Genotypic and phenotypic characterization of keratoacanthoma and possible distinguishing features from cutaneous squamous cell carcinoma

Sarita Joshi

Department of Pathology
Rikshospitalet, Oslo University Hospital
&

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Abbreviations

AK – **A**ctinic keratosis

APC/C – Anaphase –Promoting Complex/ Cyclosome

AR – Antigen retrieval

Axin2 – Axis inhibition protein 2

BUBR1 – Budding uninhibited by benzimidazoles 1

CIN – Chromosomal instability

CDK – Cyclin-dependent kinase

CGH – Comparative genomic hybridization

cSCC – Cutaneous squamous cell carcinoma

CTCE – Cycling temperature capillary electrophoresis

DMBA – 7, 12-dimethylbenz (a) anthracene

EV – Epidermodysplasia verruciformis

EMT – Epithelial-mesenchymal transition

EBV – Epstein-Barr virus

FFPE – Formalin-fixed paraffin-embedded

GEKA – Generalized eruptive KA

HNSCC – Head and neck squamous cell carcinoma

HIER – Heat-induced epitope retrieval

HPV – Human papillomavirus

IHC – Immunohistochemistry

JAK-STAT – Janus-kinases– signal transducer and activator of transcription proteins

KA – Keratoacanthoma

LEF-1– Lymphoid enhancer-binding factor 1

LOH – Loss of heterozygosity

Mad2 – Mitotic arrest deficient 2

MDM2 – Mouse double minute 2 homolog

md cSCC – moderately-differentiated cSCC

MLH1– MutL homolog 1

MSH2– MutS homolog 2

MSH6– MutS homolog 6

MSSE – Multiple self- healing squamous epithelioma

NMSC – Non-melanoma skin cancer

pd cSCC – poorly- differentiated cSCC

PUVA – Psoralens and ultraviolet A

SAC – Spindle assembly checkpoint

Shh – Sonic hedgehog

SOX9 – SRY-related HMG-box

TMA – Tissue microarray

TGFBR1– Transforming growth factor-beta receptor 1

TX+ – Solid-organ transplantation recipient

UVR – Ultra-violet rays

wd cSCC – well-differentiated cSCC

Wnt – Wingless-related integration site

Wt – Wild type

List of papers

Paper I: Tp53/p53 status in keratoacanthomas

Sarita Joshi, Aasa R.Schjølberg, Per Olaf Ekstrøm, Paula M. De Angelis, Manuela Zucknick, Solveig Norheim Andersen, Ole Petter F. Clausen

Journal of Cutaneous Pathology 2016; 43(7):571-8

Paper II: Role of the Wnt signaling pathway in keratoacanthoma

Sarita Joshi, Paula M. De Angelis, Manuela Zucknick, Aasa R.Schjølberg, Solveig Norheim Andersen, Ole Petter F. Clausen

Cancer Reports 2019; doi: 10.1002/cnr 2.1219

Paper III: Expression of spindle proteins in keratoacanthoma and squamous cell carcinoma

Sarita Joshi, Paula M. De Angelis, Manuela Zucknick, Aasa R.Schjølberg, Solveig Norheim Andersen, Ole Petter F. Clausen

Manuscript

1 Introduction

Keratoacanthoma (KA) is a common, cutaneous neoplasm on sun-exposed skin of elderly persons. The hallmark of this unique neoplasm is initial rapid growth, followed by a stable phase before it undergoes spontaneous regression. Solid-organ transplant recipients (TX+) have a higher risk of developing non-melanoma skin cancer (NMSC) and KA. A subset of KAs, especially in the early proliferation phase, may show morphological resemblance to well-differentiated cutaneous squamous cell carcinomas (wdcSCCs), posing diagnostic difficulties, thus leading to misdiagnosis and overtreatment of KA. While the biology of the development of common skin tumors like basal cell carcinoma, malignant melanoma, and cSCC has been extensively studied, there is a gap in the knowledge of the biology of KA. Characterization of KA as described in the literature relies mostly on light microscopic features and a few molecular markers, but no single marker is reliable enough to differentiate KA from cSCC. This thesis aims at a better understanding of the biology of KA, including the problem of differentiating KA from cSCC.

1.1 Keratoacanthoma

Keratoacanthoma usually presents as a solitary pink-colored nodule with a central keratin-filled crater. The lesion has a preference for sun-exposed areas of the skin of elderly persons. It proliferates rapidly within 4-6 weeks and regresses spontaneously within 3-6 months, after having a stationary phase (1, 2). Some KAs remain stationary for more than a year before they spontaneously regress.

KA has been a matter of debate for a long time, partly due to its diverse clinical nature and the lack of solid consensus on the histological diagnostic criteria. Some consider KA as a low-grade squamous cell carcinoma due to its morphological resemblance to well-differentiated cSCC (3, 4) and to rare reports of metastases (5). In the new edition of the WHO classification of skin tumors, KA is classified as a likely variant of wdcSCC because of indistinguishable histological features from cSCC (6). However, it has also been shown that KA has distinct molecular aberrations and differentially-expressed genes compared to cSCCs (7, 8). Moreover, KA has a benign clinical behavior with natural regression in most cases.

Thus, KA has a better prognosis compared to cSCC (9). However, a subset of KAs may undergo malignant transformation to cSCC and thus can be considered as a premalignant lesion (10, 11).

There is a paucity of both phenotypic and genotypic data that can contribute to a better understanding of the biology of KA. Such data might also better facilitate the differentiation of KA from SCC.

1.1.1 History

Sir Jonathan Hutchinson first described KA in 1889 as a "crateriform ulcer of the face", a form of acute epithelial cancer. McCormac and Scarff in 1936 coined the term "molluscum sebaceum" because of the lesion's histological resemblance to molluscum contagiosum. Finally, the term "keratoacanthoma" was coined by Freudenthal and promoted by Rook and Whimster in 1950 by reporting 29 cases (12).

1.1.2 Epidemiology

The true incidence of KA is very difficult to estimate, firstly because of different opinions of KA as a malignant tumor (cSCC), and secondly because of spontaneous regression and lack of distinct epidemiological studies. KA has been reported to occur at any age, but the most common solitary KA has a peak incidence between the ages of 65 to 71 years (2, 13). Some cases have been reported in children, but it is extremely rare under 20 years (14). The incidence increases with increasing age and chronic ultra-violet (UV) light exposure. Gender distribution shows male predominance probably related to outdoor work in sub-tropical areas. A Japanese and an Australian study demonstrated the incidence of KA to be 104 and 150 per 10,000 people respectively (15, 16). There is a high incidence of KA in transplant recipients and other groups with immunosuppressive therapy (17-19).

1.1.3 Risk factors

Extrinsic factors:

- **Ultraviolet radiation**: Exposure to sunlight is the most common factor implicated in the development of KA. KA is typically located on sun-exposed, hair-bearing skin of elderly, fair-skinned individuals (20). A study by Sullivan JJ and Colditz G.A in 1979 and by

Weedon et al. showed a high incidence of KA in a sub-tropical climate (16, 21). A high number of cases have also been reported after PUVA (psoralens and ultraviolet A) therapy for psoriasis, eczema, and other skin diseases (22, 23)

- **Chemical carcinogens**: Many chemical carcinogens such as 7, 12-dimethylbenz (a) anthracene (DMBA) (24), tar and pitch (25, 26), cigarettes smoking (26-28) and mineral oils (29, 30) have been implicated as risk factors of KA.

- **Trauma**: There are some reports of the development of KA in previous accidental scars and burns (31, 32). Moreover, surgical trauma such as transplant graft site (33), cryotherapy (34, 35), and smallpox vaccination site (36) is associated with the formation of keratoacanthoma.

- **Immunosuppression**: Immunosuppressive drugs such as Cyclophosphamide, Azathioprine, and Prednisolone are associated with increased risk (several-fold) of cSCC and KA as a treatment regimen in solid organ transplantation (17, 18). Kidney- transplant recipients and heart transplant recipients have a higher risk of developing skin tumors including KA than the general population (17, 19)

- **Drug exposure**: BRAF inhibitors (Vemurafenib, Dabrafenib) in the treatment of malignant melanoma (37, 38) and the Hedgehog pathway inhibitor (Vismodegib) in the treatment basal cell carcinoma (39) induce cutaneous neoplasia, including KA.

- **Human papillomavirus (HPV)**: HPV is reported to play a role in both premalignant and malignant skin tumor development, especially in immunosuppressive patients after solid organ transplantation (40, 41). However, there are contradictory reports (42-44), with positive (44, 45) and negative (43, 46) HPV DNA detection in KAs. Forslund et al. detected DNA of HPV cutaneous subtypes in 51% of KAs. Since no predominant subtypes were seen, it was assumed that HPV was not associated with development of KA. Oncogenic HPV DNA was not detected (44).

Genetic syndromes

- **Muir-Torre syndrome**: Multiple keratoacanthomas can present as a part of Muir-Torre syndrome, which is inherited in an autosomal dominant pattern. This syndrome has been attributed to a defect in mismatch repair genes *MSH2* or *MLH1* (47). Patients present with several malignancies in internal organs in addition to multiple skin lesions like KA and SCC (48).

- **Multiple KAs of Ferguson- Smith type/ Ferguson-Smith syndrome**: It is also known as multiple self- healing squamous epithelioma (MSSE), and was first described by the Scottish dermatologist Ferguson-Smith in 1934. MSSE is a rare autosomal dominant genodermatosis, exclusively reported in families of Scottish ancestry in the 2nd and 3rd decades of life. MSSE is characterized by the sudden and rapid growth of few to hundreds of KA on sun-exposed areas of the body, with signs of slow regression (12, 49, 50). It shows periodical recurrence throughout life. Both sexes are equally affected. The gene involved in this syndrome is *TGFBR1*, which is localized to chromosomal region 9q22-q31 (49, 51).

- **The Witten- Zak type of multiple keratoacanthomas**: This is a rare familial syndrome that shows combined features of both Grzybowski syndrome and Ferguson-Smith syndrome. It presents clinically with multiple small and large lesions of KAs (12, 21). The genetic aberrations involved in Witten-Zak syndrome are not known, but it is inherited in an autosomal dominant pattern (52).

- **Others**: Other disorders such as an X-linked disorder, incontinentia pigmenti, and the autosomal recessive disorder Xeroderma pigmentosum, have been associated with multiple keratoacanthomas (53, 54).

1.1.4 KA variants

- **Solitary KA**: This is the most common type of KA, located to sun-exposed skin of elderly patients (12). It may reach a size of 1-2.5 cm in diameter.

- **KA centrifugum marginatum:** A rare variant KA, characterized by a lesion that shows central healing and peripheral expanding growth. It grows up to 20 cm in diameter with simultaneous healing. It may not show spontaneous regression, or if any healing, the process may take an unusually long time. It involves the face, trunk, and extremities of middle-aged or older individuals of both sexes (12, 21).

- **Giant KA**: The tumor may grow up to 9 cm or larger. It is usually locally destructive with the invasion of the deep tissue beyond eccrine glands and is treated clinically as a squamous cell carcinoma (12, 21).

- **Subungual KA**: This type of KA shows rapid growth with nail dystrophy. It has a male predominance. The thumb or little finger is involved in 70% of cases. (12, 21).

- **Mucosal KA**: KA can infrequently occur on non-hair bearing sites like oral mucosa, bulbar conjunctiva, nasal mucosa, and genitalia and is presumed to develop from ectopic sebaceous glands on the mucosa (12).

- **Generalized eruptive KA of Grzybowski type (GEKA)**: GEKA is extremely rare. The disease clinically presents as multiple KA, comprised of hundreds to thousands of pruritic lesions with simultaneous growth and regression. The cases are sporadic without any familial pattern and occur similarly in both sexes. Middle-aged or older persons are usually affected. It may involve the face, producing masked facies and ectropion. Oral mucosa and genitalia may be affected. (12, 21, 55).

1.1.5 Phenotypic characteristics

KA is a dynamic lesion, characterized by three stages of development with distinct histopathological features.

1. **Early proliferative phase**: It is characterized by invaginations of anastomotic infundibulocystic structures containing abundant laminated keratin. In addition to the infundibular structures, the deeper part shows squamous lobules containing large pale pink cells with glassy cytoplasm often designated as ground glass appearance with trichilemmal differentiation with minimal infiltration into the surrounding stroma. Overhanging lips may or may not be fully formed at this stage. The deeper part may sometimes show nuclear atypia and mitosis (1, 56, 57). (Figure 1)

2. **Well-developed phase**: The lesion is fully formed and symmetric showing crateriform architecture as a result of the fusion of the infundibular structures and overhanging lips on both sides of the crater. Several squamous lobules with pale pink cells with abundant cytoplasm infiltrate into the deeper dermis to the level of eccrine glands. Nuclear atypia and mitosis at the periphery of the tumor containing basophilic cells may be a prominent feature in some KAs. There is an inflammatory reaction at the dermo-epidermal zone with lymphocytes, eosinophils and plasma cells. Numerous neutrophilic microabscesses are seen (1, 56, 57). (Figure 2)

3. **Regressing phase**: It is characterized by a shallow, cup-shaped lesion filled with infundibular laminated keratin. The epidermis is usually thin and keratinocytes are shrunken. No evidence of infiltrating squamous lobules is found at this stage, but remainders of some regressing squamous lobules can be seen. Heavy infiltration of inflammatory cells, often in a lichenoid pattern reaction, is present at the dermo-epidermal junction. Moderate to abundant fibrosis is the hallmark of this stage (1, 56, 57). (Figure 3)

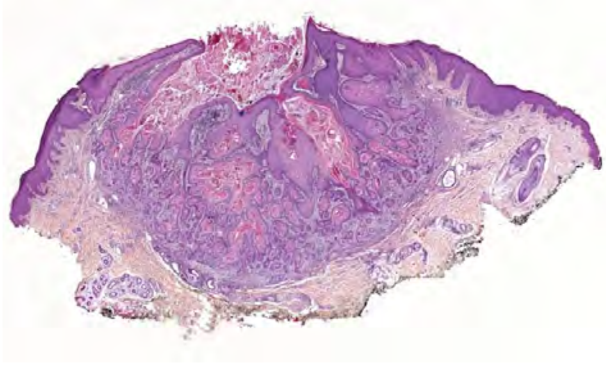

Figure 1. Early proliferative KA showing a nodular lesion with symmetric appearance, an ill-defined keratin-filled cater and an overhanging lip on one side. From the base of the crater, numerous well-differentiated squamous lobules with pale pink cells infiltrated into the sun-damaged dermis.

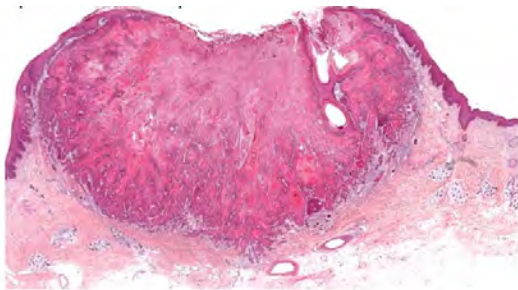

Figure 2. Mature KA showing a well-developed, symmetrical crateriform lesion with overhanging lips on both sides of the keratin-filled crator. Squamous lobules containing cells with pale pink cytoplasm and trichilemmal differentiation infiltrated into the dermis to the level of sweat glands. An inflammatory reaction is seen at the epidermal-dermal interface.

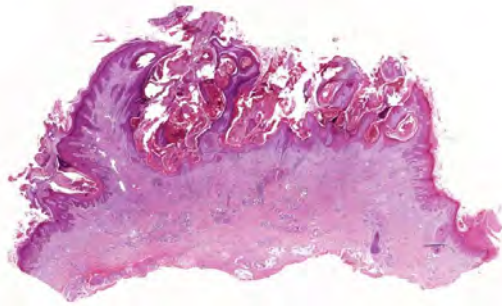

Figure 3. Late regression phase KA showing a shallow lesion filled with infundibular keratin. No infiltrating squamous lobules in the dermis. Infiltration of inflammatory cells and abundant fibrosis is seen.

Because of the resemblance between the life cycle of KA and that of the hair follicle cycle (12, 24, 58); it is claimed that KA is a hair follicle-derived tumor. Several studies tried to differentiate KA and cSCC with different cytokeratin/keratin markers (59, 60), but no study has come up with convincing results.

1.2 Cutaneous squamous cell carcinoma (cSCC)

Most cSCCs are assumed to arise from the interfollicular epidermis from precursor lesions as actinic keratosis (AK) and squamous cell carcinoma in situ (Mb Bowen), but may also develop de novo and arise from hair- follicles (1).

1.2.1 Epidemiology

cSCC is the second most prevalent non-melanoma skin cancer worldwide. The incidence rate of NMSCs has been increasing worldwide (61). The cancer registry of Norway registers cSCCs as NMSCs and registered in 2018, 1342 and 1119 new cases of NMSCs in males and females respectively. Although there are variable data from different studies, about 1,5-4% of cSCCs, metastasize to regional lymph nodes and distant sites (62-64) and up to 20% in high-

risk cSCC of head and neck (64, 65). SCC localized to ear and lips have a high metastatic rate (64).

1.2.2 Risk factors

UVR: UVR, especially UVB, induces cSCC in a multistep process, by a specific mutation in DNA, called UV signature mutation (66, 67) or by inducing immunosuppression resulting in loss of immunosurveillance on the malignant cells (68). The incidence of cSCC is highest in people with fair skin and is correlated with the geographical location. Countries near to equator like Australia and New Zealand have the highest incidence of cSCC. The prevalence of cSCC is also increasing in Europe and Northern-Europe due to recreational activities and cumulative sun exposure.

HPV: HPV 16 and HPV 18 DNA have been detected in SCC in the anogenital region, vulva, penis and in perianal regions (69). The E6/E7 oncoprotein of HPV 16 and 18 binds to p53 and RB1 proteins causing loss of their tumor suppressor functions (70, 71). The role of HPV in sunlight-induced cSCC is unclear. However, a research group has previously found the predominance of beta-papilloma HPV species 2 in cSCC in sun-exposed skin compared to normal healthy individuals (72). Patients with a rare genodermatosis, epidermodysplasia verruciformis (EV) have a high life-long risk of cSCC development when exposed to sunlight. Cutaneous HPV types 5 and 8 are often detected in cSCC of patients with EV (73-75).

Immunosuppression: Immunosuppression such as organ transplantation, has been implicated in the pathogenesis of cSCC (18, 76). HPV and Epstein-Barr virus (EBV) are associated with cSCC development in immune-deficient hosts (77, 78). A recent paper reported a high EBV DNA viral load in oral SCC and nasopharyngeal SCC (79). Patients receiving immunosuppressive treatment for rheumatoid arthritis, lymphoproliferative disorder or HIV infection have a high incidence of cSCC (80).

Chronic inflammation and chronic infection: cSCCs may rarely arise after many decades from chronic non- healing wound and post-burn scars (so-called Marjolin ulcer) (81, 82).

1.2.3 Genetic syndromes

Xeroderma pigmentosusm (XP): XP is an autosomal recessive disease with a genetic defect in DNA repair genes. Biallelic mutations of multiple genes that play a role in nucleotide

excision repair of UV-induced DNA damage are identified. XP patients have high, around 10,000 fold risk of acquiring cSCC (80, 83).

Muir-Torre syndrome: Autosomal dominant diseases due to the mutation of DNA mismatch repair genes MLH1, MSH2, MSH6. This syndrome is associated with multiple internal malignancies, especially colon cancer (HNPCC), genitourinary cancer, in addition to skin tumors like sebaceous adenoma, KA and cSCC at a very young age (80, 83).

Rothmund-Thomson syndrome: A very rare genodermatosis caused by mutation of DNA helicase *RECQL4* which plays an important role in DNA repair of the double-strand breaks. Patients usually develop NMSC at a young age in combination with poikiloderma, and osteosarcoma (80, 83).

Epidermodysplasia verruciformis (EV): EV is a rare autosomal recessive disease caused by the mutation of transmembrane channel *EVER1* and *EVER2* genes. Patients having a mutation of these genes are highly susceptible to HPV infection and developing cSCC in sun-exposed skin. (80, 83).

Dystrophic epidermolysis bullosa: This disorder is characterized by the mucosal fragility and blister formation on minor trauma. Patients show a germline mutation of the genes maintaining the structural components of the basal membrane zone in the epidermis. These patients have high penetrance for aggressive cSCC with poor outcome (80, 84)

1.2.4 Classification

We have used a 3-grade classification system as reported by Mc Kee (80) and by the Royal College of Physicians of Edinburgh (85) to assess the degree of differentiation of cSCCs.

- **Well-differentiated SCC (wd cSCC):** The tumor has infiltrating squamous lobules with clear intercellular bridges and distinct and abundant keratinization. Nuclear pleomorphism is less and there are few mitoses.
- **Moderately differentiated SCC (md cSCC):** The infiltrating squamous lobules are structurally disorganized and intercellular bridges are less evident. Keratin formation is relatively less than in wdSCC and is limited to the formation of keratin pearls, horn cyst, and scattered individual keratin cells. Nuclear pleomorphism and mitosis, including atypical mitosis, are more pronounced.

- **Poorly differentiated SCC (pd cSCC):** The tumor shows a solid sheet-like arrangement with a lack of differentiation towards squamous cells. Intercellular bridges and small foci of keratinization can on rare occasions be identified in parts of the tumor. Numerous mitoses are present, including atypical ones.

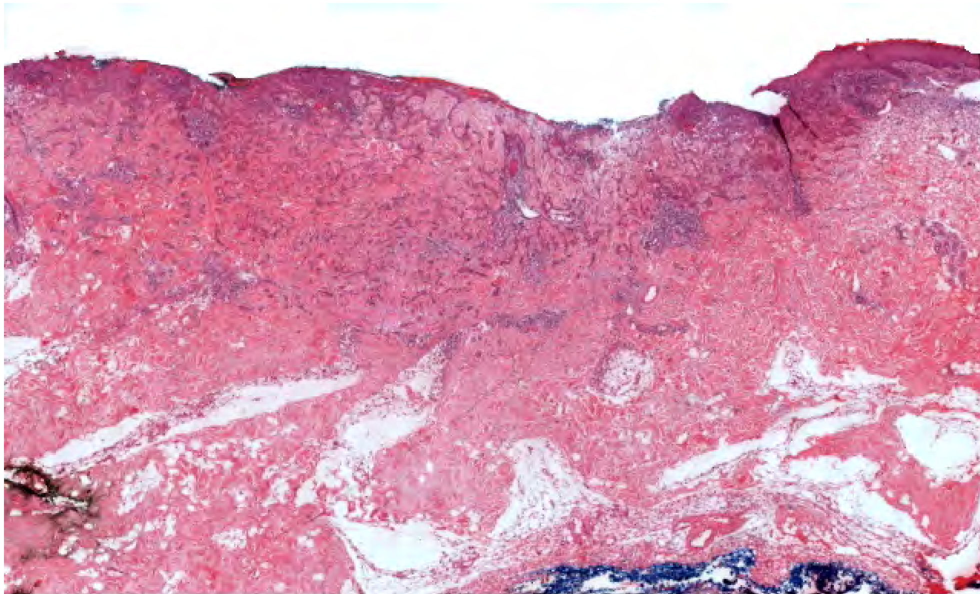

***Figure 4.** cSCC shows an ulcerative, asymmetric lesion with nests and lobules of atypical squamous cells diffusely infiltrating the dermis.*

1.3 Genomic aberrations in KA and cSCC

Over the years, several methods have been employed to gain a better understanding of the biology of KA. One of them is comparative genomic hybridization (CGH), which is a technique that detects gross chromosomal gains and losses (aberrations) simultaneously in the tumor genome in comparison to normal tissue. Chromosomal regions with aberrations may harbor putative oncogenes or tumor suppressor genes that may drive the development of neoplastic lesions. There are a few reports of CGH analyses of KA (7, 86, 87). One previous study by Clausen et al. found genomic aberrations in about 37% of cases of KAs (86) using standard CGH. A later study by the same group using the high-resolution array CGH method

revealed some degree of aberrations in all KAs and in addition, recurrent genetic aberrations that were significantly different from cSCCs (7). The loss of a region on chromosomal arm 17p corresponding to the *Tp53* gene was found in 27% of KAs and 46% of cSCCs. They found recurrent aberrations (gain) of chromosome 20, where one of the putative oncogenes *AURKA* that codes for Aurora-A is situated, occur more frequently in cSCC than in KA. The same group found high levels of recurrent chromosomal aberrations (7, 8, 10, 13, 17 and X) in cSCC, consistent with the genomic aberrations reported by Ashton et al., Boudis et al. and Salgado et al. (87-89).

Aneuploidy: Abnormal DNA content (aneuploidy) has been linked to neoplastic transformation. There are very few reports of assessment of the DNA aneuploidy status in KA (90-92). Aneuploidy incidence is lower in KA than in cSCCs and its precursors (90, 93). We have not measured ploidy status in our work.

1.4 Signaling Pathways and signaling pathway-related proteins

As described by Hanahan and Weinberg, many molecular changes occur during tumorigenesis and subsequent malignant transformation (94, 95). Depending upon the tumor type and tissue of origin, these changes can occur by different mechanisms and affect different signaling pathways. Moreover, these molecular changes can be seen in different stages of tumor development related to initiation, propagation, and invasion. A role for some of these molecular changes has been implicated in the biology of KA as well as in cSCCs.

1.4.1 p53 signaling pathway

p53: The *TP53* gene is a tumor suppressor gene, located to the short arm of chromosome 17 (17p13). The gene encodes the 53kDa protein p53, which functions as a transcription factor for target genes involved mainly in cell cycle regulation and apoptosis.

In normal cells, the level of wild-type p53 protein is low because of short half-life due to its rapid rate of destruction by ubiquitin-mediated degradation (96). Activation of the p53 protein occurs when the cell is subjected to cellular stresses like UV exposure, chemical carcinogens,

oxidative stress, or free radicals. Two major events mark the activation of p53 protein in the stressed cell; 1) Increased half-life of the protein leading to its stabilization and accumulation of the protein and 2) post-transcriptional modification resulting in its active role as a transcriptional regulator (97)(Figure 5).

Thus p53 acts as a sensor for cell damage and mediates protective responses by transcribing p53- target genes that regulate key cellular responses such as cell cycle progression, DNA-repair, apoptosis, and DNA damage checkpoints. Upon DNA damage, wild-type p53 acts as a checkpoint protein in the G1phase before DNA synthesis (98, 99). p53 is involved in the induction of a cell cycle arrest that persists until damaged DNA is repaired, at which point it is degraded (97, 98, 100). If damaged DNA is not repaired, apoptosis is induced.

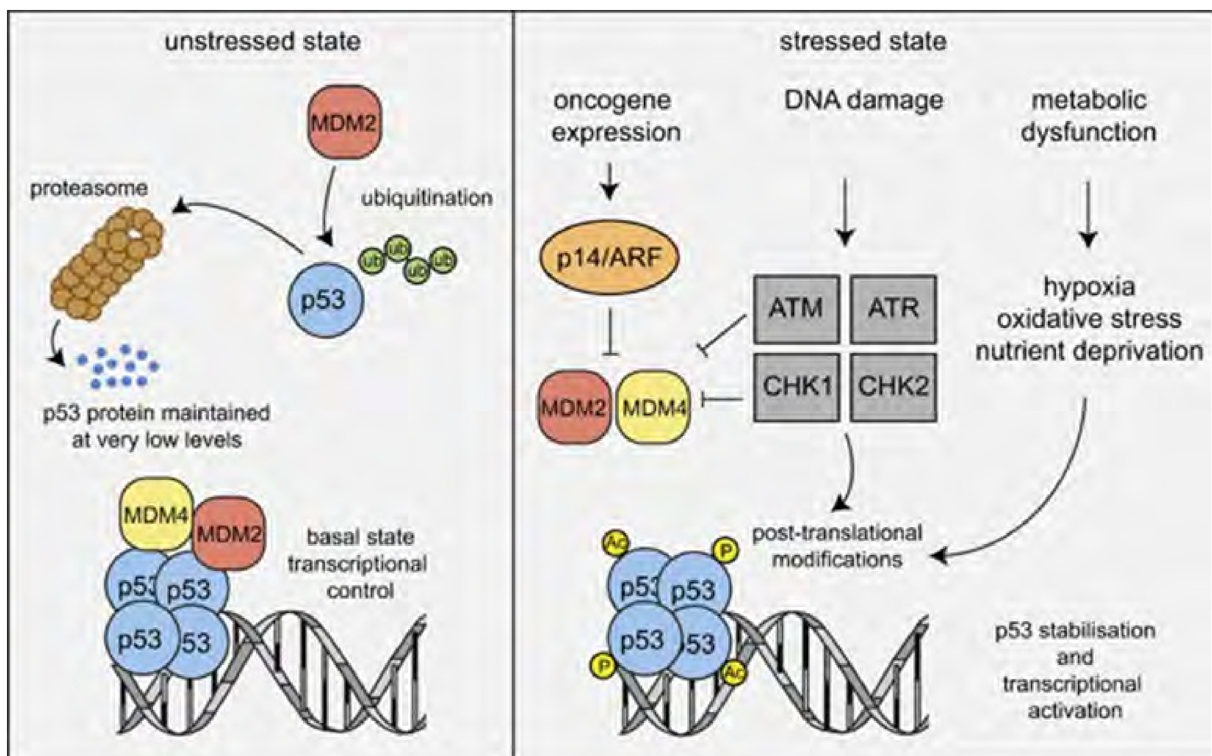

***Figure 5*: Stabilization of wt p53 in response to cellular stress and transcriptional activation leading to diverse cellular responses such as cell-cycle arrest, apoptosis, DNA repair, etc (to the right). In an unstressed state of a cell, p53 is degraded through MDM2 mediated ubiquitylation (to the left). Reproduced with permission from (97). The figure legend is modified.**

Multiple mechanisms influence the function of p53 including post-transcriptional regulation and somatic mutation of the *Tp53* gene (101-103). p53 inactivation due to gene mutation is considered an early event in cutaneous tumorigenesis (104).

Ultraviolet radiation (UVR) is the most important causative agent for the initiation of multistep cutaneous carcinogenesis. Mostly UVB (290-320) and also to some extent UVA (230-400) induce DNA damage through DNA strand breaks. The more energetic and shorter wavelength UVB induces mutation at the dipyrimidine sites; C-T and CC-TT, causing both single and double-stranded DNA breaks. The less energetic UVA releases reactive oxygen species (ROS), which ultimately induce DNA breaks (indirect DNA damage) and cause mutation in the form of G-T transversions (67, 68). These C-T and CC-TT patterns are designated as UV signature mutations (67, 105). Wild type p53 protein repairs UV-induced DNA damage. If the amount of DNA damage is too big to be repaired, wild-type p53 induces apoptosis, and the damaged cell is eliminated. When there is a mutation in the *Tp53* gene resulting in a mutated p53 protein, DNA damage cannot be repaired due to loss of wild-type p53 function, resulting in clonal expansion of the unrepaired cell. Most of the *Tp53* mutations in NMSC are UV signature mutations (67, 104).

Tp53 mutation in KA has not been extensively studied. Previously published papers were often based on the inclusion of a small number of patient samples (106-108). The genomic landscape of cSCC has not been widely studied. However some studies report *Tp53* gene mutations in around 60%-70% of cSCC (104, 109). The majority of mutations are missense mutations (point mutations), but some are nonsense mutations that lead to a complete lack of detection of p53 positivity (108, 110).

Tp53 mutation results in the accumulation of p53 protein that may be detected by Immunohistochemistry (IHC) due to the longer half-life of the mutated protein compared to wild type p53. Various studies have reported p53 positivity in KA (106, 107, 111-113), but protein levels do not always correspond to the mutational status (106, 107). Some report the pattern of expression in KA to be different from that of cSCC; KA often shows compact p53 positivity, localized to the highly proliferative peripheral zone, compared to the strong, compact and diffuse pattern of p53 positivity in cSCC (112-114).

p53 and apoptosis: One of the most critical functions of the p53 protein is the induction of apoptosis. Apoptosis is a complex process and involves extrinsic and intrinsic pathways through transcription of a series of target genes engaged in apoptosis.

In the intrinsic pathway, signals come from within the cell. The key component is the mitochondria. Upon DNA damage, p53 induces transcription of the *BAK* and *BAX* genes, which code for pro-apoptotic proteins of the Bcl-2 family. p53 also inhibits AIP (apoptosis-inducing protein) inhibitors to gear up the proapoptotic proteins. BAX proteins create a BAX tunnel on the mitochondrial membrane through which Cytochrome C is released. Cytochrome C binds with AFAP-1 in the cytosol and recruits Caspase 9 to form an apoptosome. Caspase-9 further activates Caspase- 3 that induces the nucleosome and proteasome. The nucleosome and proteasome degrade the cytoskeleton and DNA to form DNA blebs that are phagocytosed by macrophages (97, 115).

In the extrinsic pathway, apoptosis signals come from outside of the cell. Induction of three, highly tissue-specific, transmembrane proteins, Fas, DR5 and PERP, takes place upon DNA damage. Activation of the Fas receptor is pivotal in the extrinsic pathway activated by its ligand FasL produced predominantly by T cells. Through the formation of DISC (death-inducing signal complex), the activation of Caspase- 8 occurs which ultimately activates Caspase- 3. The same mechanisms as in the intrinsic pathway then proceed further to eliminate the apoptotic cells (115).

The uniqueness of spontaneous regression in KA is intriguing and has stimulated many researchers to study the apoptotic mechanisms possibly involved in the regression of KA. Several proteins of the Bcl-2 family i.e. BAK, BAX, Bcl-2, and Bcl-x are involved in programmed cell death or apoptosis (116). The p53 protein has been suggested to regulate the Bcl-2 family by upregulating *BAK* and *BAX* genes to induce apoptosis (117). *BCL2* is a proto-oncogene that is not directly related to cell proliferation but is involved in tumorigenesis by inhibiting apoptosis (118). Bcl-x is also a recognized member of the Bcl-2 family that has the same function as Bcl-2 (119). Heterodimerization of Bcl-2, Bcl-x, and BAK/BAX occurs to balance cell proliferation and cell death. The ratio between Bcl-2/Bcl-x to BAK/BAX is critical for whether a given cell promotes cell survival by inhibiting apoptosis (120). There are contradictory results of the expression of pro-apoptotic BAK and BAX and anti-apoptotic Bcl-2 and Bcl-x in KA. Increased expression of BAK and decreased expression of Bcl-x/Bcl-2 is suggested to be involved in tumor regression in KA (121, 122). Some

studies show very low levels of Bcl-2 protein in KA (123), whereas others show high expression levels of Bcl-x and BAX in KA that are not significantly different from those in cSCC (124).

The TUNEL assay, i.e. terminal deoxynucleotidyl transferase (TDT) assay, is used to measure the apoptotic indices in a tissue section via the detection of sites of fragmented DNA (125). While some studies show few TUNEL positive cells in cSCC compared to KAs (125), others show no significant difference in TUNEL positive cells in KAs and cSCCs (124).

P21: p53-mediated growth inhibition in a cell is dependent on the induction of the critical gene *CDKN1A* that codes for the WAF1/p21 protein (126, 127). The p21 protein is a cyclin-dependent kinase (CDK) inhibitor, which arrests cell cycle progression in the G1/S phase through inhibition of CDK 4,6/Cyclin D, and CDK2/Cyclin E (128, 129). Deregulation of p21 is associated with carcinogenesis (130). The role of p21 protein in KA is controversial. There are reports of both positive and negative correlations of p21 protein and p53 levels in KA (131, 132).

1.4.2 Wnt/ β-catenin signaling pathway

Different cellular signaling pathways are activated during tumor formation. These pathways cross-talk with each other and favor proliferative activities over tumor-suppressive functions in a tumor cell. One such pathway is the Wnt / β-catenin signaling pathway. It regulates cellular proliferation, differentiation, homeostasis, cell repair, and apoptosis, and is often hijacked in cancers (133-135). When Wnt signaling is not active, Wnt ligands are not bound to the frizzled receptors. A destruction complex consisting of APC, Axin, and GSK-3β is then formed and phosphorylates β-catenin that is primed to be destroyed by ubiquitin-mediated degradation. On activation of Wnt signaling, Wnt ligands bind to frizzled receptors, causing disintegration of the destruction complex through disheveled (135). Subsequently, stabilization of β-catenin occurs in the cytoplasm (136, 137). Thereafter, stabilized β-catenin translocates to the nucleus to bind with the LEF-1/TCF transcription factor to transcribe Wnt target proteins like Cyclin-D1, Sox-9, Axin 2 and others that play crucial roles in the proliferation and differentiation of normal tissues (138-140). See figure 6.

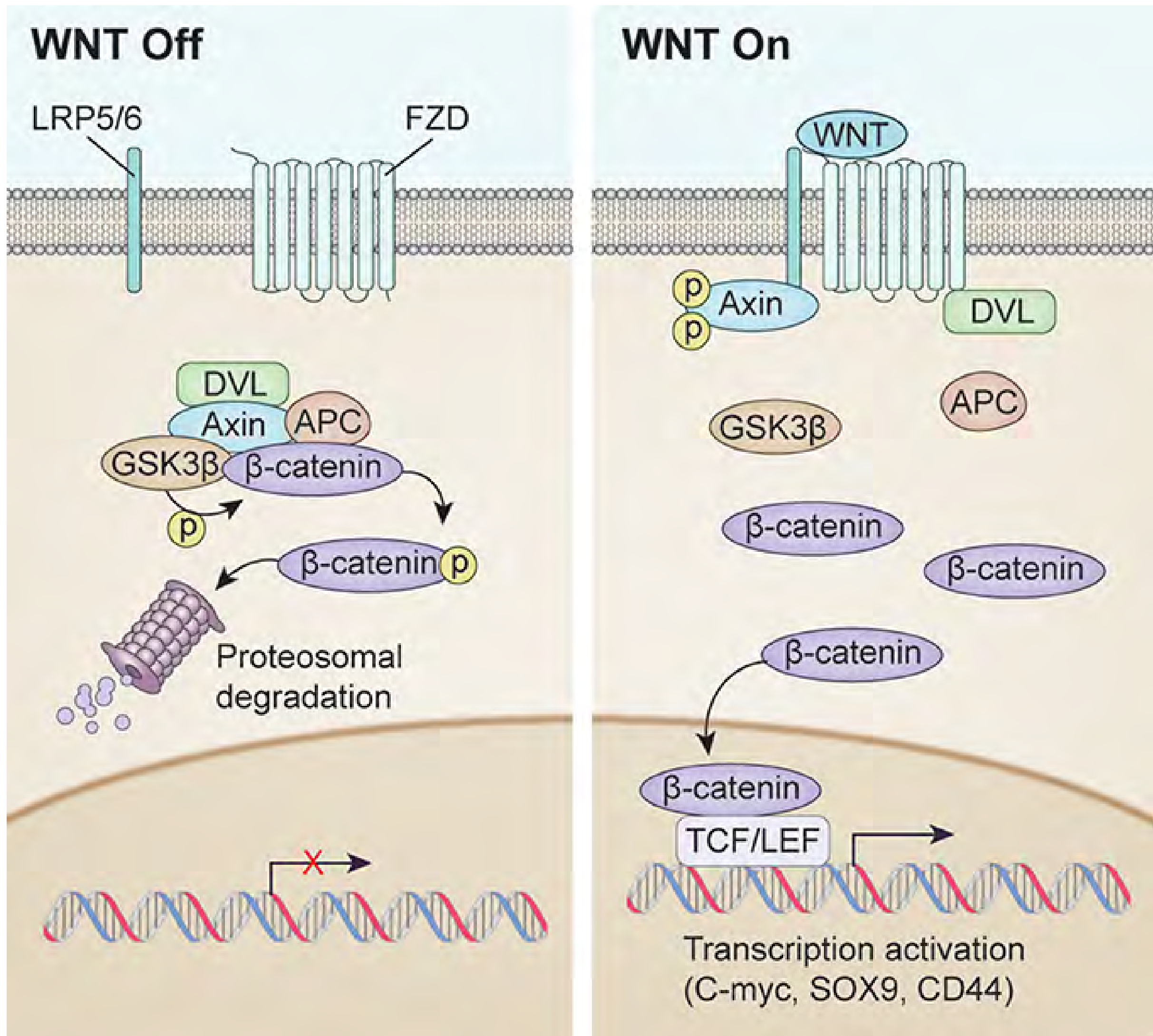

Figure 6: Wnt signaling pathway in an inactive state (left) and active state (right)

In the absence of Wnt ligands, β-catenin is phosphorylated by a destruction complex composed of Axin, APC, and GSK-β and targeted for proteasomal degradation. In the absence of β-catenin, TCF/LEF cannot transcribe the Wnt signaling protein. When the Wnt signaling is active, the destruction complex is disintegrated, so that β-catenin cannot be phosphorylated. Accumulated β-catenin is further translocated into the nucleus and bound to its cofactor TCF/LEF for transcription of Wnt targeted proteins. Reproduced with permission from (141) (open access). The figure legend is modified.

The Wnt/ β-catenin signaling pathway is involved in the genesis of the hair follicle (142, 143) and KA development is thought to recapitulate the hair follicle cycle (24, 58). Cross-talk between the Wnt signaling pathway and the retinoic acid pathway is reported to be involved in the initiation and regression of KA tumors in a murine model (144). The roles of the Wnt/ β-catenin signaling pathway have not been studied to date in human KAs.

β-catenin: β-catenin is the key molecule of the Wnt/ β-catenin signaling pathway. β-catenin nuclear accumulation and/or cytoplasmic accumulation has an oncogenic effect and is seen in various types of cancers (145-149). In most of the skin tumors, the role of β-catenin has been emphasized as an adhesion molecule. KAs show varying expression patterns of β-catenin, the majority, with similarities to the normal epidermis showing membranous positivity and are different from cSCCs (145, 150, 151). Altered expression of β-catenin i.e. reduced membrane positivity and increased nuclear intensity in oral SCC is associated with increasing histological grades of cSCC and poor prognosis (152-154).

p53 and β-catenin cross-talk with each other during physiological and pathological conditions (155, 156). While wild type p53 has an inhibitory effect on β-catenin, nuclear accumulation of β-catenin induces wild type p53 protein (157). Loss of function of p53 leads to activation of Wnt signaling in patients with Le-Fraumeni syndrome with germline mutation of the *TP53* gene (155). p53 inactivation leads to an increased level of Aha1, a co-chaperone of HSP90 protein which stimulates the Akt/GSK-3β pathway and enhances stabilization and nuclear localization of β-catenin (155).

An association between spindle protein Aurora-A and β-catenin expression was reported by Jin et al. (158). He demonstrated that the phosphorylation of β-catenin at Ser 552 and at Ser 675 by Aurora-A increases the stability and transcriptional activity of β-catenin in esophageal cancer cell lines. There is also a report of a specific inhibitor of Aurora-A, which decreases the levels of β-catenin by decreasing the phosphorylation of β- catenin in gastric cancer (159). Interaction of Aurora-A with Wnt signaling has also been reported recently by Jacobsen et al (160).

SOX-9: The SRY sex-determining region Y-box 9 (*SOX9*) is a transcription factor involved in various developmental processes. It plays a crucial role in tissue morphogenesis, survival and stem cell maintenance. In normal skin, it is expressed in the sebaceous gland, outer root sheath and the bulge region of the hair follicle and maintains homeostasis through proliferation, apoptosis, and differentiation of keratinocytes (161, 162). The expression of Sox-9 is normally regulated through the Wnt signaling pathway (163). Paradoxically, Sox-9 inhibits the Wnt/ β-catenin signaling pathway by promoting either degradation of β-catenin in the cytoplasm or degradation in the nucleus by binding competitively with TCF/LEF1 (164). However, Sox-9 is also regulated by another signaling pathway, the Shh pathway (161). Sox-9 is found to be upregulated in many epithelial cancers and is associated with poor prognosis (165-167).

Although the role of this protein has not been fully explored in skin tumors, it is reported that overexpression of Sox-9 is associated with keratinocyte-proliferation (162). Sox-9 is highly expressed in adnexal derived neoplasms (168). Shi G et al reported positive expression of Sox-9 in UVB-induced skin tumors including KA (162). Both increased and reduced expression of Sox-9 is reported in cSCCs (161, 169).

Lef-1: Lef-1 (lymphoid enhancer-binding factor-1) is a transcription factor that mediates downstream cellular effects of the Wnt / β-catenin signaling pathway (170). It plays a critical role in stem-cell maintenance and organ development (171).

Since the Wnt/ β-catenin pathway is involved in hair follicle genesis, the levels of Lef-1 are also dynamically expressed during the normal hair follicle cycle (172). Aberrant expression of Lef-1 is implicated in tumorigenesis and progression of cancer (173-175). While high expression of the protein is associated with poor prognosis in oral SCC (173) and pilomatrical carcinoma (176), loss of expression is associated with progression in sebaceous carcinoma (174). Lef-1 protein levels are differentially expressed in a murine KA model, with high levels detected in the early proliferative phase compared to the regression phase (144).

Cyclin-D1: Cyclin-D1 is a cell cycle regulatory protein, involved in cell cycle progression, especially in the G1 phase (177). Cyclin-D1 is an interconnection hub for many signaling pathways e.g. the mitogen-activated protein kinases (MAPK-ERK) pathway, phosphoinositol

3-kinase (PI3K) pathway, JAK-STAT pathway, and the Wnt signaling pathway, and others (178) (Figure 7). Imbalanced regulation of one or more proteins of these pathways or deregulation of *CCND1* gene expression influences tissue homeostasis by activating Cyclin-D1 and contributes to many proliferative diseases including cancers (179, 180). *CCND1* is a downstream target gene of the Wnt/β-catenin pathway and is related to cell proliferation (179, 181). A gain of chromosomal arm 11q that contains the *CCND1* locus is correlated to high expression of Cyclin-D1 protein in KA (182). Upregulation of Cyclin-D1 in proliferative KA and downregulation in regressive KA have been described in a mouse model (144).

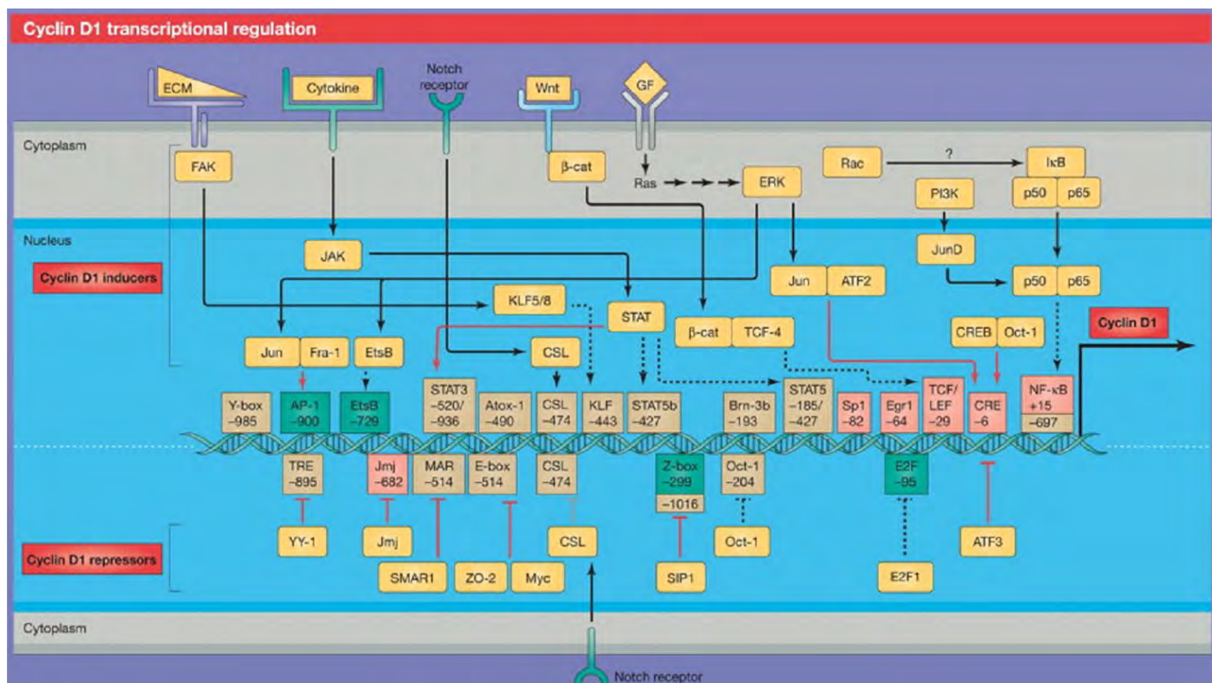

Figure 7 Cyclin D1 transcriptional regulation depicting both inducers and repressors. Reproduced with permission from (178). The figure legend is modified.

1.4.3 Mitosis and cell cycle regulatory proteins

Aurora-A: Aurora A, a member of the serine/threonine kinase family, is a mitotic protein. The primary function of Aurora-A in a normal cell is in the maturation and duplication of centrosomes, bipolar mitotic spindle assembly, alignment of chromosomes at the metaphase plate and cytokinesis (183-185).

The inactivated form of Aurora-A protein is first accumulated at the centrosome during the late S phase. A small proportion of activated Aurora-A at centrosomes during late G2 phase is required for centrosome maturation and entry into mitosis. Its further activation increases the levels in the centrosome during G2-M transition, and subsequent nuclear translocation is necessary for centrosome separation and bipolar spindle assembly in prophase. After the breakdown of the nuclear envelope, activated Aurora-A is observed at the spindle poles and migrates along with the mitotic spindles to midzone during metaphase. The Aurora-A protein levels start to decrease at the metaphase-anaphase transition and are then degraded by the anaphase-promoting complex/cyclosome (APC/C). Most of the Aurora-A protein becomes undetectable in the final stage of cytokinesis (185, 186). See figure 8.

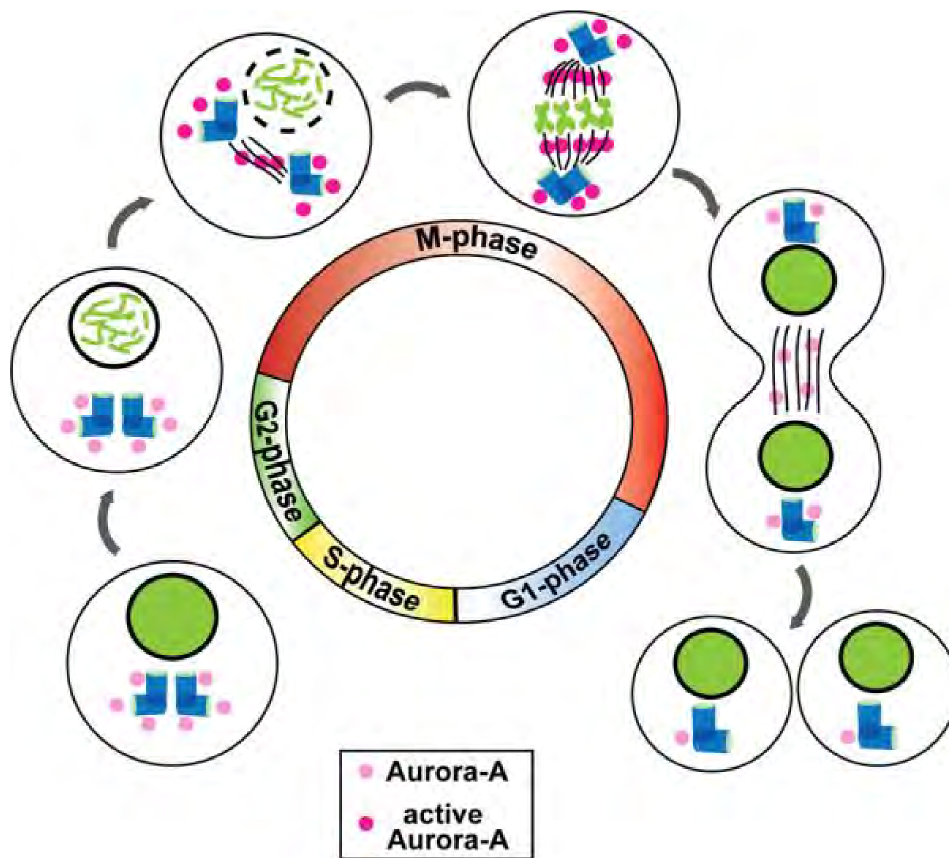

Figure 8: Activation of Aurora-A during the cell cycle

Aurora-A begins to accumulate in the S phase, but it is activated during the transition between G2 and M phases. Activated Aurora-A then migrates along the mitotic spindle towards the metaphase plate. Most of Aurora-A is inactivated and degraded before cytokinesis with very low levels of inactive Aurora-A in the early G1 phase. Reproduced with permission from (185). The figure legend is modified.

The *AURKA* gene is mapped to chromosome arm 20q13.2-3 (187). Aurora-A overexpression (both mRNA and protein levels) is a frequent occurrence in solid cancers (186, 188-190). Overexpression of Aurora-A contributes to faulty spindle formation and faulty cytokinesis (191). Cells with Aurora-A overexpression would normally be checked by the p53-RB pathway, also called the G1 checkpoint, but when the p53 pathway is defective, cells will not be checked and will continue the cell cycle leading to CIN, aneuploidy, and carcinogenesis (192, 193). There are various proposed mechanisms of overexpression of Aurora-A in a cell, see figure 9 (185, 191, 194, 195). The most described mechanism of overexpression in

cancers is chromosomal amplification of the *AURKA* gene locus (191, 196, 197). Mutations of the *AURKA* gene in all types of cancers are rarely described in the literature. Overexpression of Aurora-A is associated with tumor progression from papilloma to cSCC in a mouse model (198), and poor prognosis in HNSCC (199). Synergistic effects of Aurora-A and other proteins are also implicated in the metastasis of cSCC (198, 200).

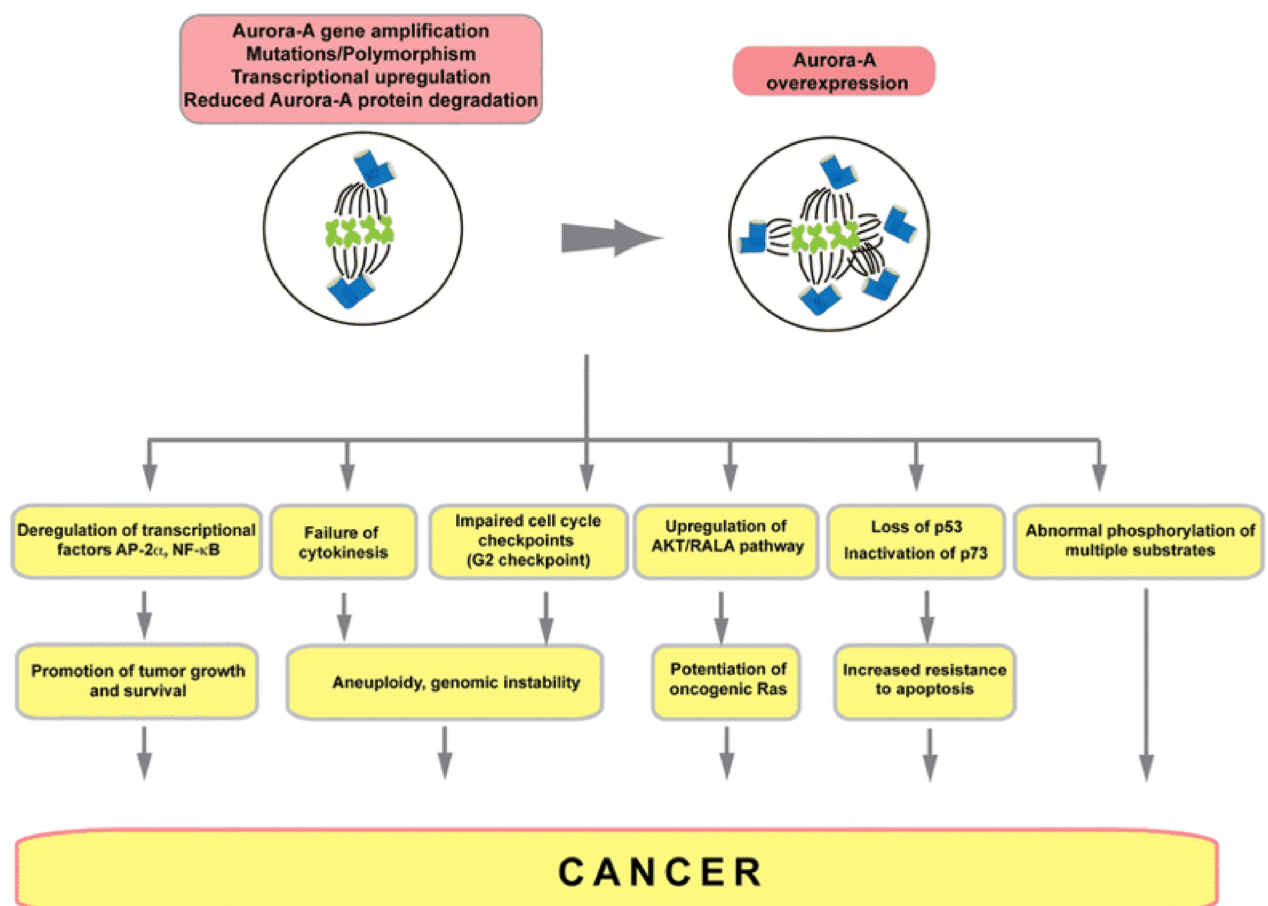

Figure 9: Multiple mechanisms of Aurora-A overexpression and its role in cancer development. Overexpression of Aurora-A may either be due to gene amplification or mutations/ polymorphism. Transcriptional upregulation or deregulated degradation also leads to Aurora-A overexpression. Reproduced with permission from (185). The figure legend is modified.

Interaction between p53 and Aurora-A: Wild type p53 protein negatively regulates Aurora-A, thus inhibiting cell proliferation (201). Conversely, Aurora-A can negatively regulate p53 (202). p53 phosphorylation by Aurora-A at serine 315 induces MDM2-mediated ubiquitination and degradation of p53, whereas phosphorylation at serine 215 inhibits its transcriptional activation (202, 203), maintaining a fine balance between the levels of p53 and Aurora-A in normal cells. This physiological feedback loop is altered either by mutation of *TP53* or deregulation of Aurora-A kinase. In a cell with *TP53* mutation, Aurora-A- induced cell damage cannot be repaired, and the cell undergoes another cycle, forming a tetraploid/aneuploid cell. Overexpression of Aurora-A, on the other hand, inhibits wild-type p53 protein (204).

Spindle assembly checkpoint (SAC): The spindle assembly checkpoint (SAC) is a critical feedback signal that ensures equal segregation of chromosomes and protects the cell from chromosomal instability (CIN). The components of the SAC include Mad1, Mad2, BUBR1 (Mad3), BUB1, BUB3, and Mps1. Mad2, BUBR1, Bub3, and Cdc20 proteins together form the mitotic checkpoint complex (MCC) that inhibits the anaphase-promoting complex/cyclosome (APC/C), a co-factor of Cdc20. Within the MCC, Mad2 and BUBR1 bind directly to Cdc20. Binding of BUBR1 to CdC20 requires all SAC proteins, whereas the interaction of Mad2 and Cdc20 requires Mad1 and Mps1. When the SAC signal is on, the MCC inactivates APC/C, thereby delaying the anaphase transition. Upon proper attachment of kinetochores, the SAC signal is satisfied. MCC then disassembles, and APC/C becomes active to promote anaphase (205-207), See figure 10. Deregulated SAC proteins Mad2 and BUBR1 are associated with poor prognosis in several cancers including oral SCC and tonsillar SCC (208-210). To date, the role of spindle checkpoint proteins has not been studied in KA.

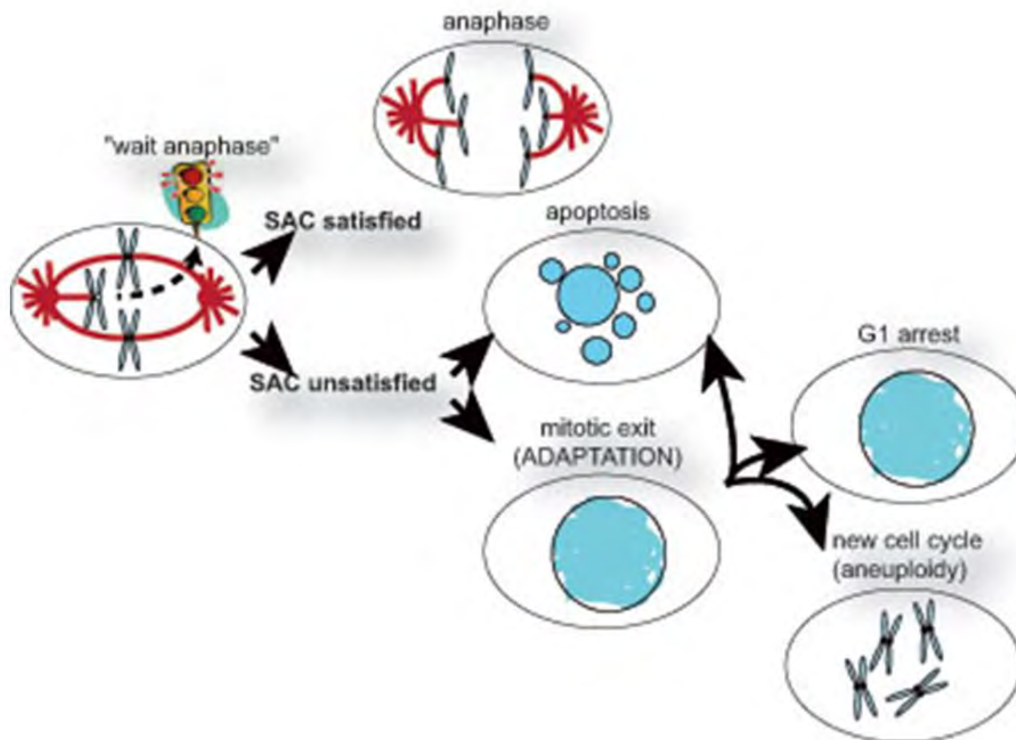

Figure 10: Cell fate on SAC activation

SAC is activated when mitotic spindles are not properly attached to the kinetochore. When SAC is satisfied, the anaphase wait signal is abolished and the cell ensures proper bipolar attachment of chromosomes and propagation of the cell cycle. When the SAC is unsatisfied, the cell can either die in mitosis or undergo mitotic slippage with different outcomes. The cell can either die by apoptosis or undergo G1 arrest or gives rise to aneuploid cells. Reproduced with permission from (211) (open access). The figure legend is modified.

Interaction between SAC proteins and p53: The mitotic checkpoint is essential for the regulation of faithful chromosomal segregation. Activation of p53 in response to spindle damage is vital for the protection of the cell either by arresting the cell cycle to allow for DNA repair or by inducing apoptosis (212). While Aurora-A and p53 have a negative feedback loop, the association between BUBR1 and p53 occurs in a positive feedback manner (212, 213). Ha GH et al, 2007 reported the phosphorylation and stabilization of p53 by BUBR1 upon spindle damage. Cells with defective BUBR1 will not be able to phosphorylate

and stabilize the p53 protein (212). Similarly, cells with deficient p53 will not be able to induce apoptosis and cells with spindle damage will proceed to the next cell cycle leading to aneuploidy (212).

Ki-67: Ki-67 is an antibody against a nuclear antigen which is associated with cell proliferation. This proliferation- associated antigen is expressed in all phases of the cell cycle except in the G0 phase, indicating that it is a biomarker of the growth fraction**.** The Ki-67 index has been used to differentiate between proliferative KA and late phase of KAs and between KAs and well-differentiated cSCCs (113, 214-216).

1.4.4 The TGF-β signaling pathway

Transforming growth factor-beta (TGF-β) is a cell surface receptor protein that induces myriads of biological signals to regulate tissue homeostasis, immune response, angiogenesis, inflammation and epithelial-mesenchymal transition (EMT). Downstream signaling occurs mainly through the SMAD, RAS, or MAPK pathways (217). Mutation of the *TGFBR1* gene has been identified as the cause for multiple KAs (MSSE or Ferguson-Smith disease) found in Scottish ancestry and leads to loss of function of the TGFBR1 protein (49). *TGFBR1* gene mutations have not been reported in sporadic KAs.

1.4.5 Ras and Raf (MAPK signaling pathway)

Vemurafenib and Dabrafenib (BRAF inhibitors) are used to treat patients with advanced malignant melanoma with *BRAF* V600E mutations with a high rate of response. However, 15-30% of patients treated with BRAF inhibitors develop cutaneous neoplasms like KA and cSCC, possibly through paradoxical activation of the Ras signaling pathway (37, 218, 219). *HRAS* mutations have been detected in a substantial number of KAs (220, 221).

1.5 Treatment of KA and SCC

KA is a challenging tumor, partly due to an unforeseeable evolution to cSCC in some cases, and to its resemblance to cSCC. Surgical removal of the primary site is the gold standard of treatment for both KA and cSCC (222, 223). Mohs micrographic surgery may be employed when tissue sparing is an issue, as is the case for large KAs and also for cSCCs localized to the head and neck region (224, 225). Intralesional methotrexate or 5-Fluorouracil injections have been tried in KA for a better cosmetic outcome (226, 227). Systemic acitretin or other retinoids have also been used as the first line of treatment, either as a monotherapy or combined therapy for multiple KAs (228) and in KA centrifugum marginatum (229, 230). Radiation therapy is the treatment of choice for recurrent cSCCs for patients who cannot undergo surgery, for cosmetic sensitive areas of head and neck, and as a combined therapy for patients with metastatic cSCC (231).

2 Aims of the study

The main aim of the study is to gain more insights into the biology of KA through studying the phenotypic and genotypic changes in KA and to relate them to different phases of development. The secondary aim is to find potential new markers to differentiate KA from cSCC.

The objectives of the study were:

Paper I: To investigate the p53/*Tp53* status during the life cycle of KA and to assess the frequencies of *Tp53* mutations with special emphasis on a possible role for the p53 protein during the development.

Paper II: To explore the idea that KA is a hair follicle-derived tumor by investigating the Wnt/β-catenin signaling pathway during the life cycle of KA.

Paper III: To study the expression of the spindle proteins Aurora-A, Mad2, and BUBR1 in KAs, and to investigate possible differences in their expression between KA and cSCC.

3 Summary of results

3.1 Paper I

Tp53/p53 status in keratoacanthomas

Sarita Joshi, Aasa R.Schjølberg, Per Olaf Ekstrøm, Paula M. De Angelis, Manuela Zucknick, Solveig Norheim Andersen, Ole Petter F. Clausen

KA has a unique feature of initial rapid growth followed by partial or complete regression after some time. We hypothesized that the pro-apoptotic p53 protein could be involved in the life cycle of KA. We analyzed the mutational frequency of *Tp53* utilizing cycling temperature capillary electrophoresis (CTCE) and p53 immunoreactivity by immunohistochemistry in a large cohort of 124 KAs. CTCE detected mutations in the hot spot regions of exons 5- 8. *Tp53* mutation status and p53 levels were analyzed for associations with developmental stages in KA that were stratified histologically, and according to the clinical age of the lesions when available, transplantation status and phenotypic characteristics (atypia, infiltration, inflammation, and fibrosis).

We detected *Tp53* mutations in a substantially larger number of KAs (ca. 40%) than described hitherto in the literature. Within the old KA subgroup based on histopathology, we found significantly more lesions without *Tp53* mutation than lesions with mutation, which may suggest the involvement of wild type p53 in the regression of KA, possibly through inducing apoptosis.

Significantly more KAs (91%) with *Tp53* mutations had moderate to very high p53 levels which reflects the accumulation of mutant p53 protein due to increased half-life compared to KAs without *Tp53* mutations. Low p53 levels (<20%) were seen more frequently in KAs without *Tp53* mutations. However, considerable high levels of p53 were also seen in KAs without detectable *Tp53* mutations, indicating that accumulation of p53 protein (wild-type) may be independent of *Tp53* mutation. High p53 levels were associated with increasing grades of atypia, especially in KAs without mutations, suggesting that atypia may not be related to *Tp53* mutations and may involve some other mechanism for this phenotype. The

frequency of *Tp53* mutation in patients with immunosuppressive treatment did not differ from patients without immunosuppressive treatment (most of the KAs were transplant patients). p21 protein is often not detected in tumors with *Tp53* mutations, but surprisingly we found high levels of p21 in KAs with *Tp53* mutations, suggesting a p53-independent pathway for the transcription of *CDKN1A*.

3.2 Paper II

Role of the Wnt signaling pathway in keratoacanthoma

Sarita Joshi, Paula M. De Angelis, Manuela Zucknick, Aasa R.Schjølberg, Solveig Norheim Andersen, Ole Petter F. Clausen

KA has a developmental life cycle with similarities to the cycle of the hair follicle. The Wnt/ β-catenin signaling pathway plays a pivotal role in the activation of the anagen phase of the hair follicle cycle. A previous study in a murine KA model showed upregulation of the Wnt/ β-catenin signaling proteins in young proliferative KAs and downregulation of the same proteins in old regressive KAs in cross-talk with the Retinoic acid pathway. To investigate whether the Wnt/ β-catenin proteins are differentially expressed in human KAs (n=67), we assessed the expression of the downstream proteins of the Wnt/ β-catenin signaling pathway utilizing immunohistochemistry. The protein levels of β-catenin, Lef-1, Sox-9, and Cyclin-D1 were analyzed for their associations with young and old KAs stratified either histologically or clinically when data were available or by the Ki-67 index.

We did not find any significant differences between the expression levels of the Wnt signaling proteins in young and old KAs. Thus we failed to recapitulate results from the animal model in human KA. However, we found high expression levels of the proliferation markers Sox-9 and Cyclin-D1 in our study, suggesting alternating mechanisms of regulation of these proteins. High expression of Sox-9 may have an inhibitory role on the Wnt signaling pathway.

3.3 Paper III

Expression of spindle proteins in keratoacanthoma and squamous cell carcinoma

Sarita Joshi, Paula M. De Angelis, Manuela Zucknick, Aasa R.Schjølberg, Solveig Norheim Andersen, Ole Petter F. Clausen

The spindle protein Aurora kinase A is highly expressed in various cancers. The SAC proteins Mad2 and BUBR1 are also aberrantly expressed in solid organ cancers including oral SCC and HNSCC. Dysfunction of spindle proteins alone is unable to transform cells, and additional inactivation of tumor suppressor genes like *Tp53* is required for cancer development. In this study, we compared the expression levels of the spindle proteins in KAs (n=25) and cSCCs (n=25). p53 positivity and the Ki-67 index were also measured and correlated with the expression levels of the spindle proteins.

Spindle proteins Aurora-A, Mad2, and BUBR1 levels were significantly higher in cSCCs compared to KAs. Well-differentiated cSCCs also showed higher levels of Mad2 than young and proliferating KAs indicating that Mad2 levels are not related to proliferation only, since there was no significant difference in Ki-67 levels between the groups. BUBR1 levels were positively correlated with p53 levels in the KA subgroup, suggesting a possibility that wt p53 have induced the increase in BUBR1 levels. The levels of Aurora-A, Mad2, and BUBR1 were correlated with Ki-67 levels in the cSCC subgroup, suggests higher proliferation in malignant cSCCs.

4 Methodological considerations

This section covers the general description, strengths, and limitations of the materials and methods chosen for this thesis work. Detailed descriptions of methods are presented in the individual papers.

4.1 Patient selection and histopathological evaluation

Patient materials with a tentative diagnosis of solitary KA were provided by the dermatology department, Oslo University Hospital, Norway, during the period 1995-2013. The majority of patients (ca. 70%) were male and ca. 80% of the patients had received solid organ transplantations. A biobank was established for the storage of fresh frozen tissue collected during the period 1995-2013, which was utilized for genetic analyses. All three articles that make up this thesis were based on the use of archival material for evaluation of histopathology and immunohistochemistry. The patient consent documents were approved by the regional committee for medical and health research ethics.

Freshly-excised specimens with a tentative diagnosis of KA (solitary) were cut into two halves. One half of the specimen was processed further for routine diagnosis, and the other half was stored at -80°C in the biobank. The diagnoses were provided by experienced pathologists.

The inclusion criteria for KAs were according to the definition and differential diagnosis by Elder et al (1). KA was characterized as a lesion with symmetrical appearance, exo-endophytic growth with a central keratin-filled crater, surrounded by two overhanging epithelial lips. From the base of the crater, well-differentiated squamous lobules with pale pink cells with ground glass appearance were infiltrating into dermis. Any such lesions not fulfilling the criteria of Elder et al were excluded. The lesions were also re-evaluated by two experienced pathologists (OPC; SJ). KAs were stratified either as young or old lesions according to phenotypic characteristics as inflammation, and fibrosis as presented in detail in our paper (232). Cytological atypia and degree of infiltration were also assessed. Lesions re-diagnosed as cSCC were excluded from Paper I and II, but included in paper III.

Particularly the proliferative phase of KA may show malignant phenotypes like atypia and infiltration which makes it difficult to differentiate KA from cSCC. We included all the lesions with symmetry irrespective of the degree of atypia and infiltration. Lesions that showed possible transformation to cSCC were excluded.

4.2 Immunohistochemistry (IHC)

IHC is the most widely used ancillary method in daily pathology practice and also in biomedical research (233, 234). The technique utilizes specific binding of antibodies to the antigen of interest (protein, macromolecule) in tissues. Although IHC is an extensively-utilized method to investigate the expression of different proteins, there are pitfalls associated with several steps of the procedure.

All three papers are based on IHC that was performed on Dako automated platforms and Ventana autostainers in our work. Autostainers are superior to manual staining because they use standardized procedures/routines and give better overall performances and less turnaround time in addition to facilitating reproducibility. Reproducibility is the ability of an experiment to be reproduced when it is repeated according to the same experimental setting and using the same procedure. However, some antibodies cannot be adapted to automated platforms, since the protocols have not been properly tested and thus manual stainings must be performed. The advantages of manual staining are increased flexibility and better optimization of specific antigen-antibody reactions.

4.2.1 Tissue fixation and processing

Proper tissue fixation is necessary for high-quality IHC results. Formaldehyde as 10% neutral buffered formalin (NBF) is the most commonly-used tissue fixative. Formalin provides not only rigidity to the tissue but also the preservation of the morphology of the original tissue. A 4 mm thick tissue biopsy needs at least 24 hours of formalin fixation. Improperly-fixed tissues or over-fixed tissues significantly impact the quality of IHC staining (235). Since KA tumors containing abundant keratin material may hamper the penetration of formalin into the tissue, our materials were fixed for at least 24 hours (or more) for optimal fixation. Formalin-fixed

tissues were then embedded in paraffin to facilitate tissue sectioning and long- term storage. A tissue section of 3-5 µm which is ideal for IHC, was used in our studies.

4.2.2 Antigen retrieval (AR)

A potential drawback of FFPE tissue is the masking of antigen epitopes due to the cross-linking of proteins to nucleic acids or between proteins by methylene bridges (236). AR is a crucial step for the visualization of antigen-antibody complexes in FFPE tissue. Previously, proteinase enzymes were used for AR. The development of heat-induced epitope retrieval (HIER) (237) has led to its being accepted as the gold standard in IHC analyses because HIER breaks the formalin cross-linking and restores the three-dimensional structure of the protein to near-native condition. HIER can be achieved by the application of autoclaving, pressure cooking, microwaving or steam heating to the tissue sections. Microwaving is the most easily available and widely-used HIER method in manual staining, whereas a water bath is used in autostainers. Pressure cooking gives a poorer staining quality compared to microwaving, whereas autoclaving is a cumbersome procedure. A proper AR also depends on the pH and composition of the AR buffer. The choice of buffers varies from antibody to antibody and the pH chosen for the AR buffer is essential for whether an antibody targets a nuclear protein or not.

High pH (pH 9) buffer was used for AR of most proteins in our studies according to the HIER protocol of Dako, except for Sox-9, where low-pH (pH 6) was used. A high pH buffer and a low pH buffer were used for β- catenin and p53, respectively, according to the HIER protocol of the Ventana (Roche) autostainer. For the spindle proteins Aurora-A, Mad2, and BUBR1, a high pH (Tris-EDTA) buffer was used as described in a previous article (238).

4.2.3 Antibody specificity and validation

The issue of antibody specificity is a major concern in IHC. Monoclonal antibodies are more specific because they target a single epitope and have a higher lot- to- lot consistency compared to polyclonal antibodies. However, polyclonal antibodies are more sensitive and robust than monoclonal antibodies because they bind to several epitopes (234). A good antibody is defined as an antibody that binds specifically to its target antigen and is sensitive and robust, as well as reproducible. In other words, it should not be cross-reactive, i.e. it

should not bind to proteins other than the intended target. At present, there are more than 350 small and large manufacturers producing antibodies for clinical and research purposes. For one target antigen, there are many candidate antibodies available. Of the thousands of commercially-available antibodies, there are some antibodies with high specificity that can be employed in IHC assays (239). Therefore, the selection of antibodies is the most crucial factor influencing IHC. The selected antibodies must be validated and optimized in the laboratory. Western blotting is widely used to validate an antibody's specificity; the blot should show a specific band at the expected molecular weight for the target antigen. However, even if an antibody performs well in Western blotting, this may not guarantee the same performance in IHC. In Western blotting, target proteins are completely denaturated and an antibody reveals a linear distributed epitope compared to a near-native, 3D state of the target epitope in IHC (239). Therefore, validation must be carried out in the context of the application. Another way of validating an antibody is to test/use more than one antibody that targets the same protein. Serial titration/ dilution of antibodies (optimization for use in staining procedures) should be done in a set of different tissue with positive and negative controls. Liquid chromatography-mass spectrometry (LC-MS) is another method that can be used to validate antibodies. The advantage of this method is its high throughput for obtaining specificity but requires an expensive mass spectrometer and highly trained personnel (240).

Western blotting (immunoblotting) is a technique where antibodies are utilized to detect target proteins from whole tissue lysates, cell cultures, and serum/plasma (241). We utilized this technique to ascertain the specificities of the Sox-9, Lef1, and Axin2 antibodies in our work. Proteins were first separated according to their molecular weights, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred by electroblotting to a nitrocellulose membrane where protein epitopes were easily accessible to antibody binding. The membrane was then blocked with 5% fat-free milk at room temperature and incubated with the primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG). Sox-9, Lef-1 and Axin 2 were then detected using an enhanced chemoluminescence system to visualize the antibody binding. One advantage of Western blotting is that it is a sensitive technique to detect the size of proteins. However, it is a time- consuming method.

We selected at least two antibodies for the same target tissue and the one that gave a high signal to noise ratio and the least background staining was chosen and used. New antibodies like Sox-9 and Lef-1 were optimized and validated utilizing Western blotting (Figure 11). Axin 2, one of the downstream proteins on the Wnt signaling pathway, was excluded from the study because it proved to be difficult to find a good Axin2 antibody; the ones tested had too much background staining in IHC and several strong non-specific bands on Western blots. Aurora-A, Mad2, and BUBR1 antibodies were previously validated in our laboratory using Western blotting (238). Other antibodies like p53, Ki-67, Cyclin-D1, and p21 that were used in our studies are commonly used in routine diagnostic pathology and are optimized and validated from time to time by the routine laboratory.

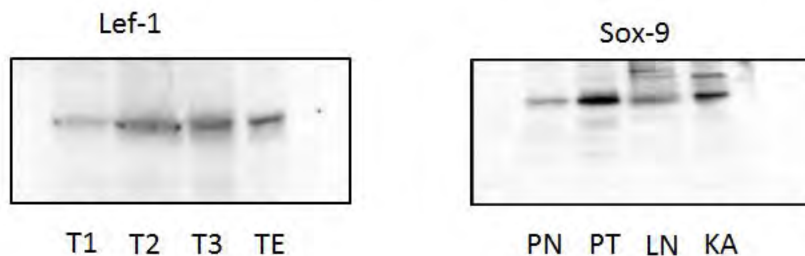

Figure 11. Western analysis with the antibody against LEF-1 (42-50 kDa) in the left with cell lysates from T1: tonsil 1, T2: tonsil 2, T3: tonsil 3, TE: testis and Sox-9 (59 kDa in the right with cell lysates from PN: normal pancreas, PT: pancreas tumor, LN: normal liver, KA: keratoacanthoma

4.2.4 Background

Background staining in IHC is due to several factors. One reason may be unspecific protein-protein interaction e.g. antibodies that bind to Fc receptors of human cells, frequently present on macrophages, granulocytes, B cells, and T cells. Other causes are binding of antibodies to endogenous molecules such as biotin or peroxide, or specific binding of an antibody to epitopes other than the target protein (cross-reactivity). Interactions of antibodies with endogenous molecules in our studies were minimized by blocking agents, e.g. the use of

hydrogen peroxide to block endogenous peroxidase activity, whereas binding of an antibody to epitopes other than target protein was hindered by using a validated antibody for IHC.

4.2.5 Detection system

Extensive use of IHC in routine diagnostic and research fields has facilitated the development of more optimal detection systems for the visualization of the antigen-antibody complex. The main detection system utilized in our work was the HRP chromogen detection system, either the Dako Envision Flex or the Ventana OptiView DAB system, a polymer-based detection system where multiple antibodies and peroxidase enzymes (HRP) are conjugated to a polymer dextran backbone which catalyzes the colorless substrate DAB (3,3' diaminobenzidine) to an insoluble brown color. This detection system gives less background and more intense staining of the tissue than earlier used ABC (Avidin-Biotin Complex) or LSAB (Labeled Streptavidin Biotin detection methods.

4.3 Quantification of immunoreactive cells

IHC-stained slides were examined using a light microscope. Quantification of immunopositive cells (scoring) was done by using 20 x magnifications. The scoring was done both quantitatively (Paper III) and semi-quantitatively (Paper I & Paper II). Two pathologists (OPC & SJ) discussed the protocol for evaluation and scoring, i.e. the cellular localization and characterization of positive and negative cells, as well as the intensity of the staining. To reduce any potential subjectivity, a research technician (AS) also scored the section in parallel with a pathologist (SJ) in Paper I and II. There was generally a strong agreement between the observers. Any disagreement about the staining data was discussed to reach a consensus. Manual scoring (quantification) of protein immunoreactivity in KA was a complex and laborious process, partly because of the abundant amount of inflammatory cells at the interface between tumor and stroma.

4.4 Cycling temperature capillary electrophoresis (CTCE)

Mutation analysis for *Tp53* (Paper I) was performed by cycling temperature capillary electrophoresis (CTCE). The tumor DNA was extracted from fresh frozen material using the

phenol/chloroform/ isoamyl alcohol (PCI) method which yielded high-quality high molecular weight DNA. The quality of the DNA was assessed using agarose gel electrophoresis. Cubit (Invitrogen) was used to measure DNA concentrations.

Briefly described, target DNA is amplified by PCR, which allows for the incorporation of a fluorescent label and a high melting domain adjacent to the target sequence. Post PCR denaturation and annealing create heteroduplexes (mutation-containing sequences) which lower the melting temperature compared to the homoduplexes (wild-type sequences). The formation of heteroduplexes improves the limit of detection of mutation. Amplified DNA migrates through a sieving matrix with an electric field under appropriate denaturing conditions. The result is allele separation due to different migration velocity through the sieving matrix. After separation of the amplified product in the capillary, the fluorescent label is detected by laser-induced fluorescence and intensity is recorded. This procedure detected the presence of gene mutations in the hot spot regions of *Tp53* exons 5- 8 in our work. The advantage of CTCE over Sanger sequencing is that it is highly sensitive with mutation detection as low as 0.1% compared with about 30% for Sanger sequencing, and it is moderately time- consuming. However, one disadvantage of this procedure is that it does not provide the exact nature of the *Tp53* mutations, i.e. the actual alteration of bases. It simply reports the presence or absence of mutations. However, individual sequences can be eluted and subjected to DNA sequencing to determine the exact nature of the mutations. This was not done by us.

4.5 Statistical analysis

In paper, I, *Tp53* mutation (yes/no), organ transplantation (yes/no), histological age of the KA (young/old) were used as nominal variables. All other variables like atypia, infiltration, inflammation, fibrosis, and clinical age of the lesions were used as ordinal variables. p53 protein expressions were also categorized into 3 groups according to the distribution in the data set as visualized by the frequency distribution histogram. We used cross-tabulation and Pearson χ^2 tests to analyze the associations between the categorical variables. For the statistical test, a two-sided p-value of <0.05 was considered to denote statistical significance.

In paper II, the histological age and the clinical age of the KAs were used as a dichotomous variable as young and old. KAs were stratified as young, when the clinical age of KA given by the patients was <5 weeks and old when the lesional age was > 3 months. The percentages of Wnt signaling protein expression; β-catenin, Lef-1, Sox-9, and Cyclin-D1 were also dichotomized according to the cut off value, which was determined using the frequency distribution histograms. Cross-tabulation and Fisher's exact test was performed to find the association between dichotomized variables. A value of 0.05 was used as the significance level; all statistical tests were performed as two-sided tests.

In paper III, the histological age of the KAs was used as a dichotomous variable (young versus old), whereas the differentiation grade of the squamous cell carcinomas was used as an ordinal variable. While the non-parametric Mann-Whitney U test was performed to compare continuous variables such as Aurora-A, Mad2, and BUBR1 between KAs and cSCCs, the Kruskal-Wallis test was used for more than two groups wd cSCC, md cSCC, and pd cSCC. For all the statistical tests, a p-value of <0.05 was considered to denote statistical significance.

Statistical considerations

In paper I, we had a large cohort (n=124) with good statistical power for all comparisons. We used the Pearson χ^2 test to test the hypothesis when the variables were nominal and categorical, which is an asymptotic test that is easy to compute, but requires a large enough sample size to ensure that the sampling distribution is approximating the assumed chi-squared distribution. In paper II we had a sufficient sample size (n=67) and the 2x2 Fisher exact test was used because its null distribution is known exactly instead of only approximately as for the χ^2 test. In Paper III, we had a limited sample size (25 cases in each category) and the statistical power may have decreased in the sub-analysis of the samples.

5 General discussion

All the papers in this thesis are based on the histopathological stratification of KAs into young proliferative and old regressive KAs according to the degrees of inflammation and extent of fibrosis. Fibrosis indeed is shown to be a better marker for the stratification of age than inflammation.

Tp53 mutation has been implicated in the development of UV-induced cutaneous neoplasms (104, 242, 243). In Paper I, we detected a significantly higher frequency of *Tp53* mutations (40%) compared to previous studies with small KA cohorts that have reported *Tp53* mutational frequencies of 0-13% (106-108). *Tp53* mutation in young KA may suggest that a mutated *Tp53* gene may play a role in tumor development in a subset of KAs. Even more interesting, substantially higher numbers of lesions have no *Tp53* mutations compared to lesions with mutations within the old KA subgroups, indicating that wild type p53 is important for regression of KA. As p53 is a pro-apoptotic protein, regression might have been brought about by the induction of apoptosis (244). Furthermore, the presence of *Tp53* mutations in some old KAs suggests that KAs with mutations are delayed on their way toward regression, possibly due to that the other allele of the *Tp53* gene is unaltered.

The tumor suppressor gene *Tp53* is critical for maintaining chromosomal stability and mitotic checkpoint integrity. p53 nuclear positivity was significantly higher in cSCC compared to KA in paper III. p53 positivity detected by IHC does not always correspond to *Tp53* mutation, but higher levels of p53 positivity reflect *Tp53* mutation, as demonstrated in **paper I** and other studies (111, 245). We did not analyze cSCCs for *Tp53* mutations, but a high frequency of *Tp53* mutations in SCCs (>70%) has been reported in the literature including cSCCs (109, 243, 246). We can infer from these studies that extremely high levels of p53 in our cSCCs are due to the accumulation of mutant p53 protein. The *Tp53* mutation frequency in cSCC is approximately double that of what we reported for KA in **paper I**. The lack of function of mutant p53 proteins has likely contributed to the overexpression of spindle proteins in cSCCs and compromised mitotic checkpoint integrity as a result.

Accumulation of p53 protein is generally associated with *Tp53* missense mutation (103, 247). This has been supported in our study with higher percentages of p53 nuclear positivity seen in

KAs with mutations. A comprehensive search in the literature reveals that both melanocytic skin cancer (MSC) and NMSC arising on sun-damaged skin have a common point mutation of *Tp53* in the hot spot regions, called the UV signature mutation (67). We analyzed exons 5-8 of the *Tp53* gene (hot spots) for mutations. We did not, however, sequence the cases that had *Tp53* mutations. We can only speculate that the majority of mutations were missense mutations since these are the most frequent type of *Tp53* mutations. We also observed p53 nuclear positivity in KAs without *Tp53* mutation possibly due to the stabilization of wild type p53 protein (100, 248). In a normal cell, IHC mostly fails to detect wild type p53 due to its rapid ubiquitination by MDM2 protein (100). However, wild type p53 may also be stabilized by cellular stress signals such as UV rays, HPV infection, oxidative stress, and interactions with other proteins, etc. (96, 248).

In paper I, individual phenotypic characteristics of KA--atypia, inflammation, infiltration, and fibrosis --were individually investigated for their associations with p53. We observed the association of higher grades of atypia with increased levels of p53 positivity in a subset of KAs without *Tp53* mutation. This result suggests that the phenotype atypia may not be directly related to *Tp53* mutation in our study in contrast to the mechanism in colorectal cancer (249, 250). Moreover, *Tp53* mutations have been reported in sun-exposed cells of normal skin without having any cellular phenotypic abnormalities (251), suggesting the involvement of other factors in the development of atypia than *Tp53* mutation in skin tumors. Some young proliferative KAs may show atypia at the periphery of squamous lobules that disappears in the regression phase, reflecting the type of atypia to be reactive and transient in nature.

Patients receiving immunosuppressive treatment after solid organ transplantation have a higher risk of developing cSCCs and KAs (17, 19). The majority of KAs in our studies were obtained from solid organ transplant recipients (paper I= 99/124 KAs, paper II= 51/67KAs, and paper III= 19/25KAs). We did not find significant differences in the frequency of *Tp53* mutation (**paper I**) or expression of proteins of the Wnt signaling pathway (**paper II**) or expression of spindle proteins (**paper III**) between KA in patients receiving immunosuppressive treatment after solid organ transplantation (TX+) and those without (TX-). Our results are in agreement with previous studies suggesting that the pathogenetic mechanisms of development may not be different between the KA in TX+ recipients and

those without TX- (7, 252). However, the lack of difference in protein expression between the TX+ and the TX- subgroups must be considered carefully, as low numbers of KAs in the TX- subgroup may give potentially unreliable statistical results.

In response to cellular stress signals and DNA damage, wt p53 protein induces the p21 protein, which leads to cell cycle arrest in order to repair DNA and to deal with stress (126). Interestingly we found that *Tp53* mutation was positively (rather than negatively) associated with higher p21 levels (>40%), which may indicate a p53-independent induction of p21, assuming that *Tp53* mutations led to loss-of-function p53 proteins as is usually the case (253). It has also been reported that p21 has both stimulatory and inhibitory functions in the cell cycle, depending on p21 expression levels, interaction with different proteins, and the localization of the protein (254).

It has long been hypothesized that KA is a hair follicle- derived tumor and that the Wnt signaling pathway may have a role in the growth of KA as it does in hair follicle development (142, 143). To investigate whether sustained proliferative Wnt signaling has a role in the proliferation of human KAs, we analyzed the expression of downstream proteins of the Wnt signaling pathway in **paper II**.

We did not find differential expression between young and old KAs in our material of any of the studied downstream proteins of the Wnt signaling pathway. Nuclear accumulation of the β-catenin protein is the hallmark of an active Wnt signaling (137, 255). We observed that 37% of KAs in our study showed nuclear accumulation of β-catenin, with or without membrane positivity. Furthermore, of the KAs that showed positive nuclear β-catenin, the majority (84%) had a low protein level (nuclear positivity of <3%). This suggests that the Wnt signaling pathway is not very active in the human KAs that we have studied. Varying patterns of β-catenin expression have been observed in KAs (145, 150, 151). Doglioni et al. showed < 10% positive nuclei for β-catenin in 4 KAs (145). Of 14 KAs studied, Fukumaru et al. showed both strong and weak positive membrane staining in some, and no membrane staining in others (151). They did not report of nuclear positivity of β-catenin. Papadavid et al. analyzed

12 KAs; none of these cases showed nuclear positivity of β-catenin (150). Our observation of strong membrane positivity of β-catenin in KAs is consistent with the results of Fukumaru et al. (151) and Doglioni et al. (145). Except for β-catenin mutation in pilomatricoma, mutation of the Wnt signaling proteins is not reported in skin tumors (255, 256).

Further confirmation of a non-active Wnt signaling pathway in our study was the low protein levels of Lef-1. Lef-1 is a transcriptional cofactor of β-catenin in the Wnt signaling pathway, (170) and high expression would have been expected if the Wnt signaling pathway had been active. These data are thus not consistent with an active Wnt signaling pathway. A non-active Wnt signaling pathway in KA may be supported by the low-moderate expression levels of Aurora-A and low levels of β-catenin. Phosphorylation of β-catenin by Aurora-A (overexpression) enhances the malignant transformation of a cell by stabilizing β-catenin and subsequent activation of the canonical Wnt signaling pathway (158, 160). We did not study the correlation between β-catenin and Aurora -A in our work, but inferred from the results that the Aurora-A expression levels might not be sufficient enough to stabilize the β-catenin in KAs.

The Wnt signaling pathway can be influenced by its down-stream proteins through a negative feedback loop as described in earlier studies (164, 257). We found high levels of the developmental transcription factor Sox-9 in our study, which was unexpected since low levels of β-catenin and Lef-1 should also result in decreased transcription of the *SOX9* gene (163). Sox-9 in KA is therefore probably regulated by a Wnt-independent pathway (161). It is unclear whether Sox-9 functions as an oncogene or tumor suppressor. Akiyama et al. (257) demonstrated an inhibitory effect of overexpression of Sox-9 on the Wnt signaling pathway, thereby inhibiting the chondrocyte proliferation. Prevostel et al. (258) reported anti-tumor (suppressor) activities of Sox-9 by binding physically to β-catenin in the intestine, thus inhibiting the activity of the oncogenic Wnt/ β-catenin signaling pathway. However, *SOX9* gene mutations do occur in colorectal cancer and are tumor-promoting (259). Additionally, overexpression of Sox-9 in a lung adenocarcinoma cell line led to a marked increase in cell proliferation, migration, and invasion (260). We speculate if high levels of Sox-9 might have exerted an inhibitory effect on the Wnt signaling pathway in KAs, thus inhibiting the proliferation. However, a pro-proliferative function of Sox-9 is reported in the skin (162).

Sox-9 acts as a downstream target of sonic hedgehog (Shh) signaling and is activated through transcriptional activator Gli 2 and has an indispensable role in the induction, development, and maintenance of hair follicles (161). Both in vitro and in vivo studies have shown that Sox-9 overexpression enhances keratinocyte proliferation (162). Upregulation of Sox-9 is reported in many adnexal skin tumors such as basal cell carcinoma, trichoepithelioma and tricholemmoma and other sebaceous tumors (168). We did not investigate the key transcription factors of the Shh pathway GLI-1 and GLI-2 or correlate its levels to Sox-9 in our work. Thus, the role of the Shh signaling pathway in KA, could be an area for further research. The exact nature of Sox-9 either as a tumor enhancer or tumor repressor in the context of KA is unclear and further complicated by the stratification issue of the age of KAs.

Cyclin-D1 levels were also high in KAs. Cyclin-D1 is a cell cycle regulatory protein and is a target gene of the Wnt signaling pathway. The transcriptional activation of this protein is related to cell proliferation (177). Cyclin-D1 levels might also be low, because of the inhibitory action of Sox-9 on the Wnt signaling pathway as described by Akiyama et al (257). High levels of Cyclin-D1 as seen in KAs, however, indicate the involvement of other signaling pathways rather than the Wnt signaling pathway in the induction of this protein (178).

On one hand, we observed a non-active Wnt signaling pathway (anti-proliferative), and on the other hand, high Cyclin-D1 levels which are pro-proliferative. The Wnt signaling pathway is complex and it interacts with many other pathways. One of the interacting partners is Shh signaling pathway. These pathways interact with each other, both positively and negatively, during hair follicle development. Wnt signaling initiates the hair – follicle placode formation, whereas Shh is active later during proliferation and maintenance of the hair follicle (261). There is also a report of inhibition of the Shh pathway on the Wnt signaling proteins through GLI-1 in colorectal cancer (262). We can speculate from these results that Wnt signaling is probably active during the initial phase of tumor formation in the KA, and that the Shh signaling pathway comes into play later during the development of KA. KA may possibly have attained a high level of Sox-9 during the development regulated through the Shh pathway.

Recent studies report an inverse relationship between wt p53 protein and β-catenin, the main regulator of the Wnt signaling pathway (155, 156). Although we have not assessed p53 levels in KAs in Paper II, one may infer from Paper I that high levels of wild type p53 in KAs, in general, keep the levels of β-catenin low or that Sox-9 helps to do that as well.

The proliferative nature of KA is further confirmed in paper III, where we found considerable levels of the spindle proteins Aurora-A, Mad2, and BUBR1 in KAs. However, we failed to see the correlation between Ki-67 and spindle proteins in the KA subgroup, despite the proliferative nature of KAs. This result strongly indicates that there are biological differences between KA and cSCC, and that the spindle proteins have different functions in KA.

Tumor regression is characterized by increased epithelial differentiation in KA (263, 264). In paper II, our hypothesis for KA regression was based on the concept of downregulation of the genes of the Wnt signaling pathway, and thus decreased expression of downstream Wnt proteins (144). We did not find low expression of Wnt signaling pathway proteins in the old KAs compared to the young ones in human KAs, as did Zito et al. in murine KAs. This may mean that the Wnt signaling pathway in human KAs does not reproduce the findings of the murine KA model. Firstly, it is not feasible to include very young KAs in the dermatology clinic, so our stratification of young and old KAs is most likely not as clear cut as it would be in experimentally-induced KAs in a laboratory setting. Secondly, the biology of KA development in humans in UVR-exposed skin may be different from that of chemically-induced KAs in mice (265). The murine KA model may not be relevant for studies of human KA.

In most human tumors with *Tp53* alterations, an inactivating point mutation of *Tp53* in one allele is followed by loss of heterozygosity (LOH) in the other (266). LOH may either be an early or a late event in tumorigenesis that together with gene mutation, leads to complete loss of function of the p53 protein. We did not analyze the KAs for LOH, nor did we perform DNA sequencing in our study. We do not know whether the other allele of the *Tp53* gene is functional or lost in KAs. Previous studies, however, have shown low LOH frequency in KA (87, 267). Moreover, the presence of *Tp53* mutations in some old regressing KAs, suggests

the presence of still intact, functional wild type p53 on the other allele, making p53 contributing to regression possible even with *Tp53* mutations.

A few papers have investigated the downstream apoptotic proteins of the p53 pathway in KA, e.g. Bcl-2, Caspase -3 and BAK proteins with inconsistent results (123, 124). Cell death markers like BAK expression and the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay used in earlier work in our group were not useful to differentiate between young and old KA (unpublished).

KAs, predominantly in their mature phases and old phases, are infiltrated with abundant inflammatory cells (57). We did not perform an immunophenotypical characterization of immune cells in KAs, but it could be speculated that there exists an association of immune-surveillance mechanisms with tumor regression (268). It is still unclear whether the immune mechanism is pro-proliferative or regressive in the context of KA (263). This would be worth investigating.

Spindle proteins, Aurora-A, Mad2, and BUBR1 are crucial for accurate segregation of chromosomes during cell division. Deregulation of these proteins leads to CIN, possibly aneuploidy and cancer development (213, 269, 270). It was thus interesting to investigate the expression of spindle proteins in cSCCs and to find out if the expression of these proteins differs from that seen in KAs. While there are few studies that have investigated genomic instability in relation to components of SAC in HNSCC and oral SCC (271, 272), there is only one study that reports the subcellular localization of Aurora-A, and SAC proteins Mad2 and BUBR1in cSCC (238). No studies of spindle protein expression have been reported for KA.

We observed significantly higher levels of Aurora-A, Mad2, and BUBR1 in cSCC compared to KAs, indicating a possible role for these proteins in malignant transformation. Significantly higher levels of Mad2 in wd-cSCCc than in early proliferative KAs further strengthen the evidence of differential expression in KA and cSCC, since they do not show significant differences in Ki-67 levels. The positive correlation between Ki-67 and spindle

proteins in the cSCC subgroup, but not in the KA subgroup suggests different biologies of the two entities or the complexity introduced by including young and old KAs together.

BUBR1 is positively correlated with wt p53 in a normal cell. Transcription of BUBR1 is controlled by wt p53, whereas BUBR1 activates wt p53 upon spindle damage. The positive correlation between BUBR1 and p53 in KA indicates the role of wt p53 for the correlation, as mutant p53 cannot transactivate BUBR1.

Higher expression of spindle proteins in cSCC may be the result of a higher level of chromosomal instability (CIN) in cSCCs than in KAs. This idea is supported by previously-published array CGH studies showing a higher frequency of recurrent chromosomal aberrations in cSCCs compared to KAs (7, 86). Generally, it is thought that gene amplification leads to increased protein levels, but this is not always the case (273). Gene expression can also be influenced by post-translational modifications (185). Deregulated degradation can also be an explanation for overexpression of a particular protein and overexpression of a protein can lead to deregulated degradation of its targets (274, 275).

KAs do not have precursors like cSCCs, but recent literature indicates that it may be regarded as precancerous lesions due to the observation that some KAs develop into cSCC (10, 11). The majority of *Tp53* mutations in cutaneous tumors of sun-exposed areas are UV-signature mutations. A recent paper revealed a high frequency of *Tp53* mutation in normal sun-exposed skin (251). There are also reports that seborrheic keratosis, a benign tumor, displays a driver mutation of *FGFR3* which is also seen in normal skin. These findings indicate that clonal expansion of driver genes does not necessarily correlate to malignant transformation (251), a finding that is relevant for KAs.

Significantly higher levels of Aurora-A, Mad2, BUBR1 and p53 in cSCCs compared to KAs support the idea of elevated chromosomal instability in cSCC leading to deregulation of crucial tumor suppressor functions and increased proliferation. Taken together, four of the molecular markers studied in this thesis, Aurora-A, Mad2, BUBR1, and p53, could possibly be used to differentiate KA from cSCC.

6 Conclusions and future perspectives

In **Paper I,** we reported a higher frequency of *Tp53* mutations in KA compared to previously-published studies, and an association of high levels of p53 protein with *Tp53* mutation that suggests a role for mutant p53 in tumor progression for a subset of KAs. The majority of old KAs lacked *Tp53* mutations suggesting the involvement of wild type p53 in tumor regression.

In **Paper II,** we did not find evidence for an active Wnt signaling pathway in the life cycle of KA. Sox-9 and Cyclin-D1 proteins seem to function in the proliferation of KA, possibly through other signaling pathways.

In **Paper III,** we reported significantly higher expressions of spindle proteins Aurora-A, Mad2, and BUBR1 in cSCCs than in KA, consistent with KA and cSCC being biologically distinct entities. These proteins can be used as potential markers of malignant progression and as diagnostic markers to differentiate between KA and cSCC.

The biology of KA is complex, but hopefully, this thesis has contributed to the ever-expanding field of keratoacanthoma biology by providing new phenotypic and genotypic knowledge.

Tumor phenotyping alone is not enough, and future studies should include genotyping of the tumors. We are collaborating with Bejing Genomic Institute (BGI), in Shenzhen, China, in order to achieve the latter. The extracted tumor DNA from fresh frozen material from about 57 KAs and 30 cSCCs and germline DNA from 15 patients has been sent to BGI for whole-genome sequencing.
Correlations of genetic aberrations, particularly on proliferation-related and regression-related pathways, to phenotypic and clinical data, will help to elucidate the biology of KAs and will also help in distinguishing KAs from cSCC. We have also extracted DNA from multiple KAs from the same patient. It will be interesting to study genetic and methylation profiles from these biopsies to see if the genetic aberrations in these lesions are similar. From the analyzed data, we also hope to find signaling pathways that may be involved in the progression of KA to cSCC (cSCC ex KA).

TGFBR1, which is localized to chromosomal region 9q22-q31 is found to be mutated in multiple KAs of Ferguson-Smith syndrome in Scottish ancestry, but *TGFBR1* gene mutation is rarely seen in cSCC. The involvement of the same gene, in a large cohort of KA, should be explored.

Till now a substantial number of KAs and cSCC have been successfully sequenced, and preliminary results confirm our previous findings of less *Tp53* mutations in old lesions compared to young ones.

The tumor microenvironment is considered to be important in the progression of cancer and metastasis. Stromal cells, tumor-associated macrophages, immune cells and vascular cells all interact in the tumor microenvironment. Particularly in the context of regression of the tumor, immunophenotyping of the inflammatory cells by flow cytometry and correlation of the results to IHC analysis and genetic studies may reveal new knowledge about the immunological mechanisms involved, if any, in the regression of KA. The phenotype fibrosis is the hallmark of old KAs. It will be interesting to study if there is any difference between the fibroblasts in benign KA which are tumor restraining vs cancer-associated fibroblasts which are tumor-promoting in cSCC in the context of the tumor microenvironment.

7 References

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Tp53/p53 status in keratoacanthomas

Sarita Joshi[1,2], Aasa R. Schjølberg[1], Per Olaf Ekstrøm[3], Paula M. De Angelis[1], Manuela Zucknick[4], Solveig Norheim Andersen[2] and Ole Petter F. Clausen[1]

[1]Department of Pathology, Oslo University Hospital, Rikshospitalet, Oslo, Norway,
[2]Institute of clinical medicine, Department of Pathology, Akershus University Hospital, Lørenskog, Norway,
[3]Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway, and
[4]Department of Biostatistics, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

Background: Keratoacanthoma (KA) is a common keratinocytic skin neoplasm that typically develops rapidly and undergoes complete spontaneous regression. As the pro-apoptotic p53 protein may be involved in the lifecycle of KA, we studied the p53 status throughout the main stages of KA that include proliferation, maturation and regression in a large series of lesions.
Methods: One-hundred and twenty-four KAs were characterized with respect to age of the lesions both clinically and histopathologically, in addition to phenotypic characteristics such as cellular atypia, infiltration, inflammation and fibrosis. *Tp53* mutations were detected by capillary electrophoresis, and p53 protein levels were assessed by immunohistochemistry.
Results: *Tp53* mutations were detected in 49 cases (39.5%) and were associated with high p53 protein levels ($p = 0.007$) and histopathologic age of the lesions ($p = 0.044$). Significant association was also seen between high p53 protein levels and atypia ($p = 0.036$), whereas the association with infiltration showed borderline significance ($p = 0.057$). High p53 protein levels were significantly associated with gene mutations in transplanted, but not in non-transplanted patients.
Conclusion: We show a high frequency of *Tp53* mutations in KAs that is associated with increased p53 levels. The results indicate a role for the p53 protein in KA development.

Keywords: keratoacanthoma, p53, *Tp53* mutation

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Sarita Joshi
Department of Pathology,
Oslo University Hospital,
Rikshospitalet, Postboks 4950
Nydalen, 0424
Oslo, Norway
Tel: +47 67969230
Fax: +47 23071511
e-mail: sarita.joshi@studmed.uio.no

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Keratoacanthoma (KA) is a common keratinocytic neoplasm that usually presents as a solitary dome-shaped nodule with a central crater. The lesion is usually localized to sun-exposed areas of the skin of elderly persons and develops rapidly within 4–6 weeks, when proliferation dominates. Following a shorter or longer stable stage, it spontaneously regresses after 3–6 months.[1,2] As some KAs may show malignant phenotypes like atypia and infiltration, particularly in the proliferative phase, some pathologists consider it to be an abortive or self-healing malignancy.[1] Patients receiving solid organ transplantation show an increased incidence of skin neoplasias including KAs.[3]

The *Tp53* gene, a tumor suppressor gene located on the short arm of chromosome 17, is the most commonly mutated gene in human neoplasms including skin neoplasias (reviews[4–6]). In normal cells, the wild-type p53 protein level is too low to be detected by immunohistochemistry because of rapid turnover. DNA damage may activate protein kinases such as ATM (ataxia-telangiectasia

mutated) to phosphorylate the p53 protein, thereby stabilizing the protein.[7] Wild-type p53 protein is a transcription factor for downstream genes that are involved in cell cycle regulation, apoptosis, senescence and DNA-repair.[7,8] Mutations of the *Tp53* gene lead to the inactivation of its tumor suppressor function, thereby promoting tumor growth.

Only a few previous studies with limited numbers of cases have analyzed the frequency of *Tp53* mutations in KAs[9–11] whereas many investigators have shown a substantial number of lesions with increased levels of p53 positivity assessed by immunohistochemistry.[9,12–16] We therefore assessed the *Tp53* mutational status in 124 KAs, as well as the p53 protein levels by immunohistochemistry to analyze for possible associations. Furthermore, correlation of these data with phenotypic characteristics of the lesions such as cellular atypia, infiltration, inflammation, fibrosis and age of lesions allowed us to assess the possible role of the p53 protein during development and regression of KAs.

We also assessed p21 protein levels by immunohistochemistry, because the *CDKN1A* gene that codes for p21 is a p53 target gene,[17] as well as levels of the proliferation marker protein that is recognized by the Ki67 antibody, and correlated these with different phenotypic variables.

Material and methods

Patient material

We analyzed 124 KAs that were resected and clinically diagnosed at the departments of Dermatology, Surgery, and Plastic Surgery at Rikshospitalet, Oslo, during 1995–2004. The majority of patients were organ transplant recipients (n = 99) who received immunosuppressive treatment; a significant number of patients were not organ transplant recipients or under immunosuppressive treatment (n = 25). The median age of the patients was 60 years. The numbers of female and male patients were 51 and 73, respectively.

Only complete excisions were included, and the fresh lesions were divided into two halves, one of which was fixed in formalin, embedded in paraffin and processed for routine histopathologic evaluation; the other half was immediately stored at −80°C for further processing.

Histopathologic evaluation and characterization of lesions

Formalin-fixed, paraffin-embedded tissue samples were cut as 5-μm thick sections and stained with hematoxylin–eosin for routine histopathologic diagnosis. According to the criteria by Elder et al. (1997), the lesions were characterized as KA by their symmetrical appearance and central keratin-filled crater with overhanging epithelial lips. From the base of the lesion, well-differentiated squamous strands with pale pink cells with ground glass appearance penetrated the dermis. Further, histopathologic evaluation was carried out with respect to the following parameters: the degree of fibrosis, inflammation, cellular atypia and infiltration. The age of the lesion as given by the patient at the time of excision was also recorded – if a realistic estimate was possible to obtain.

The degree of fibrosis, inflammation, cellular atypia and infiltration was scored as absent (0) or mild (+), moderate (++) or severe (+++); scoring was made by a single observer (OPFC), who is an experienced dermatopathologist. Infiltration was judged to be absent when the growth was expansive, moderate when several cell layers-thick, finger-like extensions were penetrating the dermis, and severe when small groups or single cells were invading the dermis. Atypia was graded according to variation in nuclear size, conformation and hyperchromasia (together defined as nuclear pleomorphism), and compared with the nuclear conformation of proliferative germinative non-neoplastic epidermal cells. Statistically four arbitrary groups were defined, one with no pleomorphism, one with mild pleomorphism (+), one with distinct pleomorphism (+++) and one group in between with moderate pleomorphism (++). Clinical age of the lesion was defined as young when reported less than 5 weeks old and old when more than 4 months (patient estimates). Histopathologic age of the lesion was defined as follows: the lesions were divided into two categories and classified as old when a moderate degree or more of fibrosis and inflammation, respectively, was seen, i.e. more than ++ in each category, whereas young lesions had either no, or mild fibrosis (0, +) and inflammation, respectively, i.e. + or less in each category, respectively.[18] About 100 lesions were tested for intraobserver reproducibility of the phenotypic parameter values with an interval of several years.

Immunohistochemistry

The 5-μm thick sections used for immunohistochemical staining were first deparaffinized in xylene, rehydrated in ethanol, rinsed in distilled H_2O and washed in phosphate buffered saline.

They were then placed in a Coplin jar with citrate buffer at pH 6 (for p53) or Tris-EDTA at pH 9 (for p21 and Ki 67) for antigen retrieval and boiled in a microwave oven (850 W). When the buffer boiled, the heat was reduced, and the jar was kept warm for 30 min. The sections were then cooled to room temperature and incubated with the primary antibodies of interest for 1 h at room temperature for all antibodies. The antibodies used were as follows: p53 anti mouse monoclonal ab (Novocastra Laboratories Ltd, Newcastle, Tyne, UK), dilution 1:50, p21 waf (ab-1) anti mouse ab (Calbiochem Merck KGaA, Darmstadt, Germany, 2014), dilution 1:20, and Ki 67 monoclonal mouse-anti Human, clone MIB 1 (Glostrup, Denmark), dilution 1:50. The antibodies were all diluted in Ventana antibody diluent.

Before staining with secondary antibodies, slides were washed with Ventana wash twice and Immune-press secondary antibody kit was used to stain the slides for 30 min. For the staining procedure, a Ventana Nexes machine (Ventana Medical Systems Inc. Tucson, AZ, USA) was used according to manufacturer's protocol. Then the slides were washed twice with the Ventana wash solution. The bound complexes were visualized with a DAB kit (Ventana). The sections were counterstained with Lillie's hematoxylin for 10 s.

For each batch of immunostained sections, one positive and one negative control were included. Positivity was scored among the germinative (basal cell-like) cells. The number of positive nuclei per 1000 germinative cells counted for each protein was registered and the resulting percentages were calculated. p53 protein expression was judged to be positive when >10% of the nuclei of the germinative cells were stained. The same cutoff value of 10% was used also for p21 analyses, whereas >20% was used for Ki 67 evaluations. The positive staining was evaluated using a light microscope.

TP53 mutation analysis

Mutation analysis for *Tp53* was performed by cycling temperature capillary electrophoresis according to Ekstrøm et al.[19–21] This procedure detected the presence of gene mutations in the hot spot regions of exons 5–8. The method was chosen because it could answer our question about the frequency of *Tp53* mutations in KAs in a reliable way with high detection sensitivity (0.1%, compared with about 30% for Sanger sequencing), and because it is only moderately time consuming.

Statistical methods

Statistical analyses were performed using the SPSS software package (version 21) for cross tabulations and Chi-square test. For all statistical tests, a p value of <0.05 was considered to denote statistical significance. Associations were analyzed between categorized p53, p21 and Ki 67 protein levels, respectively, and the following variables: *Tp53* mutational status, transplantation status, clinical and histopathologic age of the lesions, degrees of atypia, infiltration, fibrosis and inflammation. The criterion for age information given by 33 patients was not fulfilled. This group labeled 'unknown' was excluded from the statistical analyses. p53 expression was grouped as <20, 20–60 and >60%, p21 expression levels were grouped as <10, 10–40 and >40% and the Ki 67 levels were grouped as <20, 20–60, >60%. The same analyses that were carried out in the total material were also carried out separately for lesions with and without *Tp53* mutations, respectively, and in transplant recipient patients and patients without transplantation and immunosuppression, respectively.

Slide scanning for digital photos

Two slides representing different stages of KA development and three slides stained separately for p53, p21 and Ki67 were scanned using the 'Pannoramic MIDI Brightfield Scanner' (with a 20X objective) from 3DHistech Ltd. (Budapest, Hungary). Pannoramic Viewer software was used to evaluate and store digital images of the slides.

Results

Histopathology

Figures 1 and 2 depict representative KAs in the early phase and in the regression phase, respectively. Figure 1 shows a crateriform lesion with overhanging epithelial lips and well-differentiated strands of squamous cells that penetrate the dermis. The box represents the magnified area of the penetrating squamous strands. Figure 2 shows hyperplastic epidermis, severe fibrosis and inflammatory reaction in the dermis. The box represents the magnified area of fibrosis and inflammation. Intraobserver reproducibility ranged from r = 0.887 to r = 0.829.

Immunohistochemistry

One-hundred and three KAs were classified as positive for p53, and 18 lesions as negative using

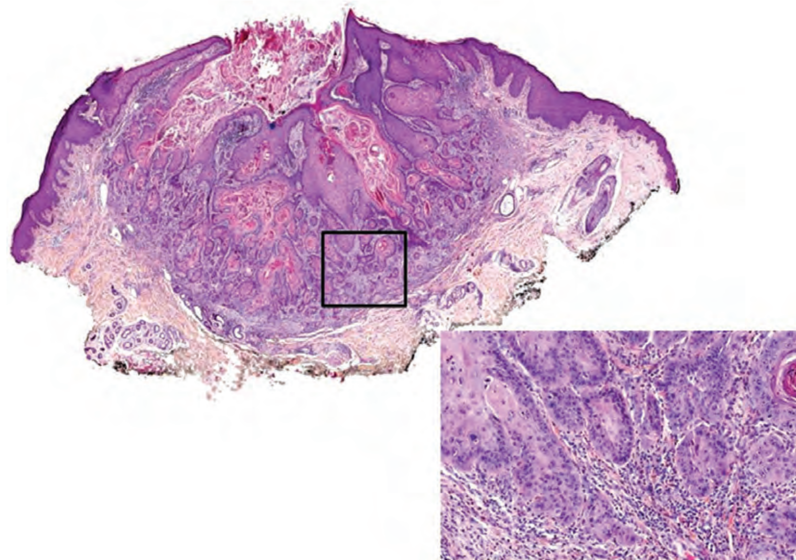

Fig. 1. Histopathological appearance of early phase of keratoacanthoma with hematoxylin/eosin (H/E) staining, scanning magnification ×20. The box represents magnified area (magnification ×40) showing penetrating squamous strands in the dermis.

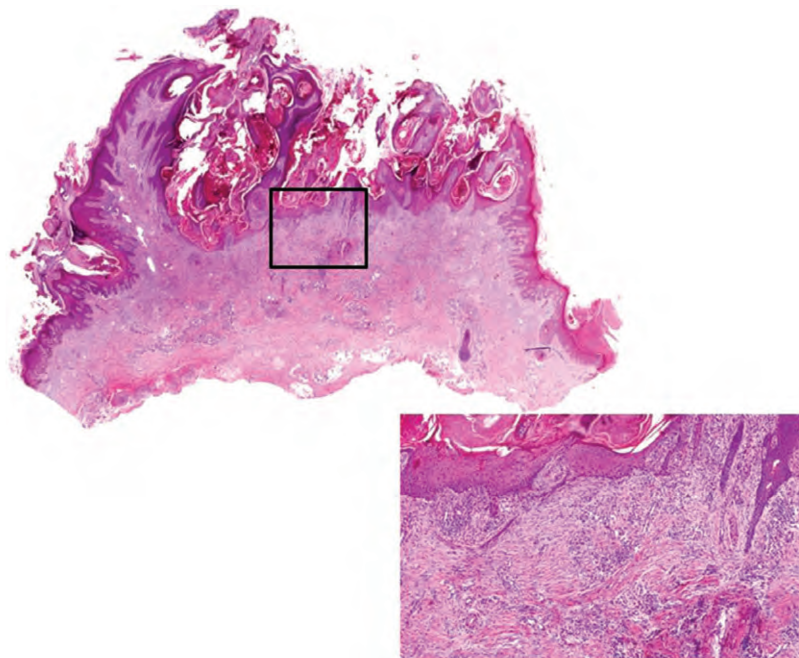

Fig. 2. Keratoacanthoma in regression phase with hematoxylin/eosin (H/E) staining, scanning magnification ×20. The box represents magnified area (magnification ×40) showing fibrosis and inflammation.

10% positivity as a cutoff (data were missing for 3 lesions). Of the 18 negative lesions, 4 were completely negative for p53 staining. The number of p53-positive lesions within each of the expression level groups is shown in Table 1. Figure 3 shows representative p53 staining mainly in the germinative layer.

Eighty-six lesions were judged to be positive for p21 and 24 negative using 10% positivity as a cutoff (data missing for 14 lesions). The number of p21-positive cases when grouped as follows: <10, 10–40 and >40%, were 24, 56 and 30, respectively. There were no completely negative cases. Figure 4 shows representative p21 staining mainly in the germinative layer.

One-hundred and six lesions were positive for Ki 67 and five negative using a 20% positivity cutoff (data missing for 13 lesions), and the number of cases within each expression level group is shown in Table 2. Figure 5 shows representative Ki67 staining.

Table 1. Frequency of p53 positivity in keratoacanthomas (n = 121) in relation to *Tp53* mutation and transplantation status

Variable	p53 < 20%*	p53 20–60%	p53 > 60%
All keratoacanthomas	29/121	50/121	42/121
Tp53 Mutation			
Yes	4/47	23/47	20/47
No	24/71	25/71	22/71
Missing†	1/3	2/3	0/3
Transplantation			
Yes	19/98	44/98	35/98
No	10/23	6/23	7/23

*This includes the 18 lesions classified as negative for p53.
†Cases excluded from the statistical analyses because of suboptimal quality.

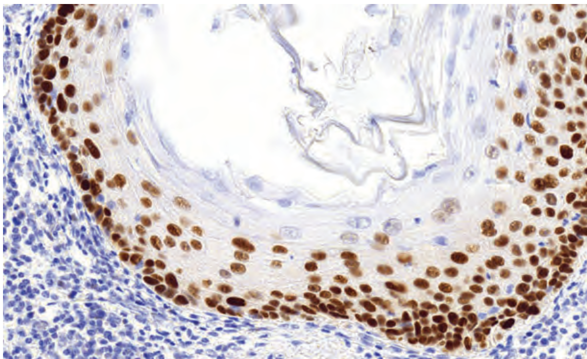

Fig. 3. p53 immunohistochemistry showing expression mainly in the germinative layer, scanning magnification ×40.

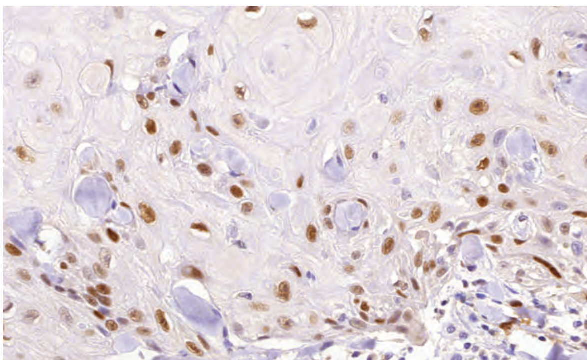

Fig. 4. p21 immunohistochemistry showing expression mainly in the germinative layer, scanning magnification ×40.

Mutation analysis

Tp53 mutations were detected in 49 out of 121 (39.5%) of KAs. Three cases were excluded because of suboptimal DNA quality (Table 1). The numbers of mutation-positive cases in transplanted and non-transplanted groups were 41 out of 97 (42.3%) and 8 out of 24 (33.3%), respectively.

Table 2. Frequency of Ki-67 positivity in keratoacanthomas (n = 111) in overall material and in relation to age of the lesions given by the patients (n = 83) and histopathological evaluated age of the lesions (n = 106)

Variable	Ki 67 < 20%	Ki 67 20–60%	Ki 67 > 60%	p value
Total material	5/111	72/111	34/111	
Age of the lesions given by the patients				0.013
Young	4/32	18/32	10/32	
Middle	1/37	30/37	6/37	
Old	0/14	6/14	8/14	
Missing	0/28	18/28	10/28	
Age of the lesions by histopathology				0.170
Young	3/42	30/42	9/42	
Old	2/64	38/64	24/64	
Missing	0/5	4/5	1/5	

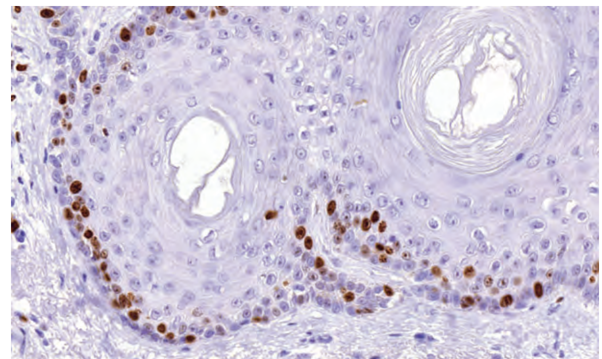

Fig. 5. Ki 67 immunohistochemistry showing expression in the germinative layer, scanning magnification ×40.

Association analyses

Significant associations between nuclear accumulation of p53 protein and *Tp53* mutational status (p = 0.007) and atypia (p = 0.036) were found, whereas borderline significance (p = 0.057) was found between p53 positivity and infiltration (Table 3). *Tp53* mutation, high-grade atypia and severe infiltration, respectively, were all associated with high levels of p53 positivity (Figs. S1–S3, Supporting information). Within the group of patients without lesional *Tp53* mutations, a similar trend of association with atypia (p = 0.029) was seen (Table 3).

Furthermore, in patients with solid organ transplantation (n = 99), p53 protein levels associated with gene mutation (p = 0.008 and Fig. S4) and infiltration (p = 0.023). In patients without immunosuppressive treatment (n = 25), the association was significant only with infiltration (p = 0.027) (Table 3).

Table 3. Association of p53 protein levels with *Tp53* mutation and phenotypic variables in keratoacanthoma

p53 levels	Tp53 mutation (p value)	TX (p value)	Age of the lesions given by the patients (p value)	Age of the lesions based on histopathology (p value)	Atypia (p value)	infiltration (p value)	Inflammation (p value)	Fibrosis (p value)
In all keratoacanthomas	0.007*	0.044*	0.769	0.115	0.036*	0.057	0.364	0.316
In keratoacanthomas with mutation	NA	0.115	0.920	0.826	0.776	0.332	0.864	0.908
In keratoacanthomas without mutation	NA	0.330	0.626	0.154	0.029*	0.313	0.187	0.390
In Keratoakanthomas with TX+	0.008*	NA	0.597	0.238	0.081	0.023*	0.145	0.652
In Keratoakanthomas with TX-	0.514	NA	0.435	0.384	0.273	0.027*	0.929	0.107

TX, solid organ transplantation with immunosupressive treatment; NA: not applicable.
*p: significant at <0.05.

Tp53 mutational status was significantly associated with histopathologic age of the lesions ($p=0.044$), whereas mutational status did not associate with the malignant phenotypes atypia and infiltration. Old KAs were significantly associated with fibrosis ($p=0.034$) (Fig. S5). High Ki67 protein expression showed significant association with age of the lesions (mature and old KAs) given by the patients ($p=0.013$). *Tp53* gene mutations were significantly associated with higher levels (>40%) of p21 protein expression ($p=<0.001$).

Discussion

Around 40% of the KAs in the present material showed *Tp53* mutations, which is a much higher percentage than previously reported. Perez et al. reported two mutations in 16 KAs (13%), Suk et al. three mutations in 49 lesions (6%),[11] whereas Kubo et al. did not detect mutations in any of five KAs.[10] Thus, we have shown that there are *Tp53* mutations in a substantial proportion of KAs, and that mutation is significantly associated with nuclear accumulation of p53 protein. Such an association could not previously be reported because of too few cases with mutation.[9,10] To the best of our knowledge, our study includes the largest number of analyzed KAs, and shows the highest frequency of mutations to date. The proportion of p53 immunopositivity in our material is consistent with what has been reported previously in the literature,[9,12,22] with the majority of tumor cells in the germinative layer having more than 20% positive nuclei.

High levels of p53 proteins are generally associated with missense mutations resulting in stabilization and increased half-lives of mutant proteins.[23,24] Thus, p53 nuclear staining in our study is partly because of mutations with subsequent accumulation of mutant proteins. This is clearly supported by the fact that p53 positivity is higher in KAs with mutations. *Tp53* gene mutations were detected by cycling temperature capillary electrophoresis[21] which is a simple, sensitive and robust method to detect the presence of mutation in the hot spot regions of exon 5–8 of the *Tp53* gene, which is located on chromosome arm 17p. As we did not sequence the cases positive for *Tp53* mutation, we do not know the types of mutations in our material, only in which exon they are located. We also found considerable p53 positivity in lesions without detectable *Tp53* mutations. As the wild-type p53 protein has a shorter half-life than mutated, it is normally not detectable by immunohistochemistry. Therefore, accumulation of p53 protein in lesions without mutation may partly be because of induction of wild-type p53 protein in response to cellular stresses, such as DNA damage because of UV radiation, viral infection or hypoxia. In addition, p53 stabilization may also be caused by binding to other proteins (for review, see articles [25–27]).

As the p53 protein is an inducer of apoptosis,[7,28] our hypothesis is that it may play an important role during the normal life cycle of KAs. Mutant p53 proteins may lead to tumor progression because they are unlikely to effectively transcribe downstream genes that are involved in cell cycle arrest and apoptosis. Cellular atypia and infiltration, typical malignant phenotypes prominent in the proliferation phase of KA, are in our material associated with high levels of p53 protein, but not with *Tp53* mutations. The association with atypia, however, is restricted to a subset of KAs without mutations, and may therefore involve wild-type p53, a mechanism which is different from that inducing DNA aneuploidy in colon cancer.[29–31]

The borderline association between high levels of p53 and infiltration may not be directly related

to mutation of *Tp53*, but cellular stress and DNA damage may trigger another mechanism for infiltrative growth in KAs. The observation that histopathologic older lesions are significantly associated with less *Tp53* mutations may indicate that mutated lesions are delayed on their way towards regression, which is consistent with a role for wild-type p53 protein during regression, possibly by inducing apoptosis. p53 function may still be present, even if only one allele is mutated or deleted, because the wild type function of p53 of the other allele may be able to transactivate pro-apoptotic target genes involved in regression. Interestingly, our research group reported that 27% of KAs had loss of the 17p13.3–17q25.3 region by array CGH (comparative genomic hybridization), where the *Tp53* gene is located,[32] consistent with a substantial number of KAs (73%) having retained a wild-type *Tp53* allele. However we cannot rule out other mechanisms of p53 inactivation for the remaining (non-mutated) *Tp53* allele in our material. The unexpected strong association between *TP53* mutation and p21 positivity strongly indicates that p21 induction occurs independently of p53.

Ki-67 is an antibody directed against a nuclear antigen that is an indirect marker of proliferation by virtue of its association with the growth fraction of tissues and tumors.[33,34] Ki-67 positivity is therefore expected to be higher in proliferating young KAs compared to mature and older lesions. We found however, high proliferation in the middle-aged and old KAs. This result is surprising, but may be explained by the fact that our stratification of old and young lesions given by the patients, which is highly subjective, does not differentiate very well between the lesions with different growth fractions and proliferative potential. Another explanation may be that some KAs have a heterogeneous growth pattern[35] showing different stages of development and proliferative areas within the same tumor.

Organ transplant recipients have an increased risk of developing systemic and cutaneous cancer than the general population.[36–38] However, the incidence of skin cancer after transplantation depends on the duration of immunosuppressive treatment and varies from 2% after 5 years to 21% after 10 years.[3] Our patient material contains significantly higher numbers of transplanted patients (n = 99 Tx+) compared to non-transplanted ones (n = 25 Tx−), and we found a higher number of lesions with *Tp53* mutations, 41 out of 97 (42.3%), in the group of transplant recipients compared with the non-transplanted group 8 out of 24 (33.3%), although the difference was not significant. Nevertheless, our reported frequencies of mutations in non-transplanted patients are much higher than previously reported. Differences in genetic aberrations in KAs from transplanted and non-transplanted patients, however, were not found in a previous study using array CGH.[32] We therefore assume that the pathogenetic mechanism of development and regression of KAs are the same in these groups. Furthermore, it was reported by Mc Gregor et al. in 1997[39] that the *Tp53* mutation spectrum and the frequency of *Tp53* mutations in cutaneous neoplasias of patients with chronic immunosuppression were remarkably similar to those seen in neoplasias from patients without immunosuppression.

The observation that the association between increased p53 positivity and atypia is the same in the group of KAs without mutation as in the total material, and lost in the group with mutations (Table 2), strongly indicates that wild-type p53 is responsible for the association. In addition the significantly higher frequencies of old KAs without *Tp53* mutation strongly support the involvement of the wild-type p53 protein in the life cycle of KAs.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Box and whiskers plot showing increased p53 levels in keratoacanthomas with Tp53 mutations.
Fig. S2. Box and whiskers plot showing that p53 levels increase with increasing levels of atypia.
Fig. S3. Box and whiskers plot showing that p53 levels increase with increasing degrees of infiltration.
Fig. S4. Box and whiskers plot showing that p53 levels are higher in the transplantation group compared to the non-transplanted group.
Fig. S5. The association between fibrosis and age of the lesions given by the patients. Fibrosis increases with increasing age of the lesions.

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Supplimentary Figures

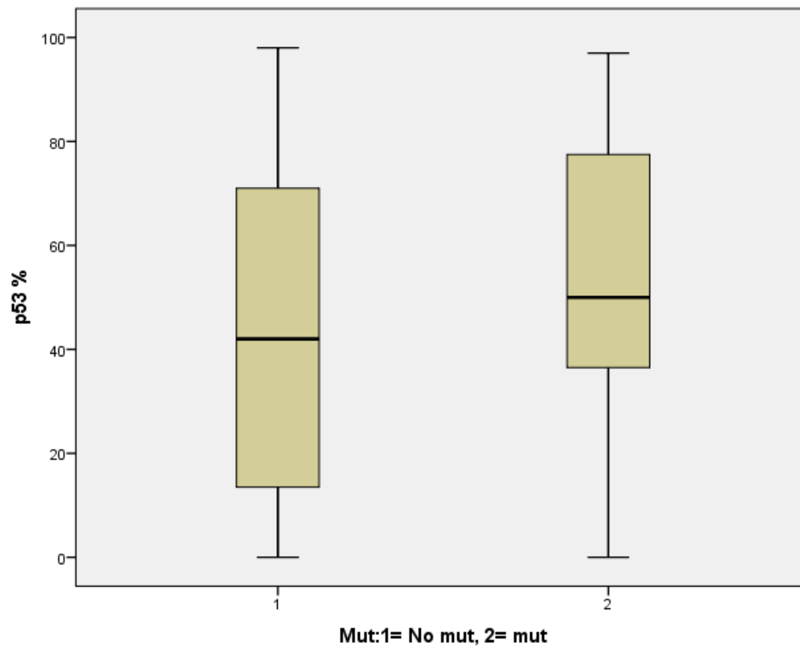

Figure S1: Box and whiskers plot showing increased p53 levels in keratoacanthomas with Tp53 mutations.

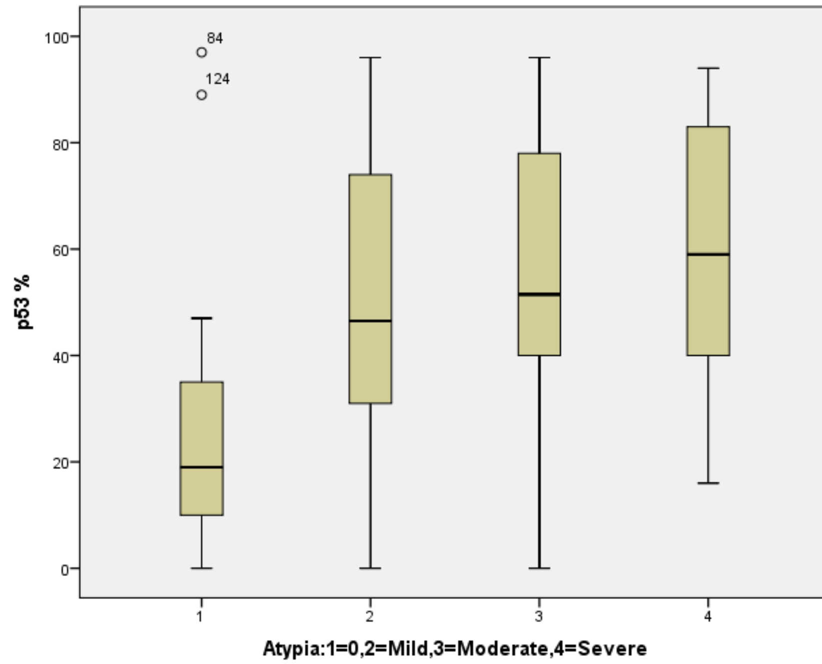

Figure S2: Box and whiskers plot showing that p53 levels increase with increasing levels of atypia.

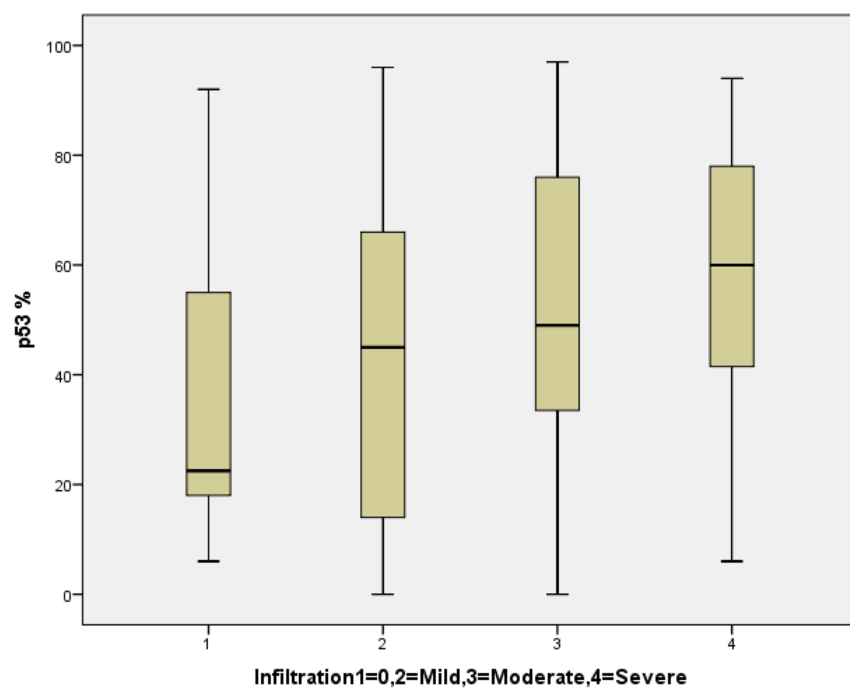

Figure S3: Box and whiskers plot showing that p53 levels increase with increasing degrees of infiltration.

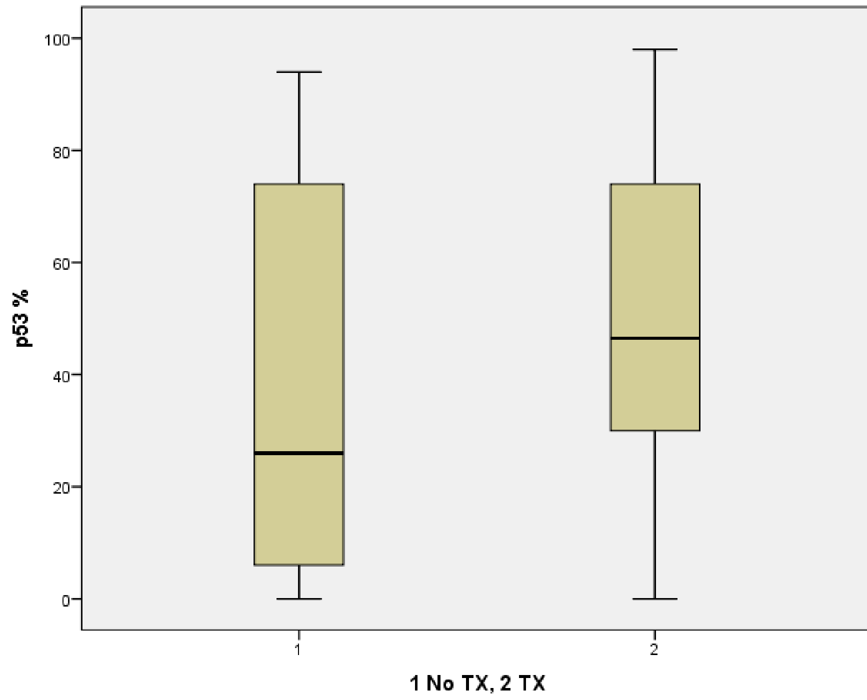

Figure S4: Box and whiskers plot showing that p53 levels are higher in the transplantation group compared to the non-transplanted group.

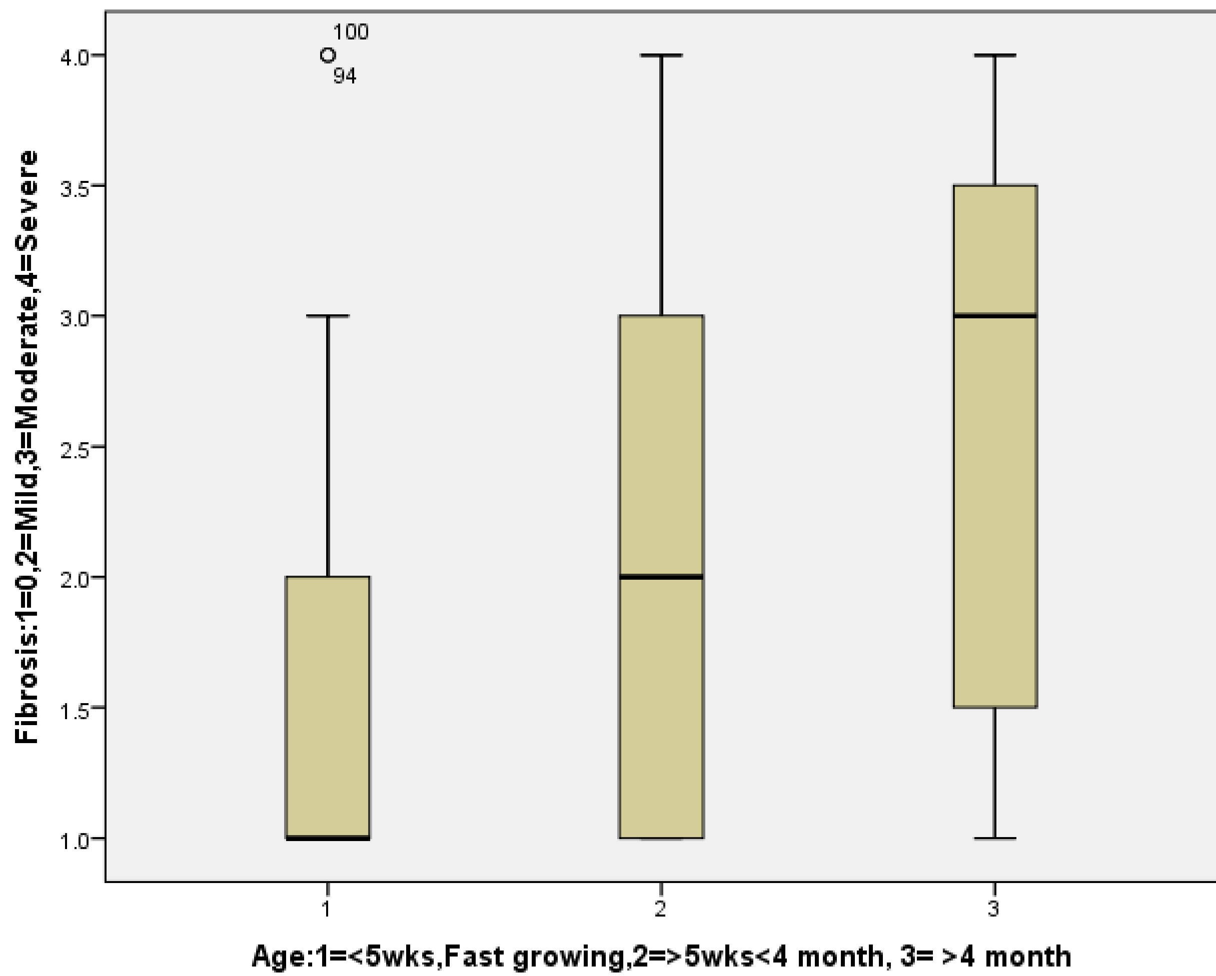

Figure S5: The association between fibrosis and age of the lesions given by the patients. Fibrosis increases with increasing age of the lesions.

II

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ORIGINAL ARTICLE

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Role of the Wnt signaling pathway in keratoacanthoma

Sarita Joshi[1,3] | Paula M. De Angelis[1] | Manuela Zucknick[2] | Aasa R. Schjølberg[1] | Solveig Norheim Andersen[3] | Ole Petter F. Clausen[1]

[1] Department of Pathology, Rikshospitalet, Oslo University Hospital, Oslo, Norway

[2] Oslo Centre for Biostatistics and Epidemiology, Department of Biostatistics, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

[3] Institute of Clinical Medicine, Department of Pathology, Akershus University Hospital, Lørenskog, Norway

Correspondence

Sarita Joshi, Department of Pathology, Oslo universitetssykehus Rikshospitalet, Postboks 4950 Nydalen, Oslo, Norway.
Email: sarita.joshi@studmed.uio.no

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Abstract

Background: Keratoacanthoma (KA) has a unique life cycle of rapid growth and spontaneous regression that shows similarities to the hair follicle cycle, which involves an active Wnt signaling during physiological regeneration. We analyzed the expression of the Wnt signaling proteins β-catenin, Lef1, Sox9, and Cyclin D1 in young and old human KAs to investigate a possible role for Wnt signaling in KAs.

Aim: To investigate the role of the Wnt/β-catenin signaling pathway in human KAs.

Methods and Results: Formalin-fixed, paraffin-embedded tissue samples of 67 KAs were analyzed for protein expression using immunohistochemistry. The majority of KAs were positive for Sox9 and Cyclin D1 but not for nuclear-localized β-catenin or Lef-1. No significant differences in protein expressions were seen between young and old KAs. However, we found a significant association between Ki67 and Cyclin D1 proteins (P= .008).

Conclusions: The Wnt signaling pathway does not appear to play a significant role in the biogenesis of human KA. Sox9 overexpression may be indicative of inhibition of Wnt signaling. Sox-9 and Cyclin D1 are proliferation markers that are most likely transactivated by alternate signaling pathways.

KEYWORDS

biomarkers, carcinogen, cell biology, heterogeneity, pathology, pathway analysis

1 | INTRODUCTION

Keratoacanthoma (KA) is presumed to originate from the hair follicle, located usually on the hair-bearing, Sun-exposed parts of elderly individuals.[1,2] Immunosuppressed patients have a higher risk of developing KA.[3,4] Some Solitary KAs undergo spontaneous regression leaving a faint scar, whereas others require excision, either conventional excision or Mohs micrographic surgery.[1] KA has a unique life cycle that shows rapid initial growth and spontaneous regression after a variable period of stable phase with similarities to the hair follicle cycle during physiological regeneration, namely, the anagen (growth) phase, catagen (regression) phase, and telogen (stable) phase.[1,5,6] The life cycle of KA, in addition to its follicular morphology, has led many to consider it as a hair follicle-derived tumor possibly regulated in the same way.

The Wnt/β-catenin signaling pathway has been postulated to be involved in hair follicle cycle regeneration.[7-11] Two plausible mechanisms have been implicated; one is the physiological "active Wnt signaling" during embryogenesis and stem cell maintenance ,[12-14] where Wnt ligands bind to frizzled receptors, thereby causing disintegration of the destruction complex composed of APC, Axin-2, and GSK-3β through disheveled. Subsequently, stabilization of β-catenin occurs in the cytoplasm[15,16] due to prevention of its ubiquitination and degradation. Thereafter, β-catenin binds to the LEF-1/TCF transcription factor in the nucleus to transcribe the Wnt target proteins Cyclin-D1, Sox-9, and others that play crucial roles in proliferation and differentiation of normal tissues.[17,18] Sox-9 also plays a role in repressing Wnt signaling.[19-21] The other mechanism is pathological "hyperactive Wnt signaling" where β–catenin is stabilized as a result of mutations in the *APC* gene, the *β -catenin* gene, or other Wnt signaling

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genes.[22-27] Thus, a role for the Wnt signaling pathway is well documented in the formation of hair-follicle-derived tumors.[24,28,29]

In a previous study by Zito G et al of carcinogen-induced KA in mice, it was observed that Wnt signaling proteins were differentially expressed in the growth and regression phases of KAs.[30] As there are few studies to date that have investigated the role of the Wnt/β-catenin signaling pathway in human KAs, we analyzed the expression of relevant Wnt signaling pathway proteins: β-catenin, Lef1, Sox9, and CyclinD1, in a series of human KAs that were stratified as young proliferating and old regressing on the basis of histological lesional age and lesional age given by the patient.

2 | MATERIALS AND METHODS

2.1 | Patient material

Formalin-fixed, paraffin-embedded (FFPE) tissue samples of 67 completely excised KAs were utilized for this study. All samples were diagnosed at the Department of Pathology during the period 1998 to 2010. Forty patients were males, and 27 were females. Fifty-one patients had received solid organ transplantation, and 16 had not. The study was approved by the Regional Ethics Committee of South-east Norway (REC# 2015/1213). Informed consent was obtained from all patients included in this study.

2.2 | Histopathologic evaluation and characterization of lesions

Sections were cut at 3- to 5-μm thickness from FFPE tissue blocks and stained with hematoxylin-eosin for routine histopathologic diagnosis. Criteria used for the diagnosis of KA and actual differential diagnoses were according to Elder et al. (1997). The KA lesions were exo-endophytic, symmetrical, crateriform containing a central keratin plug with overhanging epithelial lips or shoulders. From the center of the lesions, epithelial strands composed of pale pink cells with ground glass appearance invaded the dermis. According to the criteria, all such lesions were included, irrespective of the degree of cellular atypia or infiltrating growth. KAs with possible development of SCC were excluded.

The lesions classified as KAs were further evaluated with respect to the following parameters: the degrees of fibrosis, inflammation, cellular atypia, and infiltration. Each of these parameters was scored into three categories as absent or mild (+), moderate (++), or severe (+++) by two pathologists (O. P. F. C. and S. J.). Infiltration was judged to be absent, moderate, or severe when the growth was either expansive or finger-like, or with several cell layer-thick epithelial extensions, or small groups or single cells invading the dermis, respectively. Atypia was graded according to nuclear pleomorphism (variation in size, shape, and staining intensity of cell nuclei): absent or mild (+), moderate (++), and severe (+++). When fibrosis and inflammation were equal to or more than ++ in each

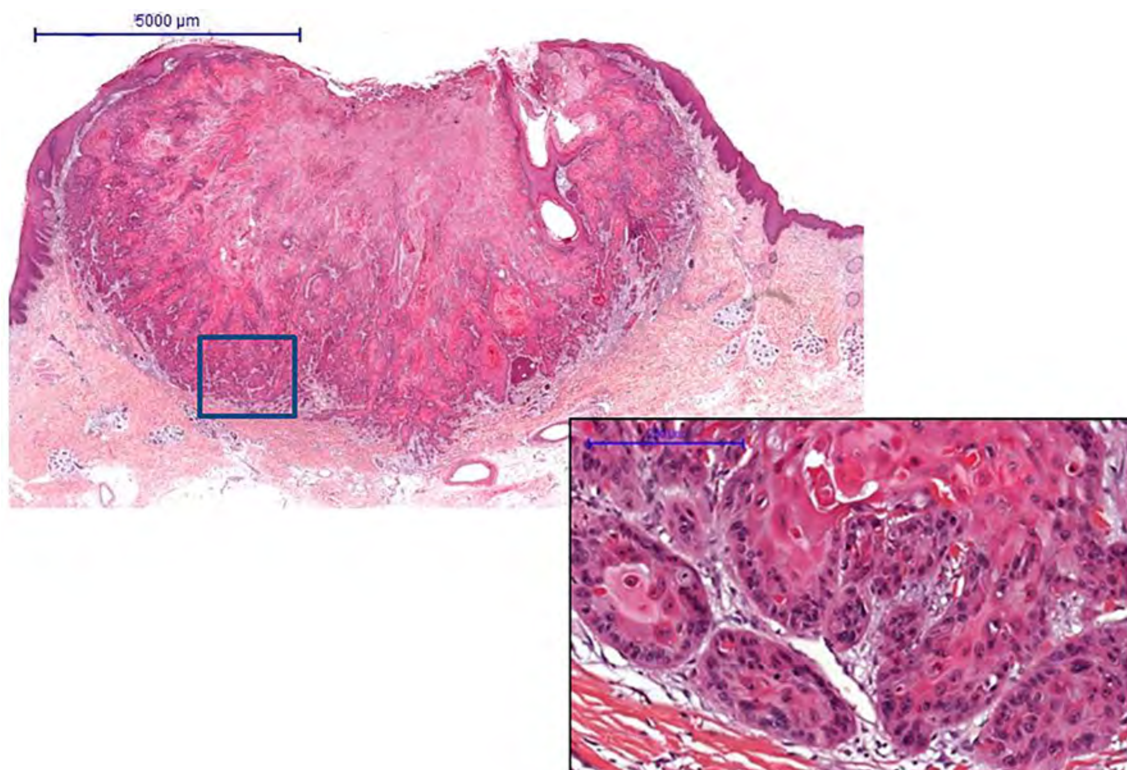

FIGURE 1 Proliferation phase of keratoacanthoma with hematoxylin/eosin staining showing symmetrical, crateriform lesion containing a central keratin plug with overhanging epithelial shoulders. The box represents the magnified field showing penetrating squamous lobules with pale pink cells in the dermis (scale bar, 5000 μm upper left, 200 μm bottom right)

category, the lesion was considered old, whereas young lesions were scored as + (Figures 1 and 2). Clinical age of the lesions given by the patients was classified as young (<5 weeks) and old (>3 months).

2.3 | Immunohistochemistry

Immunohistochemistry was done using standardized automated protocols recommended by the different autostainer manufacturers (Table S1). Three- to five-micron-thick paraffin sections were heated at 56–60°C for 15 to 30 minutes. Deparaffinized/rehydrated sections were subjected to antigen retrieval in the pretreatment module PT link Dako (3 in 1) TRS buffer, with high pH for Ki-67, Lef-1, and Cyclin-D1 and low pH for Sox-9. The visualization kit used for antibodies is given in Table S1. Lef-1 and Sox-9 antibody specificities were validated using Western blotting (data not shown). Sections were incubated with the primary antibodies for 60 minutes for Sox-9 and Lef-1 and 30 minutes for Ki-67 and Cyclin-D1 (Table 1 shows the antibodies used). The slides were incubated with the secondary antibody for 30 minutes for Sox-9 and Lef-1 and 20 minutes for Ki-67 and Cyclin-D1. DAB Chromogen was used for visualization of antigen-antibody bound complexes. The sections were counterstained with Hagen's hematoxylin, dehydrated and coverslipped.

2.4 | Scoring of immunohistochemical protein expression

The number of positive nuclei per1000 tumor cells was counted in the germinative layer for each protein, and the resulting percentages were calculated. β-catenin membrane staining was registered (qualitatively) in tumor cells and also separately in the tumor front. Nuclear positivity of β-catenin in tumor cells was counted as 1 (no staining), 2 (≤3%), and 3 (>3%).

2.5 | Statistical analyses

Statistical analyses were performed using the IBM SPSS software package, version 24 (Armonk, NY, USA). The expression levels of the proteins were dichotomized based on the distribution profiles and grouped as ≤4% and >4% for Lef1; ≤30%, >30% for Sox-9; ≤ 60%, >60 % for Ki-67; and ≤ 45% and >45% for Cyclin-D1. Since the early proliferative phase of KA is characterized by higher Ki-67 values than the later phases, lesions were also dichotomized by the Ki-67 index. Putative differences between transplanted and nontransplanted lesions were also analyzed.

Associations of expressions of β-catenin, Sox-9, Lef-1, Cyclin-D1, and age of the KA (young vs. old), Ki67 expression (high vs. low), and organ transplantation (yes vs. no) were analyzed with cross-tabulations

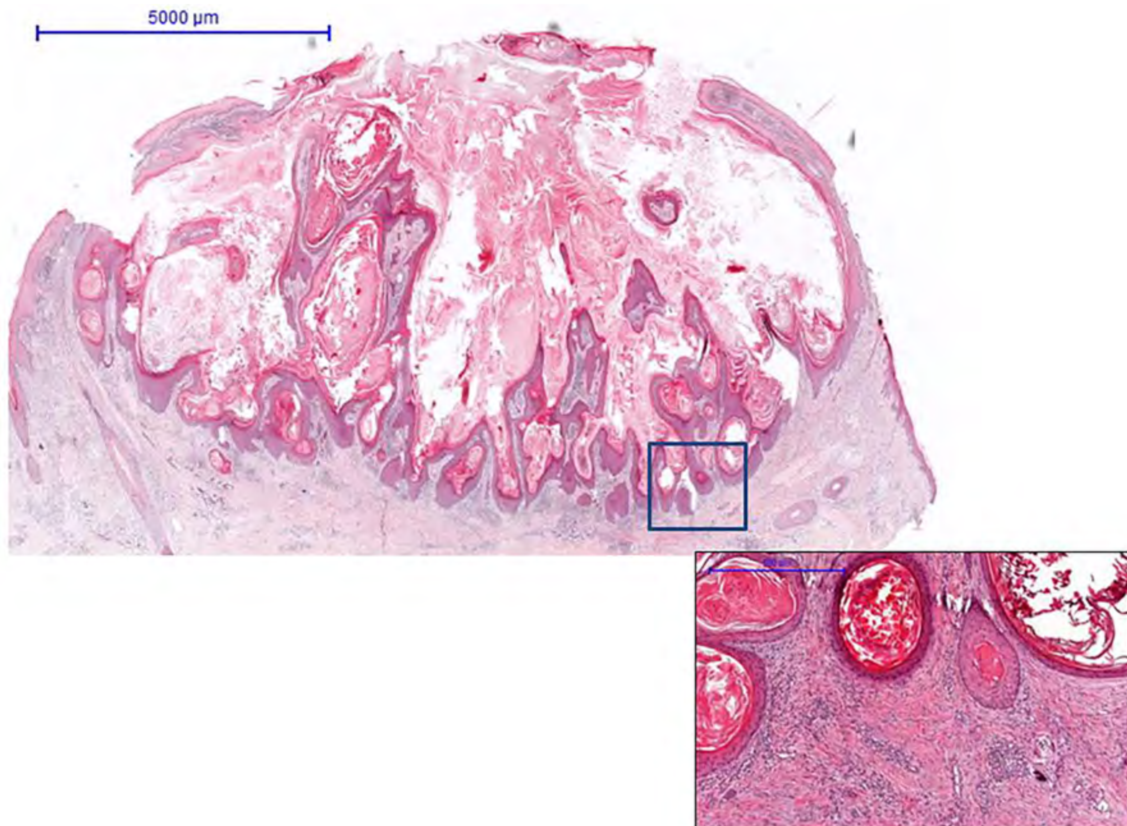

FIGURE 2 Regression phase of keratoacanthoma with hematoxylin/eosin staining. The box represents the magnified field showing fibrosis and inflammation in the dermis (scale bar, 5000 μm upper left, 500 μm bottom right)

TABLE 1 List of antibodies used in this work

Antibody	Clone	Species	Dilution	Concentration of stock solution	Concentration of working solution	pH of HIER	Company
β-catenin	14	Mouse monoclonal	RTU	1.68 μg/mL	RTU	CC1 (circa 9)	Roche (Lot: 7604242)
Lef-1	B6	Mouse monoclonal	1:50	200 μg/mL	4 μg/mL	9	Santa Cruz Biotechnology (Lot: A1617)
Sox-9	Polyclonal	Rabbit Polyclonal	1:100	0.1 mg/mL	1 μg/mL	6	Sigma Prestige antibody (Lot: F115596)
Cyclin-D1	EP12	Rabbit monoclonal	1:200	28.7 μg/mL	0.144 μg/mL	9	Cell Marque (lot: 1418115A)
Ki-67	MIB1	Mouse monoclonal	1:200	46 mg/L	230 μg/mL	9	Dako (Lot: 20040401)

Abbreviations: HIER: Heat-induced epitope retrieval; RTU: ready to use.

(Fisher's exact test). All statistical tests were two-sided, and a *P*-value of <.05 was considered to denote statistical significance.

3 | RESULTS

3.1 | Immunohistochemical expression of proteins

3.1.1 | β-catenin

Figure S1 shows the expression patterns of β-catenin in normal skin.

Membrane staining was seen in all cells of KAs (Figure 3). We scored nuclear staining in the germinative layers as positive (Figure 4), but most KAs did not show nuclear β-catenin staining. About 25/67 (37%) KAs showed nuclear β catenin expression, 22/25 had a low number of positive nuclei (≤3%) with a mean value of 1.84%, and 3/25 had >3% positive nuclei (Table 2). β-catenin was significantly associated only with Lef-1 (*P*=.022) and not with Cyclin-D1, Sox-9, or Ki-67. We did observe that β-catenin expression in the cell membrane of KAs was partially lost at the invading tumor front (Figure 5). Table S2 shows the association of β-catenin nuclear positivity with young and old KA.

3.1.2 | Sox-9

Figure S2 shows expression patterns of Sox-9 in normal skin.

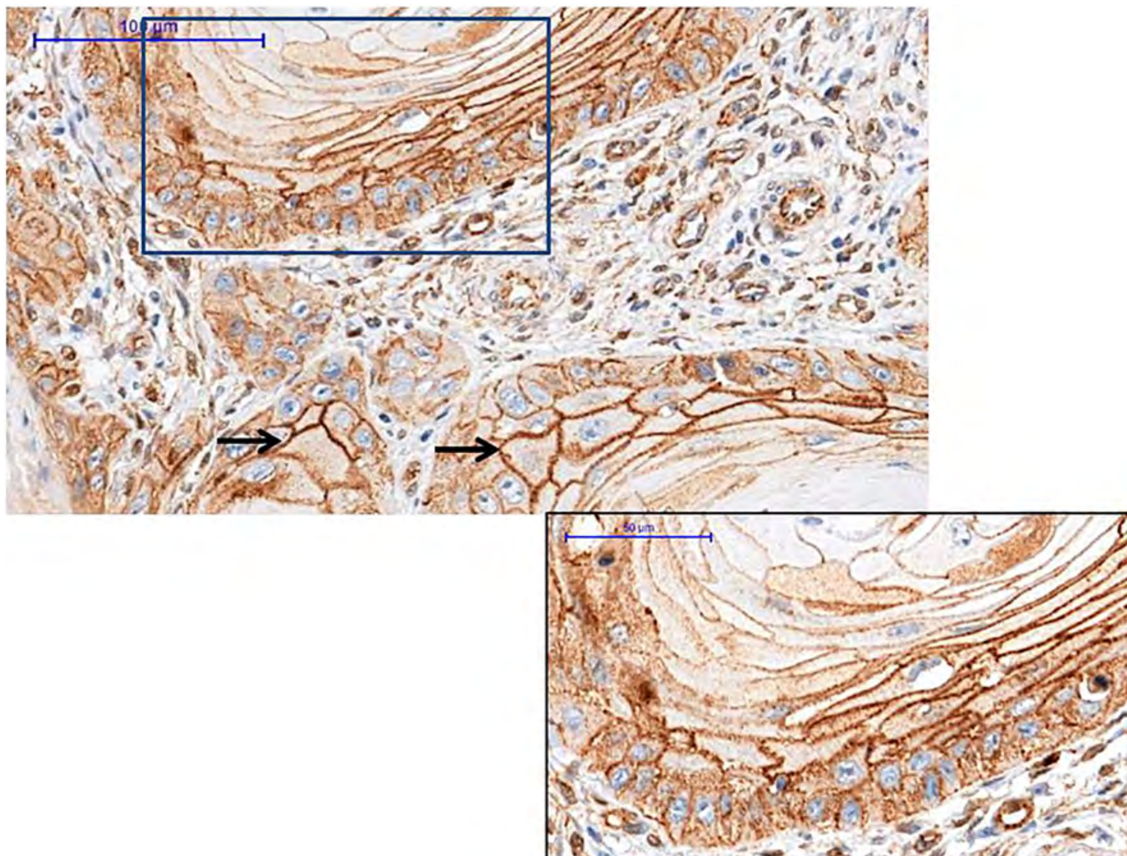

FIGURE 3 Immunohistochemistry for β-catenin shows membrane expression (arrowhead) in Keratoacanthoma (Scale bar 100 μm upper left, 50 μm bottom right)

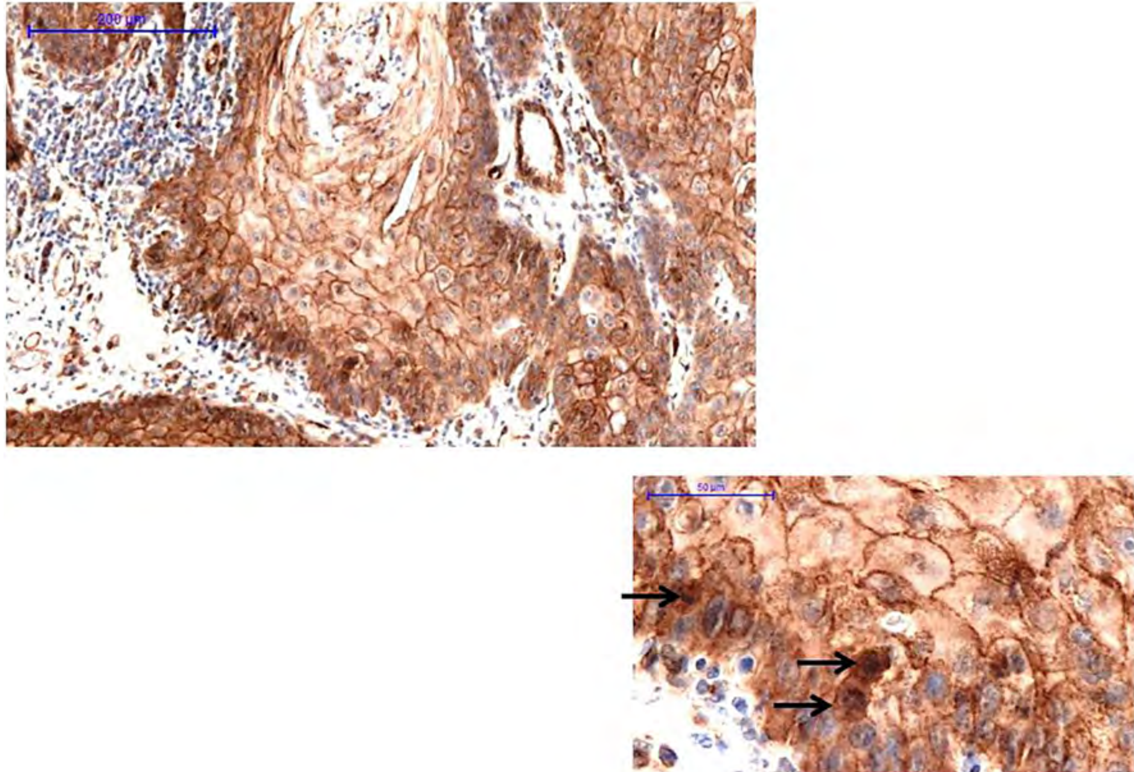

FIGURE 4 Immunohistochemistry for β-catenin shows nuclear expression (arrowhead) in Keratoacanthoma (Scale bar 200 μm upper left, 50 μm bottom right)

TABLE 2 Nuclear expression of Wnt signaling and Ki-67 proteins in KA

Protein	Nuclear positivity in total KA	Range, %	Cut off, % (Median value)	Nuclear positivity
Sox-9	65/67	5-92	≤60	54
			>60	11
Cyclin-D1	63/67	2-96	≤ 45	32
			>45	31
Lef-1	53/67	1-8	≤4	41
			>4	12
Ki-67	63/67	1-86	≤30	46
			>30	17
β-catenin	25/67	1-5	≤3	22
			>3	3

Sox9 nuclear staining was scored as positive in the germinative cells in KAs (Figure 6). The range and distribution of nuclear positivity of Sox-9 are shown in Table 2, and the distribution of dichotomized Sox-9 percentages for young and old KA is shown in Table 3.

3.1.3 | Lef-1

Figure S3 shows expression patterns of Lef-1 in normal skin.

In KAs, we scored nuclear staining in the germinative layers as positive (Figure 7). The range and distribution of nuclear positivity of Lef-1 are shown in Table 2, and the distribution of dichotomized Lef-1 percentages for young and old KA is shown in Table 3.

3.1.4 | Cyclin D1 and Ki-67

Keratinocytes in the germinative layers of KAs showed strong nuclear Cyclin D1 and Ki-67 positivity (Figures 8 and 9). The range and distribution of nuclear positivity of Cyclin-D1 and Ki-67 proteins are shown in Table 2. The distributions of Cyclin-D1 and Ki-67 percentages for young and old KAs were grouped according to the median cut-off and are shown in Table 3.

3.2 | Association of protein expressions with clinicopathological parameters

Cross-tabulation (Fisher's exact test) did not show any significant differences in expressions of the proteins β-catenin (P>.05; Table S2), Lef-1, Sox-9, and Cyclin- D1 in young versus old KAs (P>.05; Table 3) whether lesional age was defined histologically or as age given by the patients. KAs stratified by Ki-67 levels did not show any statistically significant associations with the expression of the same proteins. There were no statistically significant differences in protein expressions between transplanted (TX+) and nontransplanted patients. However, we observed a positive association between increasing levels of Cyclin-D1 and Ki-67 proteins (P=.008). We also observed that KAs with no or a low number of nuclear positivities for β-catenin showed decreased levels of Lef-1 protein (P=.022).

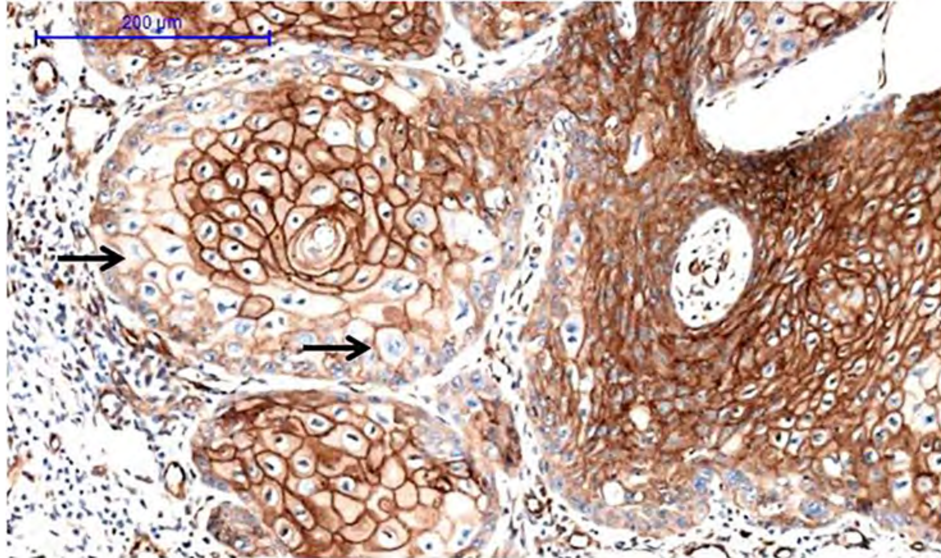

FIGURE 5 Reduced membrane staining (arrowhead) of β-catenin at the tumor front in Keratoacanthoma (scale bar 200 μm)

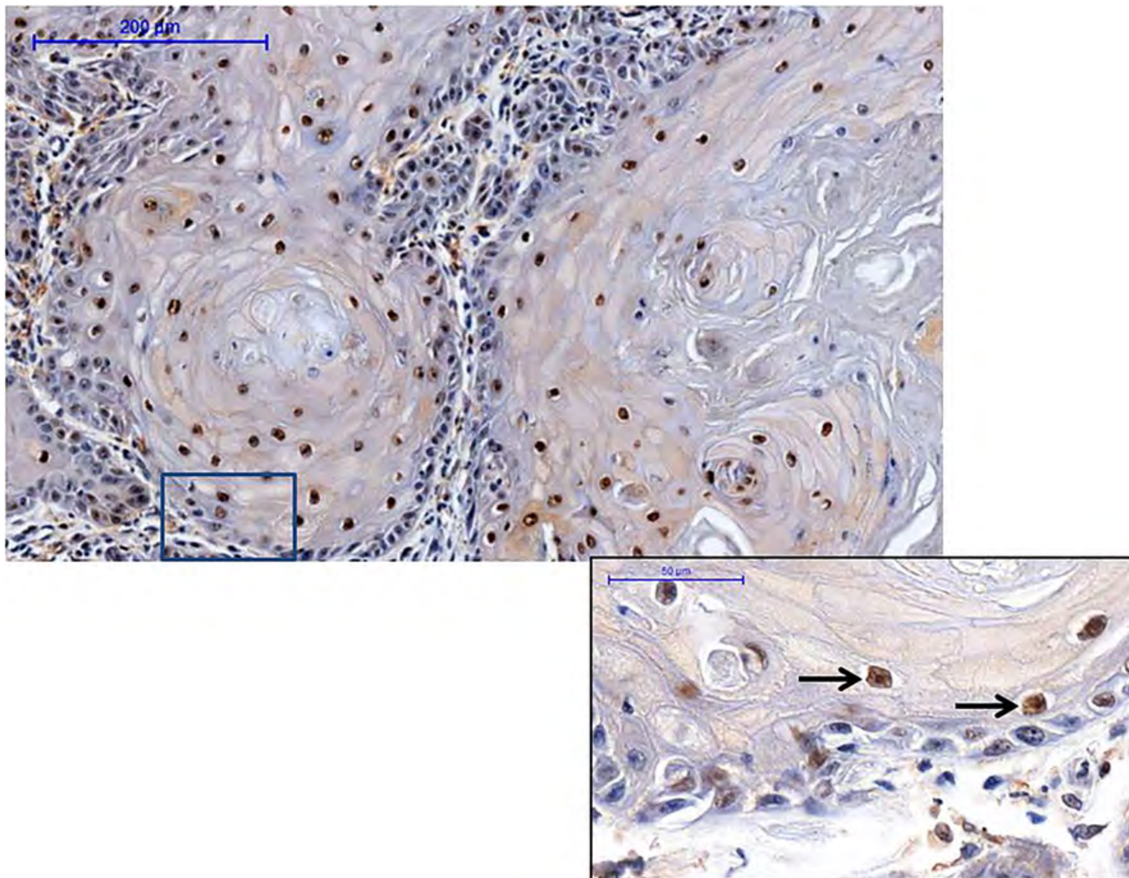

FIGURE 6 Immunohistochemistry for Sox-9 shows expression (arrowhead) in the germinative layer in keratoacanthoma (scale bar 200 μm upper left, 50 μm bottom right)

4 | DISCUSSION

The majority of human KAs in our study were positive for proliferative markers Sox9 and Cyclin D1, but not for nuclear-localized β-catenin or Lef-1. The correlation between Cyclin-D1 and Ki67 proteins was not unexpected since both are biomarkers of cell proliferation. There was no differential expression of any of these proteins between young and old KAs, whether lesional age was classified histopathologically or

TABLE 3 Associations between Wnt signaling proteins, Ki-67, and histological age of KA

Proteins	Cutoff	Histological age of KA Young (n=38)	Old (n=29)	*P*-value
Cyclin –D1	≤45%	15	17	.128
	>45%	21	10	
	Missing	2	2	
Sox-9	≤60%	28	26	.097
	>60%	9	2	
	Missing	1	1	
Lef-1	≤4%	31	23	1.000
	>4%	7	5	
	Missing	0	1	
Ki-67	≤30%	26	23	.262
	>30%	12	5	
	Missing	0	1	

Abbreviation: KA: keratoacanthoma.

estimated by the patients themselves. Additionally, there was no differential expression of these proteins between KAs dichotomized by high and low Ki-67 levels. Our results are thus not consistent with the results of a previous study of carcinogen-induced KAs in mice that showed that the Wnt pathway was activated in the proliferative phase and repressed in the regression phase of KAs.[30] There may be several explanations for this discrepancy.

The stratification of young proliferating and old regressing KAs in humans is not clear-cut, whether one considers morphologically determined lesional age, or the age of the lesion as provided by the patient. Since patients tend to contact the health care system quite a long time after the development of skin lesions, the inclusion of extremely young cases of KAs is not feasible. The longer the interval between lesional development and patient contact with the health care system, the more unreliable the age estimates. KAs may also show intralesional heterogeneity—distinct areas of growth and regression in the same KA—which has been observed by us and others.[31] Furthermore, cell death markers were not useful to differentiate between young and old KAs. We have previously used the TUNEL assay (TdT) to assess apoptotic indices in KAs, but these were very low in both growing and regressing KAs (Figures S4 and S5). Additionally, the pro-apoptotic markers BAK showed no significant differences between growing and regressing KAs (Figure S6).

Nuclear accumulation of the β-catenin protein, which suggests an active Wnt signaling[16,29] was seen in 37% of our KAs, and the majority of these had a low level of expression. Varying patterns of β-catenin expression in KAs and other skin tumors have been observed.[26-28,32-35] Doglioni et al studied four cases of KA where all had <10% positive nuclei for β-catenin, and Fukumaru et al showed strong positive membrane staining in 4 of 14 KAs studied, weak membrane staining in 7 cases, and no membrane staining in 3 cases. They did not report nuclear positivity of β-catenin. Papadavid et al analyzed 12 KAs, where 6 showed normal membrane staining and 6 showed either loss of membrane staining or coexisting membrane and cytoplasmic staining; none of these cases showed nuclear positivity of β-catenin. Strong membrane positivity of β-catenin in KAs in our study is consistent with results of Fukumaru et al[32] and Doglioni et al.[27] However, only nuclear β-catenin

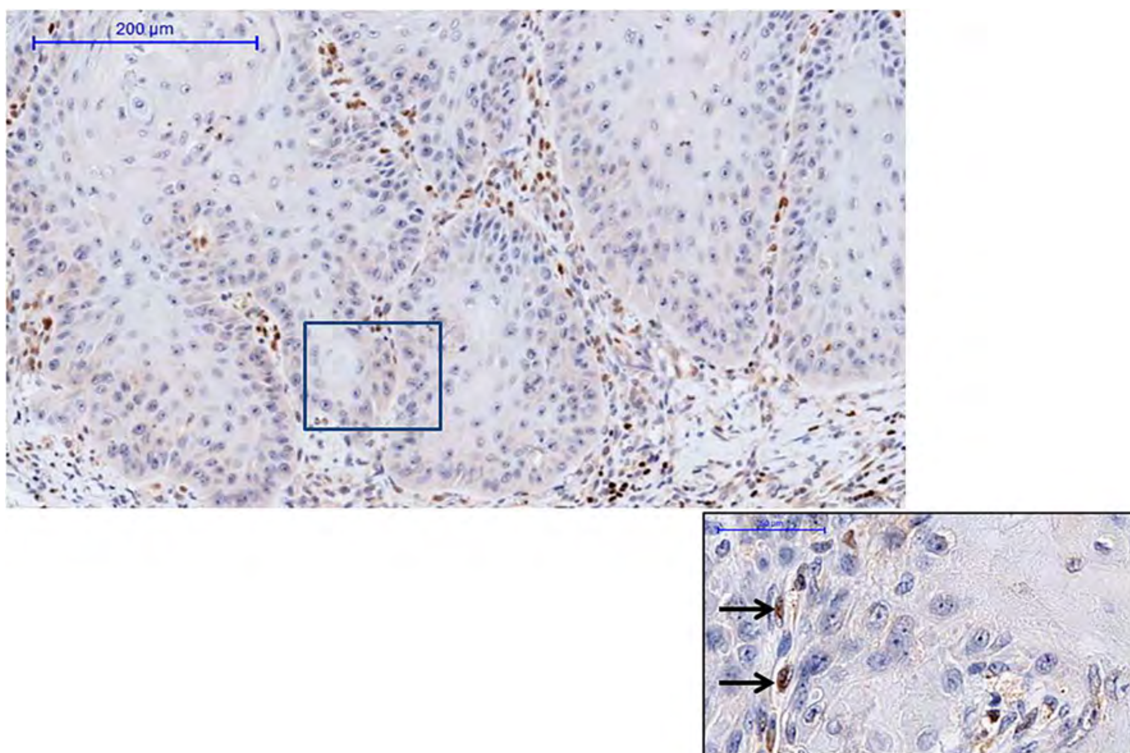

FIGURE 7 Immunohistochemistry for Lef-1 shows expression in the germinative layer in keratoacanthoma. Arrowhead represents a positive keratinocyte (scale bar 200 µm upper left, 50 µm bottom right)

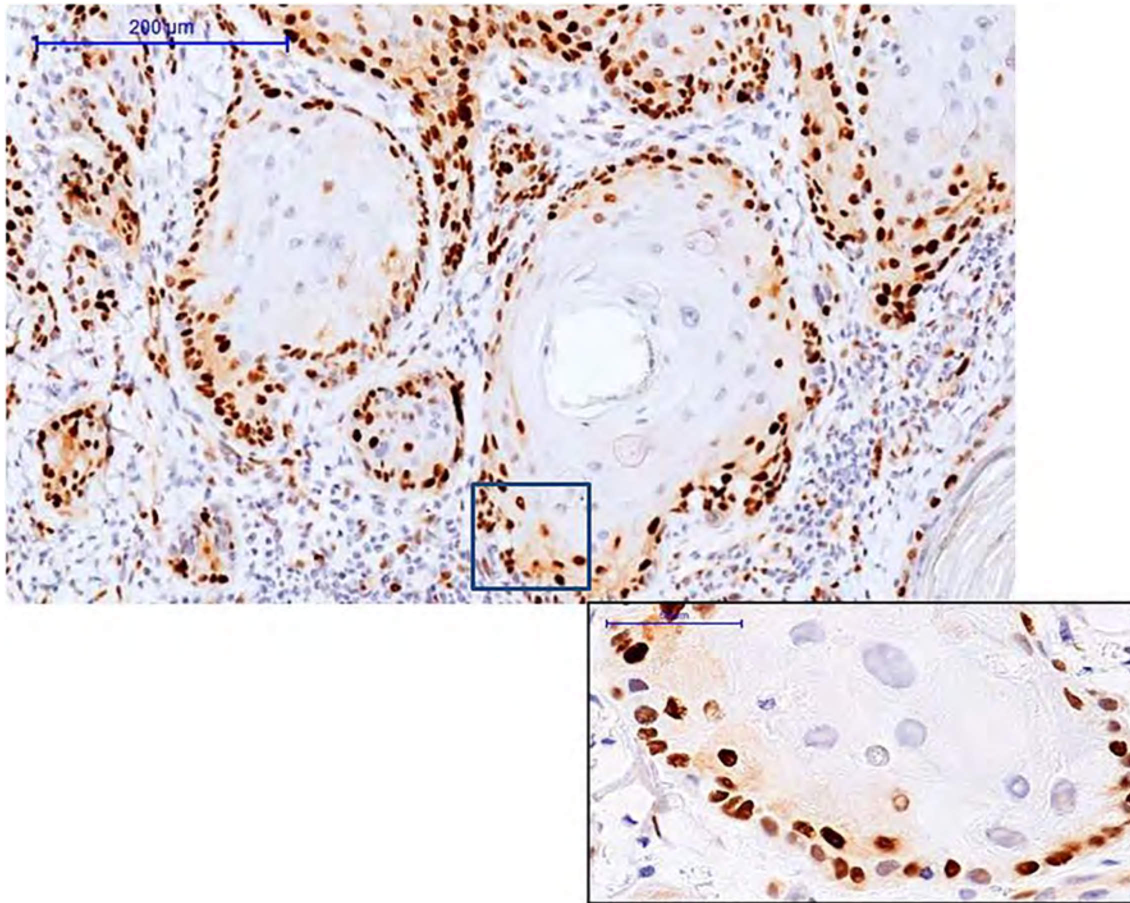

FIGURE 8 Immunohistochemistry for Cyclin-D1 shows expression mainly in the germinative layer in keratoacanthoma (scale bar 200 μm upper left, 50 μm bottom right)

positivity was considered by us to be an indicator of active Wnt signaling. Furthermore, nuclear β-catenin expression acts as a surrogate marker for *β-catenin* mutation, suggesting that the majority of KAs in our study have a wild-type *β-catenin* status. Zito G et al.[30] showed nuclear β-catenin staining in more than 98% of proliferative murine KAs and in less than 17% of regressing murine KAs. They did not, however, mention the number of human KAs that were subsequently examined for β-catenin expression or the percentage of positive nuclear β-catenin cases.

The majority of KAs (61%) showed low levels of Lef-1 expression that was associated with low levels of nuclear β-catenin. Overexpression of Lef-1 is associated with tumor progression and worse prognosis in cancer.[36,37] In skin, Lef-1 is a marker of hair matrix cells and is transactivated by stabilization of β-catenin protein or mutation in the *β-catenin* gene.[25,38] Low Lef-1 expression levels are thus not consistent with progression of KAs driven by Wnt signaling. Furthermore, one might expect low levels of Sox-9 and Cyclin-D1 proteins in KAs when the expression of upstream proteins β-catenin and Lef-1 is low. However, 97% of KAs were positive for nuclear-localized Sox-9 expression; of these, 40% showed high levels of protein expression. Sox-9 protein is implicated in tumorigenesis in various organs and has a role as both activator and repressor. On one hand, it is induced by an active Wnt signaling[39] and acts as a proliferation marker; on the other hand, Sox9 nuclear localization leads to enhancement of β-catenin phosphorylation and its eventual degradation, thus inhibiting Wnt signaling.[20] A recent immunohistochemical study also reported reduced expression of Sox-9 in cases with increased nuclear expression of β-catenin due to a mutated gene, but not in cases with a wild-type gene.[40] Our results lead us to speculate that there is an inhibitory effect of Sox-9 on activation of the Wnt signaling pathway in KAs. Since the majority of KAs were positive for Sox-9 and Cyclin-D1 expression, alternative pathways for activation of Sox9 and Cyclin-D1 are possible.[41,42]

Partial or complete loss of β-catenin membrane staining along with increasing cytoplasmic and nuclear staining of β-catenin is associated with less differentiated, aggressive clinical behavior in squamous cell carcinoma.[43,44] We observed partial loss of β-catenin membrane expression without concomitant nuclear positivity in a subset of KAs. This may indicate that reduced staining is associated with infiltration, but not with aggressive behavior in KAs.

Human KAs may be biologically different from experimentally induced murine KAs.[45] Almost all KAs developed in experimental models are induced by chemical carcinogens in contrast to mainly ultraviolet (UV)-induced KAs in humans. Duration of the life cycle and phases of KAs in experimentally induced mice obviously do not coincide with that of human KAs. The proliferative phase in KAs

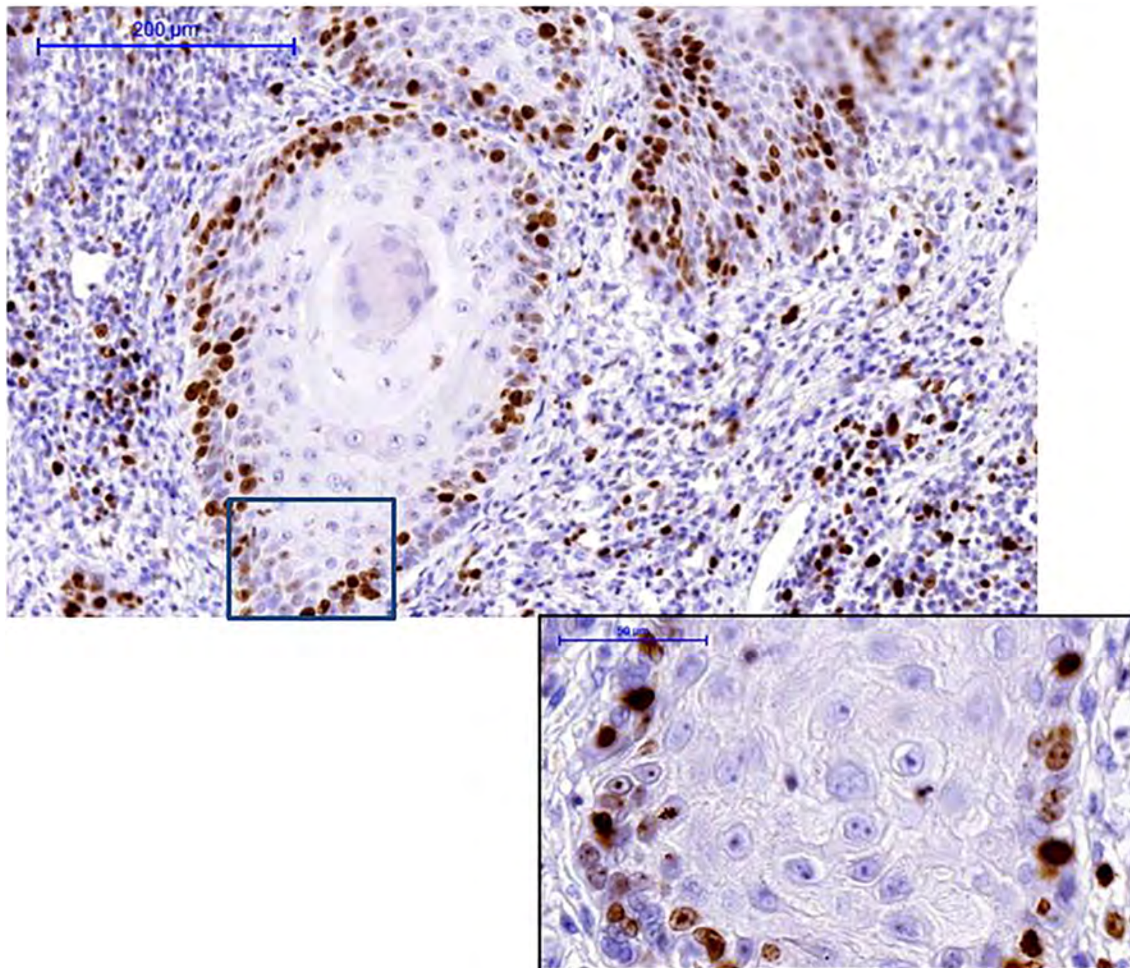

FIGURE 9 Immunohistochemistry for Ki 67 shows expression mainly in the germinative layer in Keratoacanthoma (scale bar 200 µm upper left, 50 µm bottom right)

induced by DMBA (7, 12-dimethylbenz (a) anthracene) in the mouse model was assumed to be only a few days and less than a week, whereas we do not know the exact length of the human KA phases induced by UV light. Murine KA models may thus not be relevant for studies of human KA.

In conclusion, the Wnt signaling pathway does not appear to play a significant role in the development of human KAs, although KA is considered to be a follicular-derived neoplasm.

We are currently analyzing KAs by genome sequencing in collaboration with an international institute that will help us in the future to elucidate possible molecular pathways in the life cycle of KAs.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in connection with this article.

AUTHOR'S CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, OPFC, SJ, and PDA; *Methodology*, SJ, OPFC, and PDA; *Validation*, SJ, OPFC, and PDA; *Investigation*, SJ, OPFC, PDA, and ASCH; *Formal analysis*, SJ, ASCH, and MZ; *Writing*, *Original Draft*, SJ; *Writing*, *Review*, *and Editing*, SJ, OPFC, PDA, ASCH, SNA, and MZ; *Supervision*, OPFC, and PDA; *Funding Acquisition*, OPFC.

DATA AVAILABILITY STATEMENT

The data are not publicly available due to privacy restrictions. The data that support the findings of this study are available on request from the corresponding author, S.J., OP.

ORCID

Sarita Joshi https://orcid.org/0000-0002-2853-1626

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplimentary Information

Supplementary Table 1

Platforms and visualization systems used for antibodies

Antibody	**Platform**	**Visualization**
β-catenin	Ventana Benchmark Ultra	OptiView DAB
Lef-1	Dako Autostainer plus Link 48	En Vision Flex (Code K8021)
Sox-9	Dako Autostainer plus Link 48	En Vision Flex (Code K8000)
Cyclin-D1	Dako Autostainer plus Link 48	EnVision Flex (Code K8009)
Ki-67	Dako Autostainer plus Link 48	En Vision Flex (Code K8009)

Supplementary Table 2

Association between β-catenin nuclear staining and histological age of the lesions

β-catenin staining	**Grading of β-catenin staining**	**Histological age of KA**	
		Young (n=38)	Old (n=29)
β-catenin nuclear positivity in tumor	1 (n=42)	23	19
	2 (n=22)	13	9
	3 (n=3)	2	1
	Missing	0	0
P value	0.888		

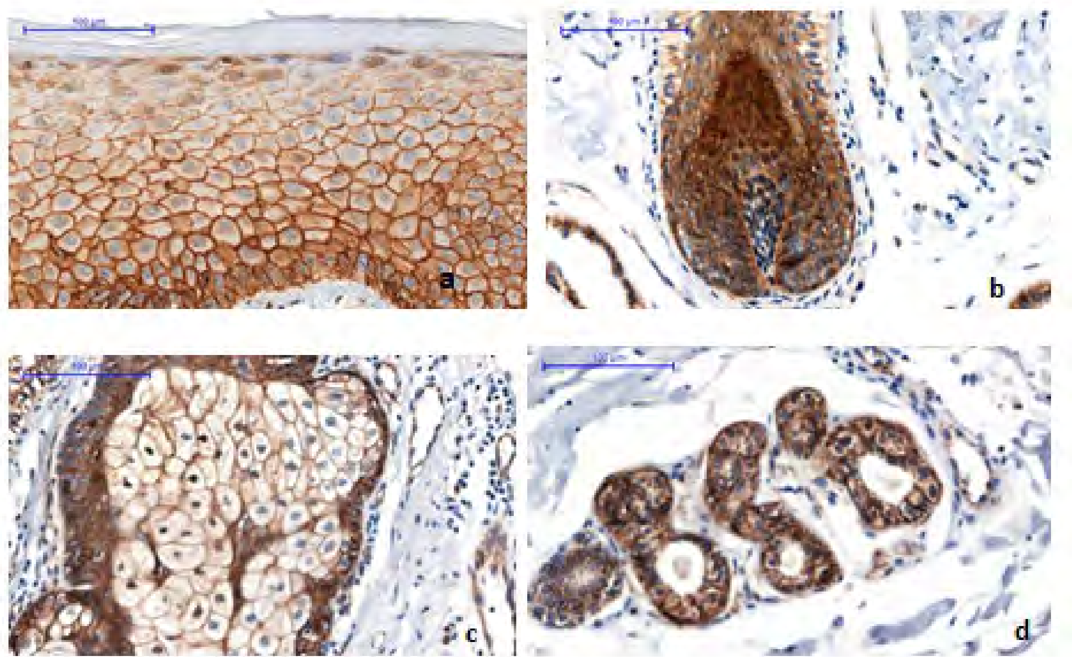

Figure S1 Immunohistochemical expression of β-catenin in normal skin

(a) Membrane expression in the epidermis and concomitant nuclear staining in some areas of the stratum granulosum

(b) Strong nuclear and cytoplasmic staining of the hair matrix cells and occasional nuclear staining of the cells in the hair papilla

(c) Intense membrane staining of sebaceous glands with occasional nuclear staining

(d) Intense membrane staining of eccrine glands

(Scale bar 100 μm)

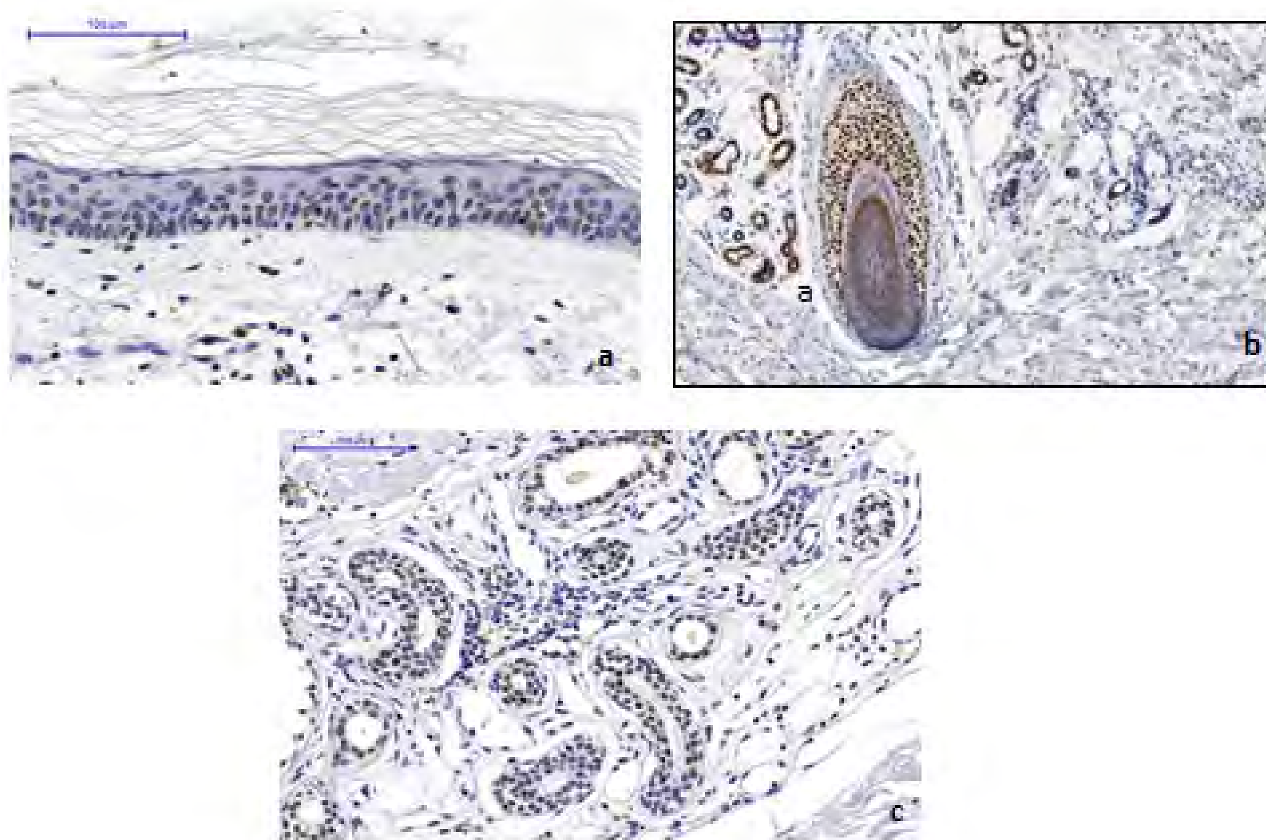

Figure S2 Immunohistochemical expression of Sox-9 in normal skin

(a) Strong nuclear positivity of occasional basal and suprabasal cells in the epidermis

(b) Strong nuclear positivity in the hair matrix cells

(c) Scattered strong positive nuclear staining in the luminal layer of eccrine glands (Scale bar 100 μm)

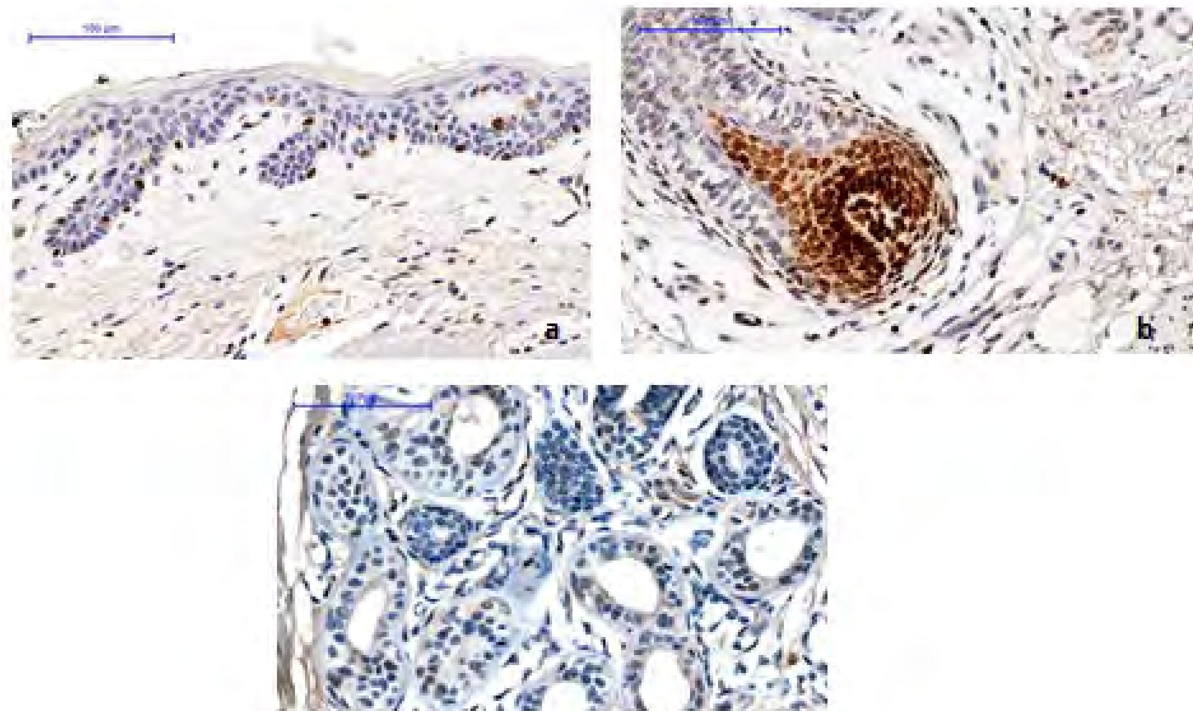

Figure S3 Lef-1 immunohistochemical expression in normal skin

(a) Occasional strong nuclear positivity of basal cells

(b) Strong nuclear staining in the hair matrix cells and the hair papilla cells

(c) Occasional moderately- stained nuclei in the outer layer and the luminal layer of eccrine glands (Scale bar 100 µm)

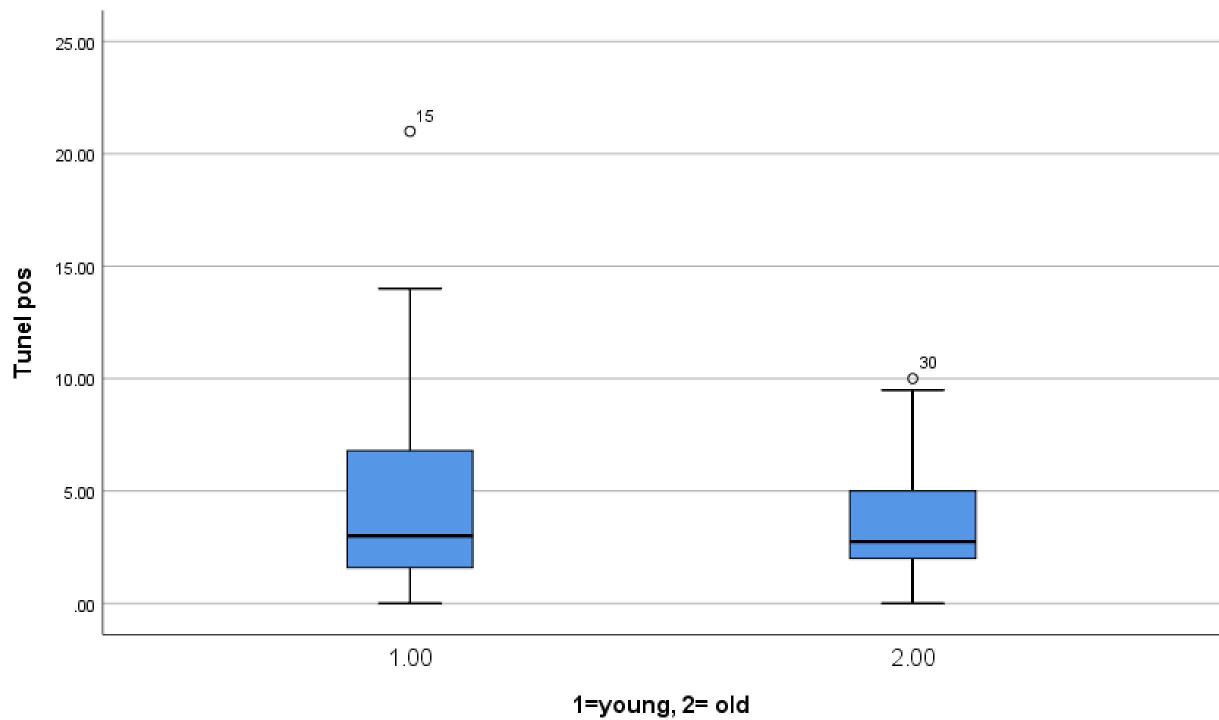

Figure S4 Box plot shows no statistical significant difference in median value of TUNEL- positive cells in young and old KAs

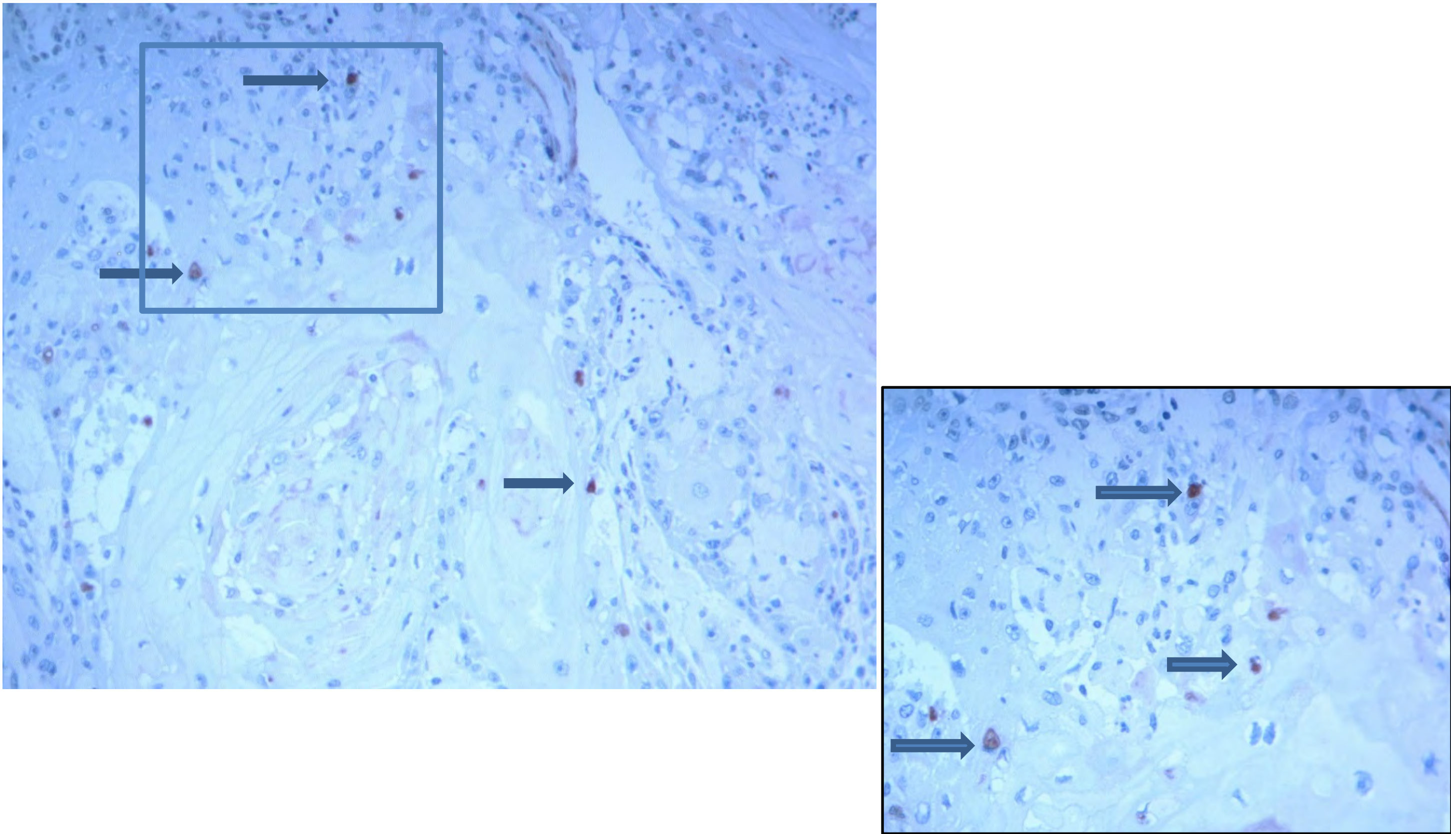

Figure S5 TUNEL immunohistochemistry. Arrowheads point to apoptotic cells (TUNEL-positive)

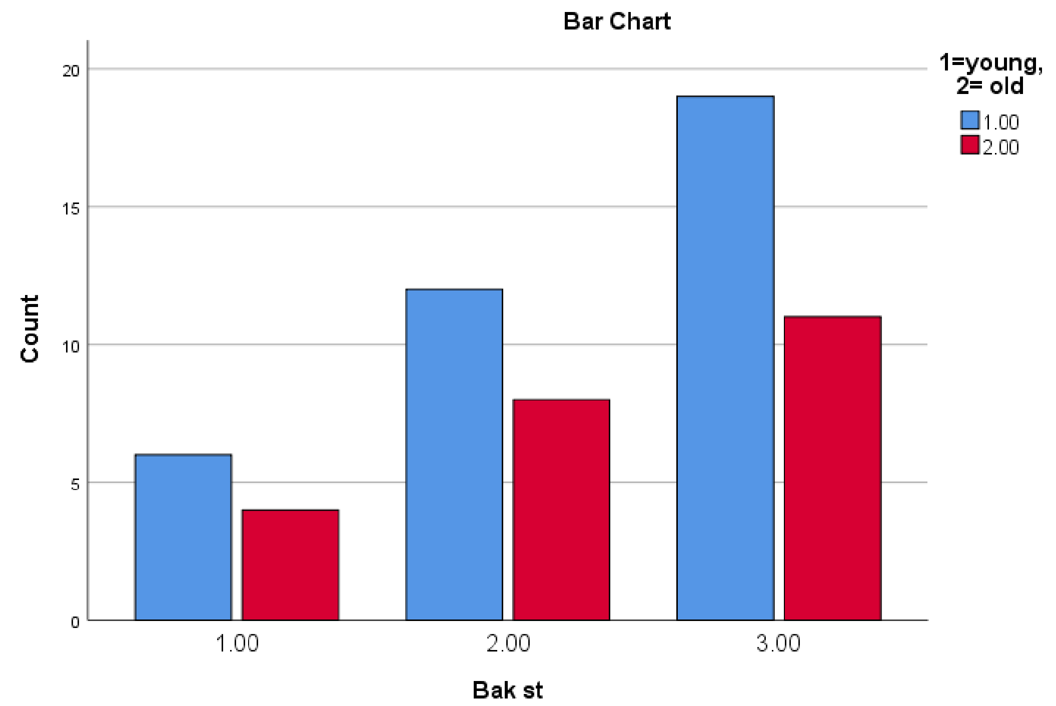

Figure S6 BAK status shows no statistical significant difference in young and old KAs

Errata list

Abbreviations for different types of corrections

Cor – correction of language

Celtf – change of page layout or text format

Page	Line	Footnote	Original text	Type of correction	Corrected text
5	8		Conducting an academic study in a unique topic ….	Cor	Conducting an academic study on a unique topic ….
5	12		… in science and her consistent guidance have enabled me ….	Cor	… in science and her consistent guidance has enabled me ….
10	26		… classification of skin tumor…	Cor	…. classification of skin tumors …
12	11		…. and smallpox vaccination site (36) are associated….	Cor	…. and smallpox vaccination site (36) is associated….
41	9		… Oslo University Hospital, Norway, during the period 1997-2013.	Cor	… Oslo University Hospital, Norway, during the period 1995-2013.
41	12		….fresh frozen tissue collected during the period 1997-2013,	Cor	…. fresh frozen tissue collected during the period 1995-2013,
48	10		In paper III, the histological age of the KAs given by patients was used as a dichotomous variable (young versus old),	Cor	In paper III, the histological age of the KAs was used as a dichotomous variable (young versus old),