the main function of the protein is that of a buffer, preventing an excessive buildup of intracellular calcium.[1] Calretinin has been shown to be present at certain times during the cell cycle, specifically in the first phase of growth (G1 phase) and mitosis associated with kinetochore, which is responsible for ensuring a normal chromosomal distribution during cell division.

Currently, immunohistochemistry for calretinin is used as a marker of mesothelial differentiation in tumors and is important in the differential histopathologic diagnosis between malignant mesothelioma and metastatic serous adenocarcinoma.[1]

Lugli et al[2] studied 5233 samples of normal and tumor tissue using immunostaining for calretinin. They reported no calretinin staining for cutaneous adnexal tumors (35 samples), angiosarcoma (3 samples), capillary hemangioma (10 samples), epithelioid hemangioma (2 samples), giant cell tumor of the tendon sheath (29 samples), glomus tumor (7 samples), Kaposi sarcoma (18 samples), lipoma (35 samples), benign histiocytoma (13 samples), dermatofibrosarcoma protuberans (3 samples), and melanocytic nevus (18 samples), among others. However, a few samples of squamous cell carcinoma and malignant melanoma were positive (7.7% and 4.7%, respectively). Another study analyzed 24 follicular cysts with hematoxylin-eosin and anti-calretinin but did not detect any immunoreactivity for calretinin.[3]

The pilosebaceous and apocrine unit contains the following structures:

1. The hair follicle itself (including the hair shaft), which can be divided into 3 segments: the infundibulum, the isthmus, and the lower segment. Only the lower segment (the mobile or transient part of the follicle) undergoes noteworthy changes during the hair growth cycle, whereas the isthmus and infundibulum (the fixed or permanent part of the follicle) remain unchanged.
2. One or more sebaceous glands
3. An apocrine gland (at certain anatomic sites)
4. Smooth muscle fibers that form the arrector pili muscle
5. The hair disk
6. Nerve fibers around the follicle

The infundibulum is lined with an epithelium almost identical to that of the epidermis, with a basal layer of palisaded columnar cells, 4 or 5 layers of keratinocytes with increasing keratinization towards the lumen of the infundibulum, a well-developed stratum granulosum made up of 1 or 2 layers of flattened cells with abundant keratohyalin granules, and a lamellar basophilic and orthokeratotic stratum corneum.

The isthmus is lined with an epithelium comprising an outer layer of palisaded keratinocytes over a thick basement membrane, 4 to 5 layers of polygonal keratinocytes containing large amounts of cytoplasm, and eosinophils that are increasingly keratinized towards the hair canal. Keratinization is only evident in the upper part of the isthmus, where the completely keratinized and desquamated inner root sheath has disappeared. Once this has happened, keratinization of the outer root sheath can take place to give rise, without the prior formation of a granular layer, to a compact and orthokeratotic eosinophilic keratin with a sawtooth or undulating edge.

The hair bulb is the deepest part of the lower segment of the hair follicle. When the follicle is in the anagen phase, this lower segment has the appearance of tongs holding the fibrocytes of the dermal papilla.

The matrix cells are the ones that give rise to several concentric cylinders of cells that form the layers of the hair shaft and the inner root sheath of the follicle. These layers are as follows, from internal to external: hair medulla, hair cortex, hair cuticle, cuticle of the inner root sheath, Huxley layer, and Henle layer. During anagen, these matrix cells of the lower segment of the hair bulb appear as immature epithelial cells that have large, round, vesicular, similarly structured nuclei with a prominent nucleolus and little cytoplasm. These cells are highly proliferative and so mitotic figures are often seen. Dendritic melanocytes are often observed between these cells and the epithelial matrix cells.

The outermost layer of the inner root sheath distinguishable as an independent layer is called the Henle layer and comprises a single layer of cells. The Henle layer contains trichohyalin granules and is the first to become keratinized where narrowing of the outer root sheath occurs. The Huxley layer, located immediately within the Henle layer, contains 2 layers of cells that begin to become loaded with trichohyalin granules and which are then lost as it becomes keratinized. The innermost layer of the inner root sheath is the cuticle, which is comprised of a single cell layer and only acquires trichohyalin granules where the Henle layer begins to lose them. These 3 layers of the inner root sheath merge at the point where they have become completely keratinized to give a single layer of amphophilic or weakly basophilic keratin, which rises vertically between

Table. Results Obtained for Calretinin Immunostaining

Tumor	Number	3[a]	2[b]	1[c]
Trichilemma/inverted follicular keratosis	15	15	0	0
Trichilemmal cyst	10	10	0	0
Basal cell carcinoma	3	1	1	1
Panfolliculoma	1	0	1	0
Trichoblastoma/trichoepithelioma	9	0	9	0
Folliculosebaceous cystic hamartoma	2	2	0	0
Pilomatricoma	2	0	2	0
Proliferative trichilemmal tumor	3	0	3	0
Infundibular cyst	2	0	2	0
Dilated pore of Winer	1	0	0	1
Acanthoma of the follicular sheath	2	0	0	2

[a]Positive immunostaining for calretinin [b]Focally positive immunostaining for calretinin [c]Negative immunostaining for calretinin

the hair cuticle on the inside and the outer root sheath on the outside, until reaching the isthmus where desquamation occurs and the layer disappears.

The hair shaft or hair originates from cells from the center of the matrix and the area above the matrix, that is, those matrix cells immediately above the tip of the dermal papilla. The matrix cells mature as they approach the surface, becoming longer and losing their nuclei. The cells of the 3 layers of the hair shaft (hair medulla, cortex, and cuticle) keratinize without trichohyalin granule formation.

Follicular differentiation is considered to occur in cutaneous adnexal tumors when the cells show, to a greater or lesser extent, any of the histologic structures of the normal hair follicle in any of the phases of the hair growth cycle.

Figure 1. Acanthoma of the follicular sheath. Strongly positive for calretinin in the epithelium of the follicular sheath (calretinin, ×10).

Materials and Methods

For this study, we selected 49 biopsy samples from different types of both benign and malignant cutaneous adnexal tumors with follicular differentiation and we classified them according to the criteria described by Requena et al.[4] Thus, we treated inverted follicular keratosis and tricholemmoma as essentially the same entity, that is, both correspond to common warts with trichilemmal differentiation and so were classed in the same group. Similarly, trichoepithelioma, desmoplastic trichoepithelioma, and cutaneous lymphadenoma (adamantinoid trichoblastoma) were considered as histopathologic variants of trichoblastoma because they exhibit differentiation towards germinal cells of the hair follicle and an abundant fibrocytic stroma that mimics the dermal papilla and the connective tissue sheaths around the follicle. The remaining cutaneous adnexal tumors with follicular differentiation in this study were classed according to the nomenclature commonly used in the literature.

First, the immunostaining pattern for calretinin was investigated in normal skin and its appendages. We then studied the immunostaining of tumor cells with anti-calretinin antibodies (a cytoplasmic marker) in each of the samples. The intensity of staining with calretinin was described semiquantitatively by assigning a value of 1, 2, or 3, and those with immunoreactivity were determined. Tumors that were negative for calretinin were assigned a value of 1, those that stained focally positive were assigned a value of 2, and those that stained clearly positive were assigned a value of 3 (Table).

Results

Of the 49 biopsies studied, 15 corresponded to tricholemmomas/inverted follicular keratosis, 10 to trichilemmal cysts, 3 to basal cell carcinomas, 1 to a panfolliculoma, 9 to trichoblastomas/trichoepitheliomas, 2 to folliculosebaceous cystic hamartomas, 2 to pilomatricomas, 3 to proliferative trichilemmal tumors, 2 to infundibular cysts, 1 to dilated pore of Winer, and 2 to acanthomas of the follicular sheath (Figure 1). All 15 cases of tricholemmoma/inverted follicular keratosis stained positive in the two-thirds of the epithelium of the superficial areas of the tumor and in the squamous eddies, with the basal cell layers clearly testing negative (Figure 2). The 10 trichilemmal cysts stained strongly positive throughout the entire cyst wall, except for the basal layer of the tumor epithelium. In the 3 basal cell carcinomas with follicular differentiation, the findings varied according to the type of follicular differentiation present. A basal cell carcinoma with adenoid-cystic differentiation exhibited a strongly positive tumor epithelium, a superficial basal cell carcinoma

that was focally positive in the outermost layer of tumor islands, and a basal cell carcinoma with matrix differentiation was completely negative for calretinin. A panfolliculoma exhibited focal positivity in the epithelium on the boundary of the cystic lumen with trichilemmal differentiation and negativity in the rest of the tumor epithelium. Nine trichoblastomas/trichoepitheliomas were studied, and focal staining was observed in the tumor epithelium. Two folliculosebaceous cystic hamartomas were strongly positive in the excretory duct of the sebaceous glands (Figure 2). Two pilomatricomas stained positive in the cell layers next to the shadow cells, which themselves were negative for calretinin as were the cells of the basal layer of the tumor epithelium (Figure 3). The cells of the tumor epithelium close to the lumen of 3 proliferative trichilemmal tumors were positive. Two infundibular cysts, a dilated pore of Winer, and 2 acanthomas of the follicular sheath exhibited no immunoreactivity for calretinin.

Discussion

The hair follicle is made up of the following concentric layers, from outermost inwards: the outer root sheath, the inner root sheath, and the hair shaft. The outer root sheath does not have a homogeneous cell population. Ultrastructural studies have identified 2 types of layer: a) an external stratum of several layers of cuboid cells containing large quantities of glycogen in their cytoplasm and b) an internal stratum formed from a single cell layer, which is in close contact with the Henle layer of the outer root sheath. This internal stratum was described by Ito[5] as the innermost layer of the outer root sheath and was denominated the *companion layer* by Orwin[6] given that it is closely related to the Henle layer.

Although it is still not completely clear, it is likely that the cells that make up the outer root sheath of the hair follicle do not originate from matrix cells of the hair bulb but rather descend from the lower edge of the isthmus or from the *bulge*, that is, the insertion point for the arrector pili muscle, during each new anagen phase until reaching the lower end of the hair follicle.[4] Thus, in each new anagen phase, the root sheaths of the hair follicle mature in opposite directions: the outer root sheath descends from the isthmus towards the deep dermis to line the inner root sheath of the lower segment whereas the cells of the inner root sheath and the hair shaft itself mature as they move upwards towards the surface. It is thought that the companion layer moves in conjunction with the maturing inner root sheath, that is, from the bulb towards the isthmus.[4] When differentiation is complete, at the upper boundary of the isthmus, both layers, the inner root sheath and the cornified companion layer, are degraded and remain in the follicular lumen, whereas trichilemmal keratinization takes place in the cells of the outer root sheath.
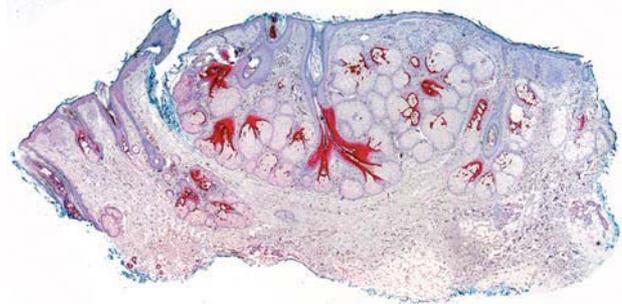
Figure 2. Sebaceous hyperplasia. Immunoreactivity of the sebaceous canal (calretinin, ×4).

Figure 3. Pilomatricoma. Immunoreactivity of the epithelium of the wall (calretinin, ×10).

A recent study by Poblet et al[7] showed that the unique morphologic and biochemical characteristics of the companion layer distinguished it from other layers of the hair follicle in the following ways:

1. The companion layer consists of a single layer of flattened cells unlike the multiple layers of cuboid cells that make up the outer root sheath.
2. The cells of the companion layer adhere to the Henle layer (through desmosomes) and not to the layers that make up the outer root sheath (intercellular spaces without desmosomes). Hnakawa et al[8] have recently reported the expression of desmoglein 1 and 3 in the companion layer of the hair follicles of mice, an observation that underlines the importance of the companion layer in the mechanism that anchors the hair to the follicle in the anagen phase.
3. The cells of the companion layer do not contain large amounts of glycogen in their cytoplasm, unlike those that form the outer root sheath. They also do not contain trichohyalin granules, unlike the cells of the inner root sheath.[9]

4. The cells of the companion layer accumulate a material similar to keratin, mainly at the level of the Henle layer.[10]

5. The cells of the companion layer express a single cytokeratin—K6hf—during cell differentiation,[11] unlike those of both the external and inner root sheath.

6. The companion layer and the cells that form the outer root sheath stain positive for immunohistochemical markers such as Ki-67,[12] plasminogen activator inhibitor type 2,[13] microtubule associated protein 2,[14] and calretinin.[5]

Several hypotheses have been put forward to explain the function of the companion layer. Orwin et al[6] postulated that it might play a role in supplying nutrients to the cells of the inner root sheath, and that it is also probably implicated in the differentiation and subsequent destruction of the hair canal. This layer might also help to maintain the integrity of both the internal and outer root sheaths of the hair follicle. Ito[10] proposed that, in view of its growth both circumferentially and perpendicularly to the hair shaft, the companion layer supports and lines the internal structures of the hair follicle like the "hoops of a barrel." Winter et al[15] found that a mutation in the companion layer-specific keratin *K6hf* gene could be a risk factor for pseudofolliculitis barbae and suggested that disruption of keratin K6hf might affect the mechanical integrity of the companion layer and its adherence to the inner root sheath.

The function of calretinin is also unknown. Calcium-dependent regulation is important for controlling the intracellular homeostasis of calcium, and it is thought that it is essential for the development of all types of hair follicles.[16] It has been shown that the calcium binding proteins are implicated in the growth and differentiation cycle and that these proteins may play an important role in the activation of stem cells at the start of follicular regeneration.[17] Some components of the S100 subfamily might not only modulate intracellular calcium homeostasis during differentiation in the follicular cycle but also have other functions such as controling the reorganization of the keratin network.[18]

In an evaluation of the immunohistochemical expression of calretinin in the diagnosis of cutaneous metastases of mesothelioma, Poblet et al[7] also found that calretinin is a selective marker of the innermost layer of the outer root sheath. The region above the hair bulb, before keratinization of the Henle layer occurs, was found to be positive but calretinin disappeared just before the inner root sheath disintegrates due to apoptosis. Calretinin immunostaining of the companion layer persists even in catagen follicles.[7]

There were 10 trichilemmal cysts among the 49 samples studied, and these were strongly positive throughout the entire thickness of the epithelium of the cyst wall. Given that calretinin immunostaining serves to identify the innermost layer of the outer root sheath or companion layer, it is reasonable to suppose that the trichilemmal cysts would be strongly positive because their walls are identical to the epithelium of the outer root sheath of the isthmus of an anagen hair follicle or to the retracted epithelial sac that represents the involution of the lower segment of a catagen follicle.

Fifteen of the tumors studied corresponded to cases of trichilemmoma/inverted follicular keratosis, in which the epithelium exhibits a similar differentiation to that of the outer root sheath of the lower segment of an anagen hair follicle. The two-thirds of the epithelium close to the surface of the lesion stained positive in these tumors, as did the squamous eddies. There is a lack of agreement about the origin of the so-called squamous eddies that are observed in the thick epithelial lobes of the tumors, although according to Draluck and Ackerman,[19] serial sections of these structures in many instances show continuity with an adjacent sebaceous gland, and so they might be hyperplastic sebaceous gland openings that are trapped within a tumor and that develop with a squamous metaplasia inside. Poblet et al[7] reported immunoreactivity for calretinin in other cutaneous cells such as those that demarcate the excretory duct of the sebaceous glands. Therefore, the immunostaining of squamous eddies for calretinin in our study supports the nature of these features as being related to sebaceous ducts, as proposed by Draluck and Ackerman (Figure 4).

Folliculosebaceous cystic hamartoma probably represents long-standing progression of trichofolliculoma lesions. It has almost all the components of a normal hair follicle, although they are aberrantly distributed. The 2 cases analyzed in this study stained positive for calretinin in the excretory duct of the sebaceous glands that comprise this hamartoma.

Panfolliculomas are made up mainly of germinative follicular cells and matrix cells, and also shows differentiation towards the outer root sheath of the hair follicle at the level of the shaft and the isthmus. They contain trichohyalin granules, compact blue-gray keratin similar to that produced by the keratinized inner root sheath, yellowish-orange refractile islands of shadow cells, and, as an expression of infundibular differentiation, small cysts lined by squamous cells. In addition, there is a well-developed granular layer with keratohyalin granules and basophilic and orthokeratotic lamellar keratin. In our study, we observed focal staining for calretinin in the cell layer that demarcates the lumen of the cysts with trichilemmal differentiation.

The diagnosis of basal cell carcinoma with follicular differentiation can be established when one or more of its components mimics a normal hair follicle structure in any of its 3 phases of the hair growth cycle. Distinction is made between basal cell carcinomas that mimic embryonic
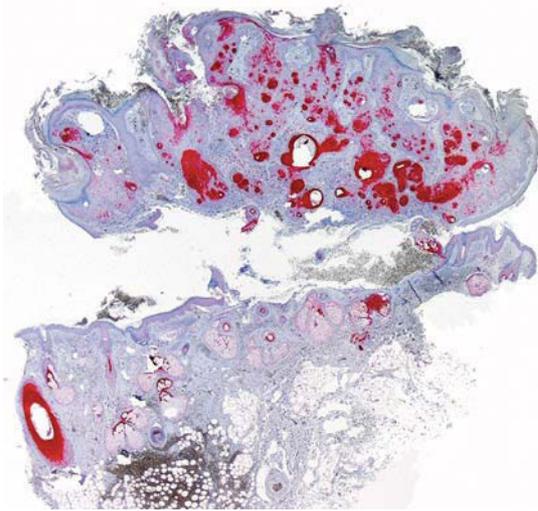
**Figure 4.** Wart with staining of the canals (calretinin, ×10).
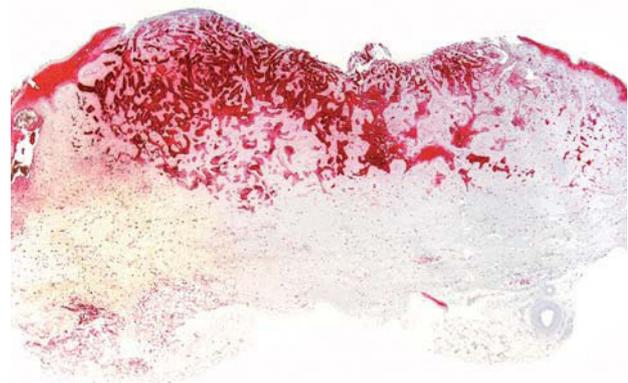
Figure 5. Desmoplastic basal cell carcinoma. Intense staining of the tumor epithelium (calretinin, ×10).

Figure 6. Trichoblastoma. Limited staining of the innermost layer that lines the cysts (calretinin, ×10).

follicular germ cells with infundibular differentiation, trichilemmal differentiation, or differentiation towards the outer root sheath, differentiation towards the inner root sheath, matrix differentiation, and anagen follicular differentiation. In our study, 3 basal cell carcinomas were included with follicular differentiation, with differing results (Figure 5). An adenoid-cystic basal cell carcinoma stained intensely positive throughout the tumor epithelium, a superficial basal cell carcinoma stained focally positive in the outermost layer of the tumor epithelium, and a basal cell carcinoma with matrix differentiation was negative for calretinin.

In the trichoblastomas/trichoepitheliomas, the degree of follicular differentiation varied from tumor to tumor, and sometimes even from section to section within the same tumor. Immunohistochemical studies have revealed a cytokeratin pattern in the epithelial islands of the tumor similar to the cells of the outermost cell layer of the outer root sheath of the normal hair follicle between the permanent segment and the upper part of the transient segment.[20] It has been shown that both trichoblastomas and basal cell carcinomas constitutively express the cytokeratins CK6hf, CK14, and CK17.[21] These findings suggest that both tumors differentiate towards the outer root sheath of the hair follicle. In our study, we analyzed 9 trichoblatomas/trichoepitheliomas and found that most of the tumor epithelium was negative for calretinin, with limited focal staining. This finding is consistent with the differentiation towards the outermost layers of the outer root sheath shown in immunohistochemical studies (Figure 6)

Pilomatricoma is a follicular tumor that is essentially made up of epithelium of the matrix and the region above the matrix. Pilomatricoma cells show a pattern of cytokeratin expression similar to that of cells of the cortex of the hair shaft and those of the outer root sheath of the normal hair follicle. In the 2 pilomatricomas included in this study, we observed staining in the cell layers close to the shadow cells but the rest of the tumor was negative, a finding which coincides with the expression of cytokeratins similar to that of the outer root sheath of the normal hair follicle (Figure 3).
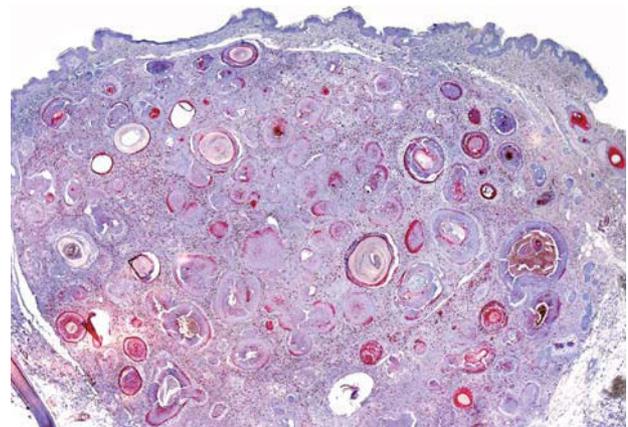
The proliferative trichilemmal tumor is a mainly solid tumor in which certain cystic structures can be discerned in a honeycomb pattern. The epithelium that lines each of these cystic structures shows histologic characteristics similar to those of the outer root sheath of the hair follicle at the level of the isthmus and to those of the lower segment of a hair follicle in an advanced catagen phase. The 3 proliferative trichilemmal tumors included in this study stained positive for calretinin in the cell layers close to the lumen of the cystic structures.

In the histologic study of the infundibular cyst, we observed a unilocular cavity lined with a multilayered

epithelium whose histologic structure was the same as that of the epidermis. Calretinin immunostaining of the infundibular cysts did not yield positive results in some of the structures that make up this cyst.

The dilated pore of Winer is made up of one or more contiguous dilated follicular infundibuli. The wall is made up of the infundibular epithelium, with a well-developed granular layer. Elongated epidermal crests project radially from the wall and penetrate the adjacent dermis. In our study, immunostaining of this lesion with calretinin was negative.

Acanthoma of the follicular sheath comprises radial epithelial lobes that are made up of cells similar to those of the epithelium of the outer root sheath at the level of the follicular isthmus and that may exhibit cell palisading in the outermost regions. The 2 samples of acanthoma of the follicular sheath included in our study stained strongly positive.

In contrast to the findings of Lugli et al,[2] who did not observe any cutaneous adnexal tumors positive for calretinin among 35 samples, some of the adnexal tumors with follicular differentiation in our study were positive for this marker, specifically towards the outer root sheath and more specifically, towards its innermost layer. One of the reasons why we saw staining of the suprabasal layers of adnexal tumors might be that calretinin participates in the cell cycle and so would participate in situations in which there is an abnormal control of the cell cycle. We also found calretinin expression in sebaceous glands, mainly in some of the corresponding excretory ducts.

Conclusions

Selective staining with calretinin helps to distinguish the companion layer of the outer root sheath, which has a different morphology and physiology to the other layers of this root sheath, and which is hard to discern with hematoxylin-eosin staining. In addition, staining identified adnexal follicular tumors or components of these tumors, with differentiation towards the outer root sheath. We could also demonstrate the participation of the sebaceous gland in some of these structures.

Future studies with calretinin may contribute to a better understanding of the pathogenesis of inflammatory diseases of the hair follicle, cutaneous adnexal tumors with follicular differentiation, and ectodermal syndromes in which hair follicle abnormalities are implicated.

Conflicts of Interest
The authors declare no conflicts of interest.

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