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Loss of ZNF750 in ocular and cutaneous sebaceous carcinomas

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Abstract

BACKGROUND: Sebaceous carcinoma (SeC) is an uncommon malignancy arising from sebaceous glands of the conjunctiva and skin. Recurrent mutations in the *ZNF750* were recently identified in ocular SeC. We assessed whether ZNF750 loss is a specific feature of ocular SeC, or a general feature of sebaceous tumors.

METHODS: Immunostaining for ZNF750 expression was performed in 54 benign and malignant sebocytic proliferations. Staining for ZNF750 was scored on a 3 tier scale: positive (>75%), partial positive (5–74%), and negative (<5%).

RESULTS: ZNF750 expression was negative in 4/11 ocular SeC, and partially positive in 4/11 ocular SeC and 6/13 cutaneous SeC. No extraocular tumors were negative. No loss was found in sebaceous adenoma or sebaceous hyperplasia. In 9 previously sequenced ocular SeCs, two lacked detectable somatic mutations in *ZNF750*, but showed complete loss of staining, indicating non-mutational inactivation of ZNF750.

CONCLUSION: We show complete loss of the ZNF750 epidermal differentiation regulator in about half of ocular SeC, highlighting the most common genetic defect in this cancer type. Loss of ZNF750 expression is seen even in tumors without truncating mutations and reduced in many of the remaining ocular and cutaneous SeC. In contrast, no ZNF750 loss was detected in benign sebaceous proliferations.

Introduction

SeC occurs in 1–2 per 1,000,000 individuals, with a disease-specific mortality rate of 3% −6.7%[2,3]. SeC can arise from sebaceous glands at any anatomic site, but 39% of cases occur on the eyelid[2]. Regardless of anatomic site, SeC has been considered a potential cutaneous marker for Muir-Torre syndrome, a cancer syndrome caused by germline mutations in mismatch repair pathway components *MLH1*, *MSH2*, *MSH6*, and *PMS2*[4]. However, two recent studies have shown ocular SeC is genetically distinct, lacking DNA mismatch repair

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defects (either somatic or germline) or UV signature mutations[1,5]. Instead, ocular SeC appears to harbor a high frequency of truncating mutations in *ZNF750*, compared to extraocular SeC[1].

The ZNF750 transcription factor regulates KLF4 to coordinate terminal keratinocyte differentiation[6]. While specific alleles are associated with psoriasis and seborrheic dermatitis[7], the pathogenesis appears to involve keratinocyte dysmaturation, rather than abnormal sebocyte homeostasis. Although ZNF750 inactivation has been reported in specific subsets of squamous cell carcinoma[8], the gene is not considered a major tumor suppressor in skin cancer. While truncating mutations in ZNF750 in ocular SeC were recently detected, the extent and frequency of protein loss remains unknown. To our knowledge, loss of function of ZNF750 has not been assessed in sebaceous carcinoma prior to this. We used immunohistochemistry to query whether ZNF750 protein expression is frequently lost in ocular and cutaneous SeC, what mechanisms might produce such loss, and whether this tumor suppressor is generally inactivated in sebaceous neoplasms.

Methods

With IRB approval from our institutional committee on human research, we searched the pathology archives for prior specimens of ocular SeC, cutaneous SeC, sebaceous adenomas, and sebaceous hyperplasia. 15 consecutive cases of sebaceous hyperplasia, sebaceous adenoma, and 13 cutaneous (i.e. non-ocular) SeC with additional tissue available in the associated paraffin block were collected from our dermatopatholgoy archives. The diagnosis was confirmed by a board certified dermatopathologist. Cases of sebaceous carcinoma in which sebaceous differentiation was not clearly evident in routine sections were additionally stained with adipophilin (Cell Marque, Rocklin, CA; predilute on Leica Bond) to confirm the diagnosis. Eleven cases of ocular sebaceous carcinoma with additional tissue available in the associated paraffin block were obtained from the *** Department of Pathology. The diagnosis was confirmed by three pathologists (JPN, DAS, MB). Formalin-fixed paraffin-embedded (FFPE) sections of 4 μm thickness from each of the tumors were stained with ZNF750 (polyclonal, Millipore-Sigma catalog # HPA021573, Sigma-Aldrich, St. Louis, MO; 1:80 dilution and pH6 antigen retrieval). ZNF immunohistochemical studies were scored as negative (<5% tumor cells positive), partially positive (5–75% tumor cells positive) and positive (>75% tumor cells positive). Only nuclear staining was counted as positive. Both immature basaloid sebocytes and mature lipidized sebocytes were counted in the staining assessment. For ZNF750 staining, keratinocytes in the epidermis and/or conjunctival epithelium served as positive internal controls, and inflammatory and stromal cells served as negative internal controls.

DNA Sequencing

Exome sequencing was performed previously on some of the ocular sebaceous carcinomas in a prior study as previously described.[1] Briefly, tissue sections were macrodissected to collect the desired regions. DNA was then extracted using the Qiagen QIAamp DNA FFPE Tissue Kit for DNA. Extracted DNA was quantified using a Qubit fluorometer. DNA-seq libraries were captured to exome regions using xGen Exome Research Panel v1.0 (IDT), and

libraries were prepared using the KAPA Hyper prep kit. DNA libraries were sequenced to a target depth of 200x for tumor sample, 100x for normal samples on the Illumina HiSeq platform.

Results

Patient demographic information and tumor location are listed in tables 1 and 2. Sebaceous carcinomas occurred in older adults (mean 75; range 49–96) with no gender discrepancy. The mean age was similar for sebaceous hyperplasia (mean 68, range 38–89) and sebaceous adenomas (mean 71, range 55–88). Sebaceous hyperplasia had a 1.5:1 female to male ratio, and sebaceous adenomas had a 3:1 male to female ratio.

ZNF750 is expressed in normal sebaceous glands and epidermis with a pattern of increasing expression from less differentiated basal epithelial cells to mature sebocytes and keratinocytes (Figure 1A). ZNF750 staining was negative in 36% (4/11) of ocular SeC (Figure 2), partially positive in 36%, and positive in 27%. Negative tumors were frequently poorly differentiated (Table 2). Cutaneous SeC (Figure 3) stained positively for ZNF750 in 54% (7/13), partially positive in 46%, and none were negative. All sebaceous adenomas and sebaceous hyperplasia were uniformly positive with a pattern similar to that seen in normal sebaceous glands with increasing nuclear expression of ZNF750 as germinative sebocytes transition to mature sebocytes (Figures 1,4).

Sequencing data for *ZNF750* was available for 9 of the 11 ocular SeCs, 7 of which harbored somatic mutations (Table 2). For the two samples in which no *ZNF750* mutations were detected, protein expression was completely lost.

Discussion

Cutaneous SeC acquire large mutation burdens, almost exclusively because of defects in DNA mismatch repair or from chronic sun damage. SeC with DNA mismatch repair genotypes are generally more well-differentiated tumors, while the UV signature SeC tend to be more poorly differentiated with infiltrative features[1]. Interestingly, ocular SeC lack evidence of inactivated DNA mismatch repair proteins, which has been previously regarded as the major contributor to sebaceous carcinoma development. Recent exome sequencing analysis has shown that ocular SeC have frequent mutations in the *ZNF750* tumor suppressor gene, as well as frequent *TP53* mutations and occasional *Notch1/2* mutations.[1] Previous studies using targeted next generation sequencing panels on ocular SeC had also shown frequent *TP53* mutations and infrequent *Notch1* mutations, and additionally found frequent *Rb1* mutations.[5,9] Ocular SeC which had mutations in both *TP53* and *Rb1* were more likely to recur.[9] Given these new genomic data, we further explored involvement of ZNF750 in sebaceous tumors at the protein expression level and found that dysregulation and loss of ZNF750 is frequent in malignant sebaceous neoplasms, particularly ocular SeC, and retains a normal expression pattern in sebaceous hyperplasia and sebaceous adenomas.

Multiple mechanisms appear to collaborate to attenuate ZNF750 function in ocular SeC. Of the 9 tumors for which sequence data is available, four contain truncating mutations. Two of these (samples 6 and 9) show loss of heterozygosity retaining only the mutated allele, based

on the number of sequencing reads of each copy. Both samples demonstrate partial loss of ZNF750 staining. Interestingly, both samples harboring wild-type *ZNF750* (3 and 8) show complete loss of staining, suggesting that epigenetic silencing operates in cases where protein function is not compromised by mutation. This observation is similar to that for mismatch repair gene inactivation in MSI-class sebaceous carcinoma, where mismatch repair protein can be lost in the absence of a corresponding truncating mutation. Therefore immunohistochemical staining, as described in this paper, can provide a more comprehensive assessment of whether ZNF750 has been affected through mutation or epigenetic modifications in the pathogenesis of sebaceous tumors. Future studies assessing the role of epigenetic modifications in ZNF750, as well as upstream and downstream proteins in the ZNF750 pathway will be helpful to further understand the molecular pathogenesis of ocular SeC.

The results of ZNF750 staining in this study indicate such staining may be useful in the diagnosis of sebaceous tumors. Benign sebaceous tumors exhibit a typical pattern of ZNF750 expression with minimal expression in germinative sebocytes and positive nuclear expression in mature lipidized sebocytes (figure 4). This pattern can be seen in well-differentiated SeC, so it cannot exclude SeC. However, frequently there is abnormal expression of ZNF750 in germinative cells in SeC (figures 5 and 6). When present, this aberrant expression could serve as a clue to malignant transformation in which ZNF750 expression has been upregulated as a tumor suppressor in germinative cells of SeC. Alternatively, loss ZNF750 expression in mature sebocytes can also serve as a clue to malignant transformation, particular in ocular SeC where complete loss of expression can be seen (figure 2).

ZNF750 has also been reported as recurrently mutated in esophageal SCC[8–11], but its inactivation is not common in SCC arising at other anatomic sites. The discovery that ZNF750 inactivation is a general feature of ocular SeC is particularly intriguing because of this SeC subtype's low mutation rates, often less than 100 mutations per exome. Therefore a combinatorial loss of *TP53* and/or *Rb1* in combination with *ZNF750* may be sufficient to initiate tumor formation in periocular sebocytes, a low threshold that could explain the predilection of SeC for eyelid skin (~39% overall).

In contrast to the patterns detected with *ZNF750*, *NOTCH1* mutations are infrequent in ocular SeC, but common in extraocular SeC[1]. Like ZNF750, Notch receptors play a tumor suppressive role in keratinocytes[12]. Therefore, we speculate it is possible that inactivation of one of these genes and its downstream targets is required for tumorigenesis in sebocytes, depending on the cell of origin and its anatomic site. As a tumor suppressor, *ZNF750* cannot be directly inactivated for therapeutic purposes[13]. However downstream targets of ZNF750 have been recently discovered[14,15], elucidating possible alternative targeting strategies for this pathway. Reduced *ZNF750* expression also predicts more aggressive esophageal SCC[16], making it possible that detecting its loss in SeC may help identify the small proportion of tumors that recur and metastasize. Additional study correlating ZNF750 expression with clinical follow up data is necessary to assess if ZNF750 truly correlates with clinical outcomes.

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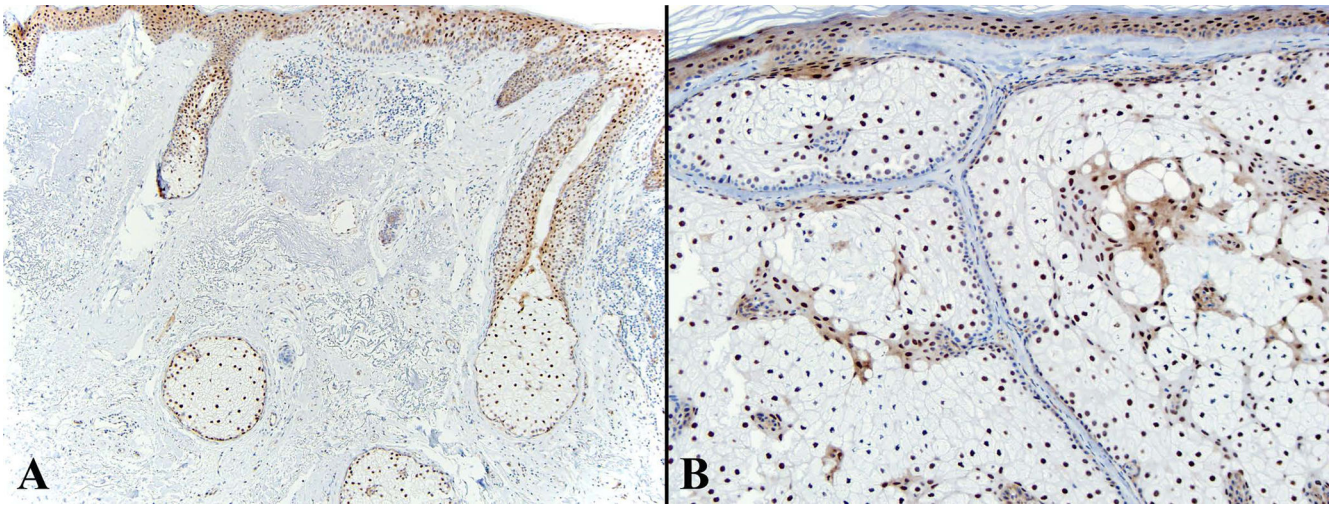

Figure 1.

ZNF750 expression in normal skin (A) and sebaceous hyperplasia (B).ZNF750 is increasingly expressed in the nuclei of keratinocytes in the epidermis and sebocytes in sebaceous glands as cells differentiate into corneocytes and mature sebocytes.A- ZNF750 40x, B- A- ZNF750 100x.

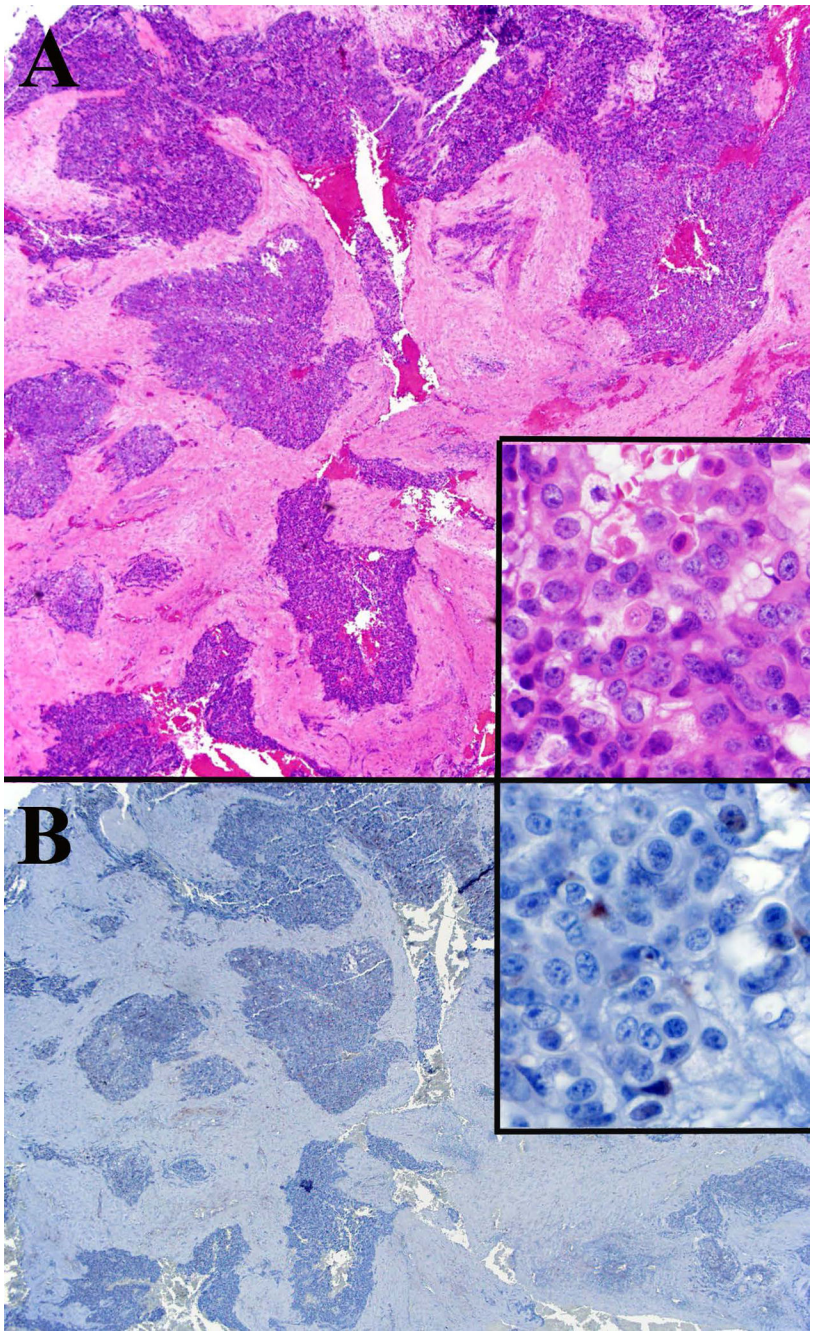

Figure 2.

Ocular sebaceous carcinoma with negative ZNF750 staining. A. Low power view of the tumor shows a large basaloid neoplasm with irregular nodules of cells that have focal sebaceous differentiation (inset).H&E 20x, inset 600x. B. ZNF750 staining is negative in the tumor cells (inset).ZNF750 stain 20x, inset 600x.

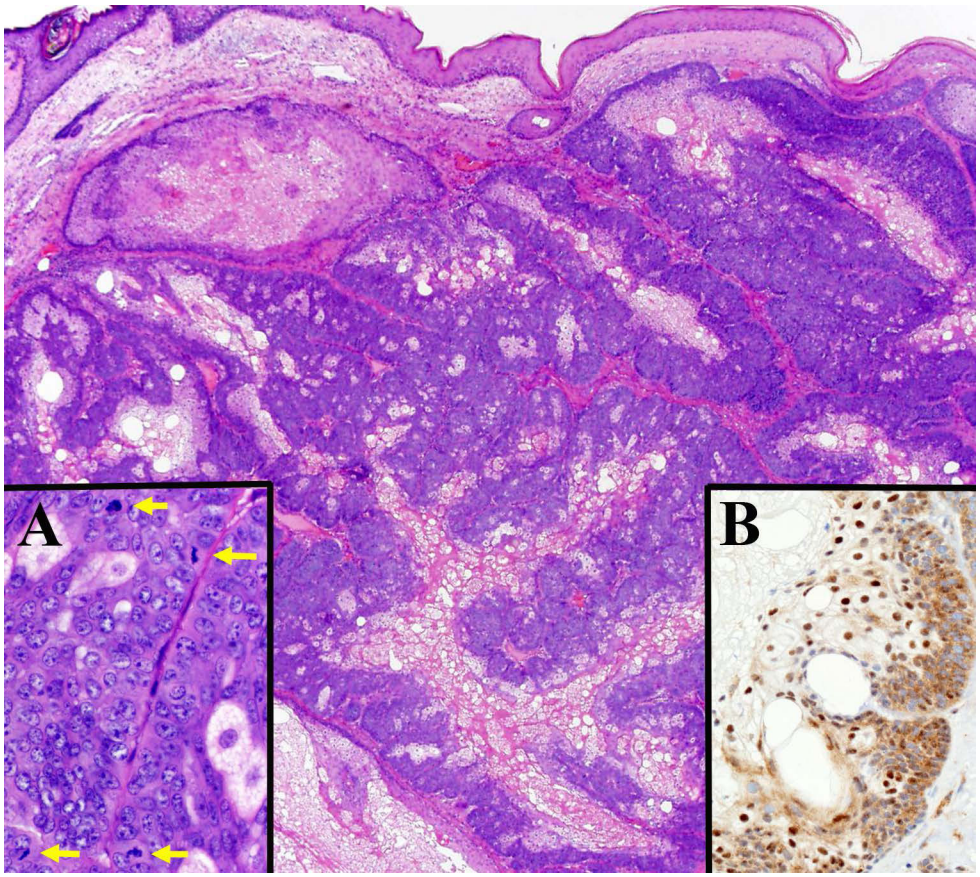

Figure 3.

Cutaneous sebaceous carcinoma with positive ZNF750 staining. A. Low power view of the tumor shows large collections of both germinative and mature sebocytes with large pleomorphic nuclei and frequent mitotic figures (inset A-yellow arrows). H&E 20x, inset H&E 600x. Inset B shows ZNF750 staining is weakly positive in the nuclei of the germinative cells and strongly positive in the mature sebocytes in this cutaneous sebaceous carcinoma.ZNF750 stain 100x.

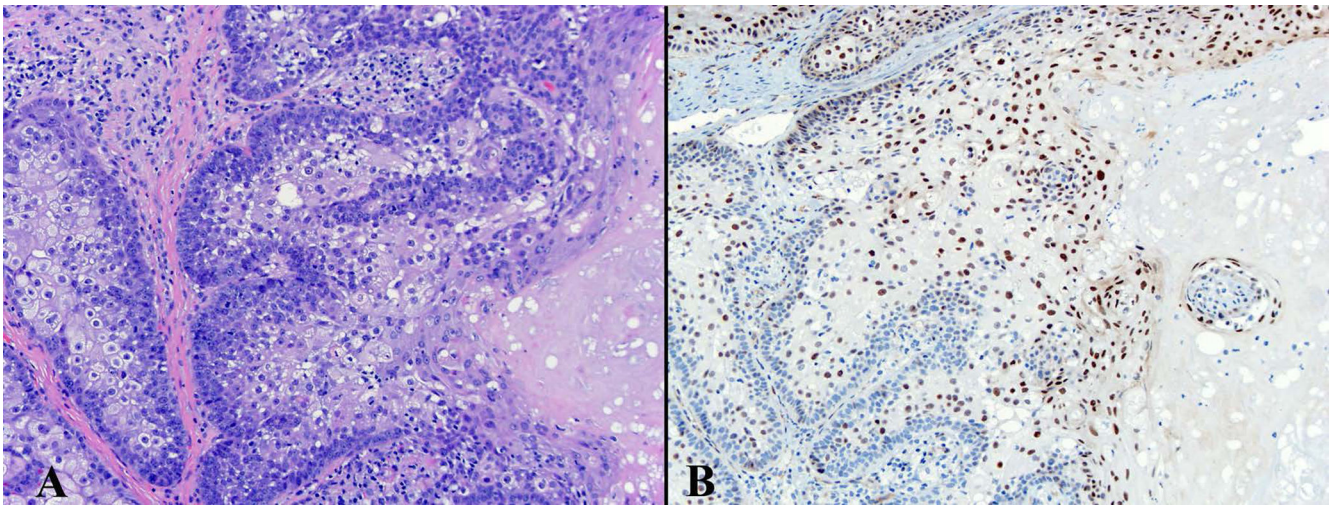

Figure 4.

Sebaceous adenomas all express ZNF750 in a pattern similar to that observed in normal sebaceous glands and sebaceous hyperplasia with increasing expression as germinative sebocytes differentiate to mature sebocytes. A- H&E 200x, B-ZNF750 200x.

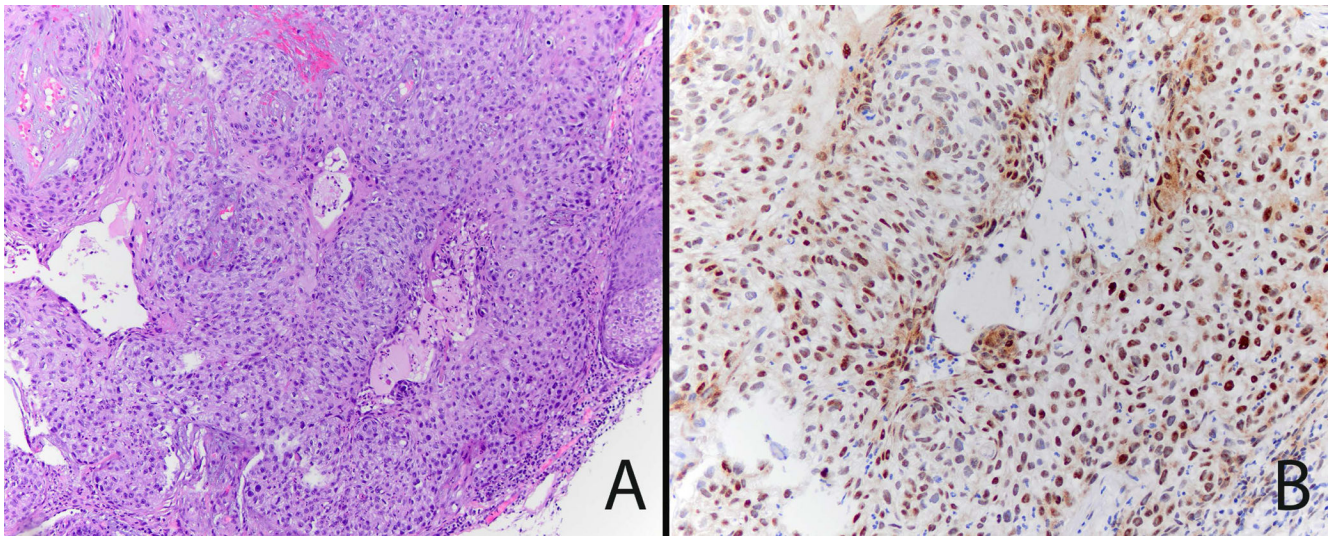

Figure 5.

Poor-moderately differentiated cutaneous SeC with abnormal expression of ZNF750 in immature sebocytes.A- H&E 400x, B- ZNF750 400x.

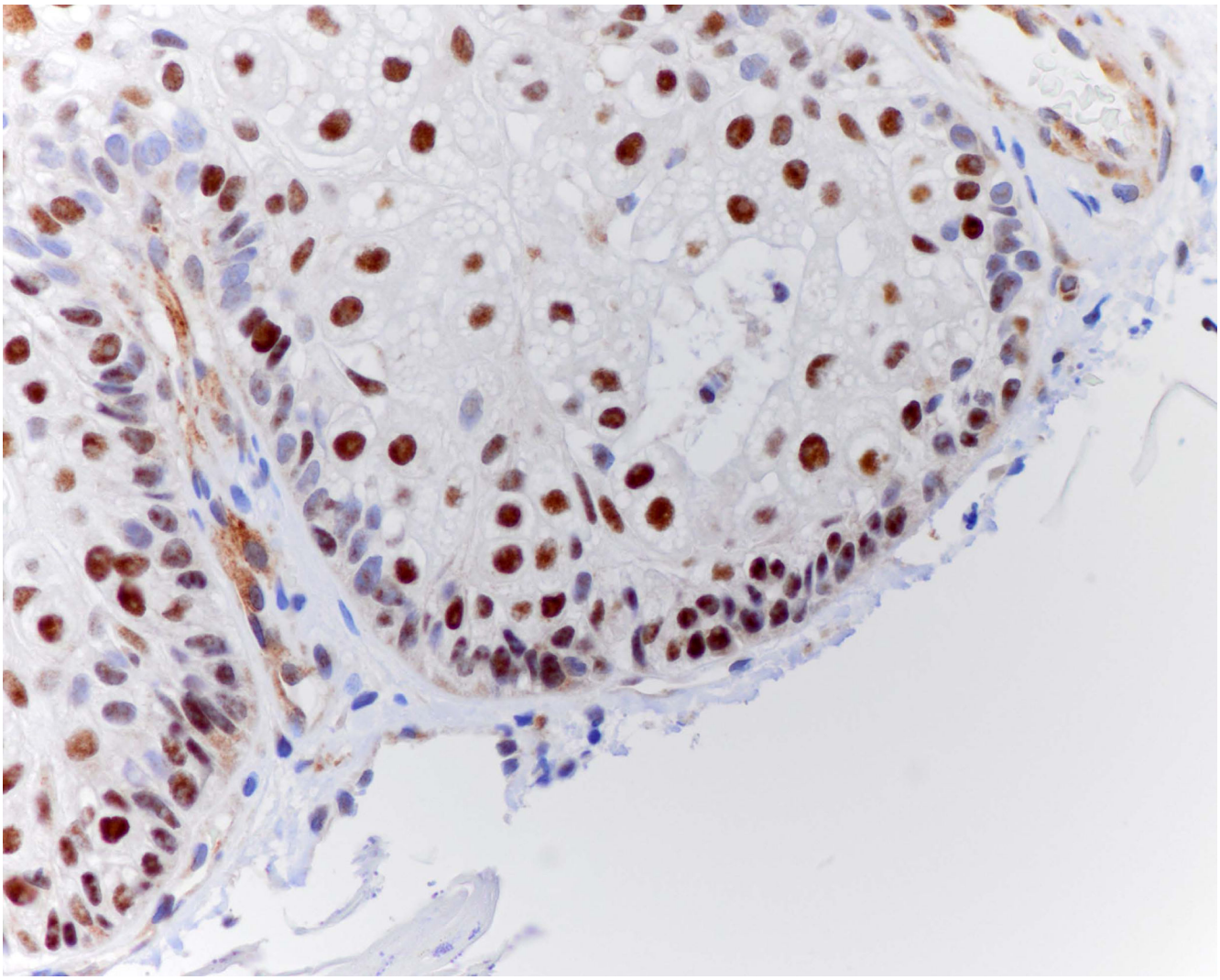

Figure 6.
Abnormal expression of ZNF750 in some of the germinative cells in well differentiated cutaneous SeC. ZNF750 600x.

Table 1.

Demographics and ZNF750 staining results for sebaceous hyperplasia and adenomas

Sebaceous Adenoma	Biopsy site	Age/gender	ZNF750 Stain
1	Right posterior neck	72/M	positive
2	Left nasal sidewall	79/M	positive
3	Left nasal ala	59/M	positive
4	Right cheek	75/M	positive
5	Left face	67/F	positive
6	Left post-auricular	64/M	positive
7	Left neck	70/M	positive
8	Right cheek	88/F	positive
9	Right upper cutaneous lip	55/F	positive
10	Left upper arm	73/F	positive
11	Right neck	75/M	positive
12	Left lower eyelid	69/M	positive
13	Right mid upper back	80/M	positive
14	Left temple	64/M	positive
15	Nasal root	72/M	positive
Sebaceous Hyperplasia			
1	Right posterior shoulder	54/M	positive
2	Dorsal nose	58/M	positive
3	Left forearm	68/M	positive
4	Left temple	73/M	positive
5	Right cheek	38/M	positive
6	Left mid cheek	55/F	positive
7	Right eyebrow	71/F	positive
8	Left nasal ala	77/F	positive
9	Medial forehead	52/F	positive
10	Left eyebrow	68/F	positive
11	Glabella	89/F	positive
12	Left forehead	78/F	positive
13	Left clavicle	73/F	positive
14	Right malar cheek	72/M	positive
15	Right medial cheek	88/F	positive

Table 2.

Demographics, ZNF750 staining results for sebaceous carcinomas

Cutaneous SeC	Site	Age/gender	Histopathologic pattern	ZNF750 staining	Mutation analysis
1	Posterior scalp	80/M	Well-diff	positive	N/A
2	Right nose	96/M	Poor-mod diff	partial positive	N/A
3	Left ear concha	70/F	Mod-diff	partial positive	N/A
4	Tip of nose	75/M	Mod-well diff	Partial positive	N/A
5	Nose	64/F	Poor-mod diff	partial positive	N/A
6	Left neck	88/F	Mod diff	positive	N/A
7	Left preauricular area	66/M	Poor-mod	positive	N/A
8	Scalp	81/F	Mod-diff	partial positive	N/A
9	Left ear	80/M	Mod-diff	positive	N/A
10	Glabella	49/M	Well-diff	Partial positive	N/A
11	Left lower cutaneous lip	84/M	Mod-diff	positive	N/A
12	Right cheek	79/M	Mod-diff	partial positive	N/A
13	Right shoulder	78/F	Poor-mod diff, infiltrative	partial positive	N/A
Ocular SeC					
1	eyelid	73/F	Mod-diff, infiltrative	partial positive	p.E154delinsE
2	eyelid	71/F	Mod-diff	partial positive	p.C27R
3	eyelid	66/M	Mod-poorly diff	negative	Wild type
4	eyelid	78/F	Mod-poorly diff, infiltrative	negative	N/A
5	eyelid	69/F	Mod-poorly diff	positive	p.Y205Kfs*163
6	eyelid	80/M	Mod-poorly diff, infiltrative	partial positive	p.Q279*
7	eyelid	75/F	Mod diff	negative	p.S52*
8	eyelid	71/M	Mod-poorly diff, infiltrative	negative	Wild type
9	eyelid	74/M	Mod-well diff	partial positive	p.K221Lfs*146
10	eyelid	80/F	Mod-diff, infiltrative	positive	p.C34L
11*	eyelid	80/F	In situ carcinoma only	positive	N/A

*Sebaceous carcinoma in situ; Well-diff= well differentiated; Mod-diff= moderately differentiated; Poor-mod diff= poor to moderately differentiated