Universitat Autònoma de Barcelona

Progression pathways of squamous cell carcinoma associated with actinic damage: From cancer field to actinic keratosis and invasive squamous cell carcinoma

Xavier Sáenz Sardà

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Progression pathways of squamous cell carcinoma associated with actinic damage: From cancer field to actinic keratosis and invasive squamous cell carcinoma.

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Dedicated to

My PhD director, Maite Fernández Figueras, who has been a source of true inspiration during both my medical training and my pathology training.

and

Dr. Oller, because this thesis would have never been started without his obstinate altruism.

Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.

Sir Winston Churchill, House of Commons, Nov 10, 1942.

Contents

List of papers

Papers included in the thesis

I. M.T. Fernández-Figueras, C. Carrato, X. Sáenz, L. Puig, E. Musulén, C. Ferrándiz, A. Ariza. Actinic keratosis with atypical basal cells (AK I) is the most common lesion associated with invasive squamous cell carcinoma of the skin. J Eur Acad Dermatol Venereol. 2014 Oct; 29(5):991-7.

II. X. Sáenz-Sardà, C. Carrato, L. Pérez-Roca, L. Puig, C. Ferrándiz, A. Ariza, M.T. Fernandez-Figueras. Epithelial-to-mesenchymal transition contributes to invasion in squamous cell carcinomas originated from actinic keratosis through the differentiated pathway, whereas proliferation plays a more significant role in the classical pathway. J Eur Acad Dermatol Venereol. 2018 Apr; 32(4):581-586.

III. MT Fernández-Figueras, X Sáenz, P Vargas, CT Thompson, C Carrato, L Puig, C Ferrándiz MD, A Ariza MD. The depth of follicular extension in actinic keratosis correlates with the depth of invasion in squamous cell carcinoma: implication for clinical treatment. J Eur Acad Dermatol Venereol. 2018 Oct; 32(10):1657-1661.

Related works:

- Actinic keratosis, spread throughout the adnexal epithelium and transformation to invasive squamous cell carcinoma. Oral communication at XL National meeting of the Spanish Group of Dermatopathology, Málaga, Spain. 7 & 8 Nov 2014.
- A Progression of actinic keratosis to infiltrating squamous cell carcinoma of the skin: Importance of follicular epithelium involving. XII Congress of Catalan Society of Pathology. Sitges, Catalonia. 3 & 4 June 2016.
- Review of 202 cases confirming the predominance of the differentiated pathway in the origin of cutaneous iSCC, with direct emergence of infiltrative tumor from areas of differentiated intraepidermal neoplasia. External Rotation in Lund University Hospital, Sweden. 1 February 2016-2 May 2016.
- An alteration in the epithelial-mesenchymal transition can explain the development of invasive squamous cell carcinomas from actinic keratosis with atypia limited to the basal layer (AK I). 25th EADV Congress in Vienna, Austria. 28 September-2 October 2016 (Oral presentation).
- Follicular involvement in sun-related iSCC. Oral communication at 20th joint Meeting of the International Society of Dermatopathology in Orlando, March 2017.

Abbreviations

AK	Actinic keratosis
BCC	Basal cell carcinoma
CIN	Cervical intraepithelial neoplasia
CISH	Chromogenic in situ hybridization
CP	Classical pathway
DP	Differentiated pathway
EMT	Epithelial–mesenchymal transition
HFSCs	Hair follicle stem cells
HPV	Human papillomavirus
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
IQR	Interquartile range
ISH	In situ hybridization
NMSC	Non-melanoma skin cancer
KIN	Keratinocyte intraepidermal neoplasia
PDPN	Podoplanin
PDT	Photodynamic therapy
PTEN	Phosphatase and tensin homolog

SCC	Squamous cell carcinoma
cSCC	Cutaneous squamous cell carcinoma
iSCC	Invasive squamous cell carcinoma
SD	Standard deviation
TMA	Tissue microarray
TP	Tumor protein
UV	Ultra-violet
5FU	5%-fluorouracil

Thesis overview

Study	Question	Results and implications
I	Progression from actinic keratosis to invasive squamous cell carcinoma of the skin was described after the development of full thickness epidermal neoplasia. Can an invasive squamous cell carcinoma arise from a proliferation limited in the basal layer?	In 63.8% of the cases (totally assessed, 196 skin biopsies), atypical cells were just found in the basal layer; a direct invasion from proliferating basal atypical keratinocytes was therefore the most common form of progression. This may potentially entail different treatment implications.
Lund	Can the results of the first study be reproduced and verified?	The results after reviewing 79 cases were in concordance with the findings of the first study. In 68.4% of the cases atypical cells were just found in the basal layer. Therefore, a progression in two different pathways was confirmed.
II	Do differences between these two pathways exist? And if so, which are they?	Epithelial-mesenchymal transition participates in the transformation from actinic keratosis limited to the basal (DP) whereas a higher proliferative capacity facilitates intraepidermal extension in the CP.
mi-RNA	Is there any effect on mi-RNA profiling?	Tumors arising from a DP express higher levels of miRNA-31 than those arising from a CP.
III	Does follicular extension play any role in the invasive squamous cell carcinoma pathogenesis?	Follicular extension was found in 25.9% of the cases and can be a source of recurrence, especially under superficial treatment modalities. This may potentially entail different treatment implications.

Resumen en castellano

La incidencia del carcinoma escamoso infiltrante cutáneo, el segundo cáncer más frecuente en humanos, está aumentando muy rápidamente y su mortalidad es equiparable a la del melanoma en algunas regiones geográficas, lo que constituye un grave problema sanitario.

Se pensaba que la progresión de una queratosis actínica (AK) a carcinoma escamoso infiltrante de piel ocurría siempre y cuando la neoplasia intra-epidérmica (lesión precursora "in situ"/displasia/AK) ocupara todo el espesor de la epidermis como ocurre en la vía clásica descrita en el cáncer de cérvix. Sin embargo, el carcinoma escamoso infiltrante cutáneo puede aparecer directamente desde una displasia que sólo ocupe el tercio inferior de la epidermis (AK I). Esta segunda vía de progresión se ha descrito en el carcinoma escamoso de vulva y de cavidad oral (vía diferenciada del cáncer), cuyo comportamiento biológico es además más agresivo que el de la vía clásica.

Esta tesis empezó revisando todos los casos correspondientes a biopsias quirúrgicas, obtenidas mediante el BioBanco del Hospital Germans Trias y Pujol, de tres años consecutivos correspondientes al período 2004-2007. Seleccionamos 503 casos de carcinoma escamoso infiltrante cutáneo, de los que finalmente estudiamos 196 que cumplían todas las características necesarias para su correcta evaluación y que fueron revisados por tres patólogos.

La mayor parte mostraron AK I en superficie (125/196, 63.8%), en los bordes (141/181, 77.9%) o en ambas (105/181, 58%), concluyendo así que la invasión directa desde queratosis actínicas con displasia limitada a la capa basal / AK I (vía diferenciada) es la forma más frecuente de progresión a carcinoma escamoso infiltrante cutáneo. Por otro lado, la progresión siguiendo la vía clásica fue demostrada en el resto de los casos. Por lo tanto todas las queratosis actínicas, sin tener en cuenta el

grosor de su extensión o grado (AK I, AK II o AK III) son potencialmente precursoras de carcinoma escamoso infiltrante. Este estudio fue el primero que se propuso investigar la prevalencia de la vía clásica y diferenciada en la transformación de la AK en carcinoma escamoso infiltrante aportando evidencias de su existencia.

Los hallazgos han sido publicados en la revista European Academy of Dermatology and Venereology (M.T. Fernández-Figueras, C. Carrato, X. Sáenz, L. Puig, E. Musulen, C. Ferrándiz, A. Ariza. Actinic keratosis with atypical basal cells (AK I) is the most common lesion associated with invasive squamous cell carcinoma of the skin. J Eur Acad Dermatol Venereol. 2014 Oct; 29(5):991-7.).

En una estancia de tres meses en el Hospital Universitario de Lund se analizaron 202 casos de carcinoma escamoso infiltrante cutáneo provenientes todos ellos del año 2015. Aplicando los criterios establecidos en el estudio anterior se incluyeron para estudio 79 casos. En esta ocasión, se identificaron un 68.4% de AK I en superficie y un 74.7% en los márgenes. Estos datos consolidan la vía diferenciada como la principal en la génesis del carcinoma escamoso infiltrante cutáneo. Dado que los factores geográficos, pero también el incremento de la exposición solar de tipo vacacional, son conocidos por contribuir al desarrollo de las queratosis actínicas, se analizó el porcentaje de elastosis solar sobre población sueca hallándose estos sorprendentemente elevados pues el 44.3 % de los casos presentaban dano severo.

La siguiente etapa del estudio consistió en la realización de una matriz de tejidos o tissue microarray (TMA), mediante la revisión sistemática de las biopsias, ya estudiadas para la realización de la primera fase del estudio, con el propósito de seleccionar aquellos casos que contenían suficiente tejido representativo. Una vez segregados los casos con carcinoma escamoso infiltrante cutáneo con AK I en superficie (vía diferenciada) de los que presentan AK III (vía clásica) se marcaron en todos ellos áreas de lesión en superficie, lesión en profundidad, extensión por el epitelio anexial y campo de cancerización obteniendo 3 cores de cada zona. También se procedió a la realización de dos TMAs de muestras procedentes de diagnósticos de AK sin carcinoma escamoso infiltrante marcando en ellas lesión y campo de cancerización. En total se realizaron ocho TMA que supusieron 756 cores a evaluar. Entre otros, dicho TMA permitió el estudio de la extensión de la atipia a través del epitelio folicular y el estudio de las moléculas involucradas en las dos vías de progresión desde la AK al iSCC principalmente mediante técnicas de inmunohistoquímica, pero también de técnicas de CISH para el estudio de miRNA (miRNA-21 y miRNA-31). Por lo que respecta a ésta última, no se han encontrado diferencias significativas en la expresión de miRNA21; sin embargo, los tumores surgidos por vía diferenciada expresan mayores niveles de miRNA31 tanto en su intensidad como en su extensión. Este estudio se encuentra pendiente de publicación.

Para explorar las diferencias entre las dos vías de progresión en la patogénesis de las AK nos centramos especialmente en los marcadores Ki67, p53, p16 así como en las moléculas implicadas en la transición epitelio-mesenquimal (vimentina, E-cadherina, β-catenina y D2-40). Los carcinomas escamosos infiltrantes originados por vía diferenciada mostraron un índice de proliferación (Ki67) significativamente menor (30% vs 46%, P = 0.003) y una expresión significativamente menor (P<0.001) de las moléculas implicadas en la transición epitelio-mesénquima (vimentina, E-cadherina y β-catenina).

Por lo tanto, se concluyó que la transición epitelio-mesénquima participa en la transformación de AK I en carcinoma escamoso infiltrante (vía diferenciada) mientras que una capacidad proliferativa mayor facilita la extensión intra-epidérmica en la vía clásica. También se hallaron diferencias significativas en cuanto a la expresión de CD31 (angiogénesis) y MMP (metaloproteinasas) hallándose estos marcadores elevados en los tumores que progresan por vía diferenciada, lo que junto con la transición epitelio-mesenquima podría facilitar la progresión local.

Una parte de estos resultados han sido publicados en la revista European Academy of Dermatology and Venereology (X. Sáenz-Sardà, C. Carrato, L. Pérez-Roca, L. Puig, C. Ferrándiz, A. Ariza, M.-T. Fernández-Figueras. Epithelial-to-mesenchymal transition contributes to invasion in squamous cell carcinomas originated from actinic keratosis through the differentiated pathway, whereas proliferation plays a more significant role in the classical pathway. J Eur Acad Dermatol Venereol. 2018 Apr; 32(4):581-586).

La extensión de la atipia queratinocitaria, ya sean de tipo AK I o AK III, por el epitelio de los anejos, especialmente a través de los folículos pilosos, puede constituir un mecanismo adicional en la génesis del iSCC, siendo un elemento facilitador especialmente en la lesiones de tipo AK I. Dicha extensión es una de las características de las queratosis actínicas proliferativas y su importancia recae especialmente en el hecho de ser resistentes a ciertos tratamientos locales, debido a la profundidad a la que pueden encontrarse y a que es difícil reconocerlas para su diagnóstico mediante técnicas de imagen en vivo.

En nuestro primer artículo, se observó que en 52 casos (26.5%) existía una extensión en profundidad de la atipia siguiendo la basal del epitelial de los anejos cutáneos y que el 75% de ellos aparecían en el contexto de una AK I. Por consiguiente, la progresión del tumor hacia los anejos podría ser una de las principales formas de desarrollo de iSCC sobre AK I. En consecuencia, se estudió en los caos de nuestra serie la relación entre la extensión folicular de queratinocitos atípicos en lesiones de tipo queratosis actínica y el desarrollo de carcinomas escamosos con origen en la pared folicular. También se correlacionaron la profundidad de la extensión folicular con la profundidad de infiltración en milímetros y en niveles anatómicos (infundibular, itsmico o subitsmico). Se observó que la extensión folicular estaba presente en el 25.9% de los casos y, de ellos, la infiltración del carcinoma escamoso directamente adyacente a la basal folicular estaba presente en el 58% de los casos, correlacionándose el riesgo de desarrollar carcinoma escamoso con la profundidad de la extensión folicular. Además, la profundidad de la extensión folicular de las queratosis actínicas se correlaciona la profundidad de invasión del carcinoma escamoso asociado, indistintamente de la vía de progresión que lo origine. En consecuencia, seria altamente recomendable indicar la profundidad de la extensión folicular en el diagnóstico histológico de biopsias incisionales, dado el riesgo de recurrencia e infiltración que ello implica, así como las derivadas terapéuticas que conlleva.

Los hallazgos han sido publicados en la revista European Academy of Dermatology and Venereology (MT Fernández-Figueras, X Sáenz, P Vargas, CT Thompson, C Carrato, L Puig, C Ferrándiz MD, A Ariza MD. The depth of follicular extension in actinic keratosis correlates with the depth of invasion in squamous cell carcinoma: implication for clinical treatment. J Eur Acad Dermatol Venereol. 2018 Oct; 32(10):1657-1661).

Consideramos que la serie de estudios que conforman esta tesis proporcionan conocimiento nuevo sobre las vías de progresión del carcinoma escamoso cutáneo y de sus lesiones precursoras. Se ha establecido que existen al menos dos vías de progresión de carcinoma escamoso a queratosis actínica, se ha introducido el concepto de vía diferenciada en la carcinogénesis cutánea, se han hallado bases moleculares que explican la progresión a través de ambas vías y se ha constatado el riesgo de la extensión folicular en la queratosis actínica. Todos estos estudios han supuesto en algunos ámbitos un cambio de paradigma y tienen relevancia tanto en el diagnóstico como en el tratamiento de las queratosis actínicas.

Sammanfattning på svenska

Incidensen av infiltrativ skivepitelcancer i huden, den näst vanligaste cancerformen hos människan, ökar mycket snabbt och dödligheten är jämförbar med den för melanom i vissa geografiska regioner. Detta utgör ett allvarligt hälsoproblem.

Man har tidigare trott att progressionen av aktinisk keratos (AK) till infiltrativt skivepitelkarcinom i huden sker under förutsättning att den intraepidermala dysplasin/den aktiniska keratosen upptar hela epidermis tjocklek, enligt principen för den s.k. klassiska utvecklingsvägen beskriven vid livmoderhalscancer. Det är emellertid känt att kutan infiltrativ skivepitelcancer kan uppkomma direkt från en dysplasi som endast upptar den nedre tredjedelen av epidermis (AK I). Denna andra utvecklingsväg har beskrivits i skivepitelkarcinom i vulva och i munhålan (differentierad cancer-utvecklingsväg), vars biologiska beteende också är mer aggressiv än den klassiska vägen.

Detta avhandlingsarbete omfattar flera olika studier, projekt 1-3.

Projekt 1.
Innefattade en granskning av alla kirurgiska biopsier erhållna genom Germans Trias i Pujol sjukhus Biobank under tre år: perioden 2004-2007. Vi valde 503 kutana fall av infiltrativ skivepitelcancer, varav vi slutligen valde ut 196 fall som uppfyllde alla nödvändiga egenskaper för att kunna utvärderas korrekt och som därefter granskades av tre patologer.

De flesta proven visade AK I i ytan (125/196, 63,8%), i kanterna (141/181, 77,9%) eller både och (105/181, 58%). Från detta kunde man dra slutsatsen att direkt invasion från aktiniska keratoser med begränsad dysplasi vid basalcell-skiktet/AKI (differentierad cancer-utvecklingsväg) är den vanligaste formen av progression till kutan infiltrativ cancer. Man kunde i de övriga fallen se progression ut efter den klassiska vägen.

Utfallet visade att alla aktiniska keratoser, oberoende av tjocklek eller grad (AK I, AK II o AK III) är potentiella prekursorer till infiltrativ skivepitelcancer. Denna studie är den första i sitt slag och den syftar till att undersöka prevalensen av den klassiska och differentierade cancer-utvecklingsvägen i omvandlingen av AK till infiltrerande skivepitelcancer.

Resultaten i projekt 1 har publicerats i tidskriften European Academy of Dermatology and Venereology (M.T. Fernández-Figueras, C. Carrato, X. Sáenz, L. Puig, E. Musulen, C. Ferrándiz, A. Ariza. Actinic keratosis with atypical basal cells (AK I) is the most common lesion associated with invasive squamous cell carcinoma of the skin. J Eur Acad Dermatol Venereol. 2014 Oct; 29(5):991-7.).

Projekt Lund.

Under en tre månaders vistelse på Patologkliniken i Lund analyserades 202 fall av kutant infiltrativt skivepitelkarcinom, alla från 2015. Vid tillämpning av de kriterier som fastställdes i föregående studie inkluderades 79 fall för studien.

Den här gången identifierades, vid sidan av cancerlesionen, 68,4% av AK I på ytan och 74,7% i kanterna. Dessa data konsoliderar sålunda den differentierade cancer-utvecklingsvägen som den huvudsakliga vägen i uppkomsten av kutant infiltrativt skivepitelkarcinom. Eftersom geografiska faktorer men också ökningen av semester-relaterad solexponering är kända för att bidra till utvecklingen av aktiniska keratoser, analyserades andelen av solar elastos. Förekomsten av solar elastos hos den svenska befolkningen visade sig förvånansvärt hög, då det i 44.3 % av fallen hittades svår skada.

Projekt 2.

När fallen med kutant invasiv skivepitelcancer med AK I på ytan (differentierad utvecklingsväg) hade skiljts ut från de med AK III (klassisk utvecklingsväg), utfördes en tissue microarray (TMA) genom systematisk bedömning av de biopsier, som redan var studerade i studiens första fas, med syftet att välja de mest representativa fallen. I samtliga biopsier märktes områden av ytskada, djup lesion, förlängning genom adnex-epitel och cancer-utvecklingsfält, vilket gav 3 TMA-kolvar från varje ställe. Vi gjorde också TMA från prover med AK-diagnos utan iSCC, och 2 TMA-kolvar gjordes här, från lesions- och canceriseringsfältet markerades. Totalt åtta TMA-klotsar gjordes med totalt 756 kolvar att utvärdera. Bland andra, det nämnda TMA tillät studium av omfattningen av atypi genom follikulära epitelet och studiet av molekyler som ingår i de två progressions vägar från AK till iSCC huvudsakligen genom immunohistokemi tekniker men även CISH tekniker för studien av mikro-RNA (miRNA-21 och miRNA-31). Det fanns ingen statistisk signifikant skillnad i uttrycktet av miRNA21. Dock tumörerna som framkom genom differentierad utvecklingsväg uttryckte en högre nivå av miRNA31 både i intensitet och utbredning. Den senare är igång, i väntan på publicering.

För att utforska skillnaderna mellan de två skilda utvecklingsvägarna i uppkomsten av infiltrativ skivepitelcancer gjordes immunhistokemisk infärgning av proverna – här fokuserade vi speciellt på markörerna Ki67, p53, p16 samt de molekylerna som är involverade i den epitelial-mesenkymala övergången (vimentin, E-cadherin, β-katenin och D2-40).

Infiltrerande skivepitelkarcinom som bedömts uppkomma genom den differentierade utvecklingsvägen visade ett proliferationsindex (Ki67) som var signifikant lägre än det i den klassiska vägen (30 % vs 46 %, p = 0,003) och hade ett signifikant lägre uttryck (P <0,001) av de molekyler

som är involverade i den epiteliala-mesenkymala transformationen (vimentin, E-cadherin och β-catenin).

Därför kunde vi dra slutsatsen att den epiteliala-mesenkymala transformationen är involverad i omvandlingen av AK I till infiltrerande skivepitelkarcinom (den differentierade utvecklingsvägen) medan en högre proliferativ förmåga underlättar intraepidermal tumörutveckling och -utbredning via den klassiska vägen. Det fanns också en statistiskt signifikant skillnad avseende uttrycket av CD31 (kärlnybildning) och MMP (matrixmetalloproteinases). Dessa markörer var förhöjda endast i tumörer med en differentierad utvecklingsväg. Sammantaget visar detta att den epiteliala-mesenkymala transformationen kan underlätta lokal progression.

Merparten av resultaten har publicerats i tidskriften European Academy of Dermatology and Venereology (X. Saenz-Sarda, C. Carrato, L. Perez-Roca, L. Puig, C. Ferrandiz, A. Ariza,M.-T. Fernandez-Figueras. Epithelial-to-mesenchymal transition contributes to invasion in squamous cell carcinomas originated from actinic keratosis through the differentiated pathway, whereas proliferation plays a more significant role in the classical pathway. J Eur Acad Dermatol Venereol. 2018 Apr; 32(4):581-586).

Projekt 3.

Redan i den första artikeln noterades att det bör tas hänsyn till att utbredningen av dysplasierna, oavsett typ AK I eller AK III, genom adnex-epitel (särskilt genom hårfolliklar) kan utgöra en ytterligare mekanism i ISCC patogenesen och att det är en progress-faciliterande faktor speciellt vid AK I- typ lesioner. Påtagligt utbredda AK beskrivs som proliferativa aktiniska keratoser och dessa kan vara resistenta mot behandling på grund av att de kan ligga delvis djupt ner i huden och att de kan vara svåra att identifiera diagnostiskt med användning av in vivo avbildningstekniker.

I den första artikeln observerades att det i 52 fall (26,5%) fanns en utbredning av dysplasin ner genom adnexa och att 75 % av dem förelåg i samband med en AK I. Man kan av detta dra slutsatsen att tumörprogression till adnexstrukturer i huden kan det vara ett av de centrala sätten på vilket ett iSCC utvecklas ur AK I. Baserat på dessa fynd studerades i den tidigare beskrivna serien av fall sambandet mellan follikulär utbredning av atypiska keratinocyter vid aktinisk keratos och utveckling av skivepitelkarcinom med ursprung i den follikulära väggen. Härvid korrelerades också djupet av den follikulära utbredningen med infiltrations-djupet, med hjälp av Breslow-index, tjockleken och nivån (inom follikelenheten) för atypierna - infundibulum, isthmus eller subisthmus. Det observerades att follikulär utbredning fanns i 25,9% av fallen och normalt sträckte de sig till det understa segmentet. Skivepitelkarcinom med infiltration sågs i direkt anslutning till det nedre follikulära segmentet i 58 % av fallen vilket korrelerade med djupet av den follikulära utbredningen. Därför konstaterades att djupet på spridningen av atypiska keratinocyter i aktiniska keratoser korrelerar med utvecklingen av djupet av invasionen av skivepitelcancer, oavsett vilken progressionsväg denna malignisering inträffade. Det är därför viktigt att djupet av den follikulära utbredningen påpekas när den histopatologiska diagnosen anges, då risken för återfall också ligger till grund för val av terapeutiska alternativ, specifikt för att förhindra utvecklingen av infiltrativ skivepitelcancer i de fall där en ytbehandling är vald.

Resultaten har publicerats i tidskriften European Academy of Dermatology and Venereology (MT Fernández-Figueras, X Saenz, P Vargas, CT Thompson, C Carrato, L Puig, C Ferrándiz MD, A Ariza MD. The depth of follicular extension in actinic keratosis correlates with the depth of invasion in squamous cell carcinoma: implication for clinical treatment. J Eur Acad Dermatol Venereol. 2018 Oct; 32(10):1657-1661).

Vi anser att den serie studier som utgör denna avhandling ger ny kunskap om vägarna för kutan invasiv skivepitelcancer och dess prekursorskador. Det har fastställts att 1) det finns minst två utvecklingsvägar från aktinisk keratos till skivepitelcancer, 2) konceptet med en differentierad utvecklingsväg vid kutan karcinogenes har införts, 3) molekylära indikatörer förklarar progressionen genom båda utvecklingsvägarna och även risken för tillväxt genom hårfolliklar i aktinisk keratos. Alla dessa studier har lett till ett paradigmskifte på vissa områden och är relevanta både vid diagnos och behandling av aktinisk keratos.

Resum en català

La incidència del carcinoma escamós infiltrant cutani, el segon càncer més freqüent en humans, està augmentant molt ràpidament i la seva mortalitat és equiparable a la del melanoma en algunes regions geogràfiques, la qual cosa constitueix un greu problema sanitari.

Es pensava que la progressió d'una queratosi actínica (AK) a carcinoma escamós infiltrant de pell, succeïa en tant en quan la neoplasia intraepidèrmica (lesió precursora "in situ"/displasia/AK) ocupés tot l'espessor de l'epidermis tal i com succeeix en la via clàssica descrita en el càncer de cèrvix. No obstant, és conegut que el carcinoma escamós infiltrant cutani pot aparèixer directament des de una displàsia que només ocupi el terç inferior de l'epidermis (AK I). Aquesta altra via de progressió ha estat descrita en el carcinoma escamós de vulva i de cavitat oral (via diferenciada del càncer), que té a més un comportament biològic més agressiu que el del descrit a la via clàssica.

Aquesta tesi començà revisant tots els casos corresponents a les biòpsies quirúrgiques, obtingudes mitjançant el BioBanc de l'Hospital Germans Trias i Pujol, de tres anys consecutius corresponents al període 2004-2007. Es varen seleccionar 503 casos de carcinoma escamós infiltrant cutanis dels quals finalment es varen estudiar 196 que complien totes les característiques necessàries per a la seva correcta avaluació i que varen ser revisats per tres patòlegs.

La major part varen mostrar AK I en superfície (125/196, 63.8%), a les vores (141/181, 77.9%) o en ambdues (105/181, 58%), concloent per tant que la invasió directa des de queratosis actíniques amb displàsia limitada a la basal/AK I (via diferenciada) és la forma més freqüent de progressió a carcinoma escamós infiltrant cutani. D'altra banda, la progressió seguint la via clàssica va ser demostrada en els casos restants.

Per tant, totes les queratosis actíniques, sense tenir en compte el gruix de la seva extensió o grau (AK I, AKII o AK III) són potencialment precursores de carcinoma escamós infiltrant. Aquest estudi va ser el primer en proposar-se estudiar la prevalença de la via clàssica i diferenciada en la transformació de la AK en carcinoma escamós infiltrant aportant evidències de la seva existència.

Les troballes van ser publicades a la revista European Academy of Dermatology and Venereology (M.T. Fernández-Figueras, C. Carrato, X. Saenz, L. Puig, E. Musulen, C. Ferrandiz, A. Ariza. Actinic keratosis with atypical basal cells (AK I) is the most common lesion associated with invasive squamous cell carcinoma of the skin. J Eur Acad Dermatol Venereol. 2014 Oct; 29(5):991-7.).

Durant una estada de tres mesos a l'Hospital Universitari de Lund es varen analitzar 202 casos de carcinoma escamós infiltrant cutani provinents tots ells de l'any 2015. Aplicant els criteris establerts en l'estudi anterior es van incloure finalment 79 casos. Aquest cop es van identificar un 68.4% de AK I en superfície i un 74.7% en les vores. Aquestes dades consoliden doncs la via diferenciada com la principal en la gènesis del carcinoma escamós infiltrant cutani. Donat que els factors geogràfics però també l´increment de l'exposició solar de tipus vacacional són coneguts per contribuir al desenvolupament de les queratosis actíniques es va analitzar el percentatge d'elastosi solar sobra la població sueca trobant-se aquestes xifres sorprenentment elevades doncs el 44.3% dels casos presentaven dany sever.

La següent etapa de l´estudi va consistir en la realització d´una matriu de teixits o tissue microarray (TMA), mitjancant la revisó sistemàtica de les biòpsies ja estudiades per a la realització de la primera fase del estudi, amb el propòsit de seleccionar aquells casos qe contenien suficient teixit representatiu. Un cop segregats els casos amb carcinoma escamós infiltrant cutani amb AK I en superfície (via diferenciada) dels casos que presentaven AK III (via clàssica) es varen marcar les àrees de de lesió superficial, lesió en profunditat, extensió a través de l'epiteli annexial i camp de cancerització obtenint tres cores/mostres de cada zona. També es va procedir a realitzar dos TMAs de mostres procedents de diagnòstics de AK sense carcinoma escamós infiltrant marcant en aquestes lesió i camp de cancerització. En total es van realitzar vuit TMAs amb un total de 756 cores a avaluar. Entre d'altres aquest TMA va permetre l'estudi de l´extensió de l´atípia a través de l´epiteli fol·licular i l'estudi de les molècules implicades en les dues vies de progressió des de AK a carcinoma escamós infiltrant principalment mitjançant tècniques d'immunohistoquímica però també de tècniques CISH per l'estudi de micro-RNA (miRNA-21 y miRNA-31). Pel que fa a aquesta última, no s´han trobat diferències significatives en l´expressió de miRNA21; no obstant, els tumors sorgits per via diferenciada expressen majors nivells de miRNA31 tant pel que fa a la intensitat com en l extensió. Aquest estudi es troba pendent de publicació.

Amb el propòsit d'explorar les diferències entre les dues vies de progressió en la patogènesi de les AK ens vam centrar especialment en els marcadors Ki67, p53 i p16 així com en les molècules implicades en la transició epiteli-mesènquima (vimentina, E-caderina, β-catenina i D2-40). Els carcinomes escamosos infiltrants originats per via diferenciada van mostrar un índex de proliferació (Ki67) significativament menor (30% vs 46%, P = 0.003) així com una expresió significativament menor (P<0.001) de les molècules implicades en la transició epiteli-mesènquima (vimentina, E-cadherina y β-catenina). Per tant es va

concloure que la transició epiteli-mesènquima participa en la transformació de AK I en carcinoma escamós infiltrant (via diferenciada) mentre que una capacitat proliferativa major facilita l´ extensió intraepidèrmica en la via clàssica. També es van trobar diferències significatives pel que fa a l expressió de CD31 (angiogènesis) i MMP (metaloproteinasses) trobant-se aquests marcadors elevats en els tumors que progressen per via diferenciada, que juntament amb la transició epiteli-mesènquima podrien actuar com a facilitadors de la progressió local.

Una part dels resultats han sigut publicats a la revista European Academy of Dermatology and Venereology (X. Saenz-Sarda, C. Carrato, L. Perez-Roca, L. Puig, C. Ferrandiz, A. Ariza,M.-T. Fernandez-Figueras. Epithelial-to-mesenchymal transition contributes to invasion in squamous cell carcinomas originated from actinic keratosis through the differentiated pathway, whereas proliferation plays a more significant role in the classical pathway. J Eur Acad Dermatol Venereol. 2018 Apr; 32(4):581-586).

L´extensió de la atípia queratinocitaria, ja siguin de tipus AK I o AK III, per l'epiteli dels annexes, especialment a través dels fol·licles pilosos, poden constituir un mecanisme addicional en la gènesi del carcinoma escamós infiltrant sent-ne un element facilitador especialment en les lesions de tipus AK I. Aquest tipus d'extensió és una de les característiques de les queratosis actíniques proliferatives i la seva importància recau en el fet de ser resistents a tractaments locals degut a la profunditat a la que poden trobar-se i al fet que és difícil reconèixer-les en el moment del diagnòstic mitjançant tècniques d´imatge in vivo.

En el nostre primer article es va observar que en 52 casos (26.5%) existia una extensió en profunditat de la atípia seguint la basal del epiteli dels annexos cutanis i que el 75% d'aquestes apareixien en el context d'una AK I. Per tant, la progressió del tumor pels annexes podria ser una de les principals formes de desenvolupament del carcinoma escamós infiltrant sobre AK I. Per aquest motiu es va estudiar en els casos de la nostre serie la relació entre l'extensió fol·licular de queratinòcits atípics en les lesions de tipus queratosis actínica i el desenvolupament de carcinomes escamosos amb origen a la paret fol·licular. Es van correlacionar també la profunditat de l'extensió fol·licular amb la profunditat d'infiltració en milímetres i en nivells anatòmics (infundibular, ístmica o subístmica). Es va observar que l'extensió fol·licular estava present en el 25.9% dels casos i, d´entre ells, l´infiltració del carcinoma escamós directament adjacent a la basal fol.licular estava present en el 58% dels casos, correlacionant-se el risc de desenvolupar carcinoma escamós amb la profunditat de l´extensió fol·licular. A més a més, la profunditat de l´extensió fol·licular de les queratòsis actíniques es correlaciona la profunditat d´invasió del carcinoma escamós associat, independentment de la via de progressió que l´origini. En conseqüencia , seria altament recomenable indicar la profunditat de l´extensió fol·licular en el diagnòstic histológic de biòpsies incisionals donat el risc de recurrència i infiltració que això implica, així com les derivades terapeutiques que implica.

Els resultats d´aquest tercer treball han estat publicats a la revista European Academy of Dermatology and Venereology (MT Fernández-Figueras, X Saenz, P Vargas, CT Thompson, C Carrato, L Puig, C Ferrándiz MD, A Ariza MD. The depth of follicular extension in actinic keratosis correlates with the depth of invasion in squamous cell carcinoma: implication for clinical treatment. J Eur Acad Dermatol Venereol. 2018 Oct; 32(10):1657-1661).

Considerem que la serie d'estudis que conformen aquesta tesi proporcionen coneixament nou sobre les vies de progressió del carcinoma escamós cutani i de les seves lesions precursores. S'ha establert que existeixen al menys dos vies de progressió de carcinoma escamós a queratosi actínica, s'ha introduït el concepte de via diferenciada en la carcinogènesi cutania, s'han trobat bases mol.leculars que expliquen la progresió a través d'ambdues vies i s'ha constat el risc de l'extensió fol·licular en la queratosi actínica. Tots aquests estudis han suposat en alguns àmbits un canvi de paradigma i tenen rellevància tant en el diagnòstic com en el tractament de les queratosis actíniques.

1. - Introduction

1.1. - The skin

The skin is only a few millimeters thick; however, it is the heaviest and largest organ. The skin can weigh between 3.5 and 10 kilograms and have a surface area of 1.5 to 2 square meters depending on the individual. (1)

The skin's primary function, among others, is to protect the organism from deleterious physical, chemical or microbiological environmental impacts, as well as maintaining temperature and the electrolyte and fluid balance. The skin is also a huge and highly active biofactory for the synthesis, processing and metabolism of a wide range of structural proteins, glycans, lipids and signaling molecules. The skin is furthermore an integral component of the immune, nervous and endocrine systems contributing to the overall homeo- stasis of the body. (2)

The skin can be divided in three different layers. The first and most superficial one is called the epidermis. It is mainly made up of keratinocytes that are firmly stuck together; it also contains other types of cells like melanocytes, lymphocytes, Langerhans cells and Merkel cells.(1) It constantly renews itself. New cells are made in the lower layers of the epidermis and progress to the surface in a process that takes place within four weeks. The epidermis varies in thickness on different parts of the body. It is in the deepest part of the epidermis,

where the melanocytes are located, that melanin is produced, which gives the skin its color. (3)

The middle layer, located under the epidermis, is called the dermis. It contains blood and lymph vessels, hair follicles and glands that produce sweat, which helps regulate body temperature, and sebum, which helps keep the skin from drying out. These glandular fluids reach the skin's surface through the pores. (1,3)

The deepest layer is called subcutis, hypodermis or subcutaneous fat tissue. The subcutis is rich in fat and connective tissue, and some hormones are produced there. The most well-known is vitamin D, an essential vitamin made when the skin is exposed to sunlight.(1)

Exposure to ultra-violet (UV) radiation, especially UV-B, is the most common cause for genetic abnormalities in the skin.(4) UV radiation is both a mutagen and a non-specific damaging agent acting as tumor initiator and tumor promoter, therefore an important modifiable risk factor. Nonetheless, UV also benefits human health by mediating natural synthesis of vitamin D among others. (5)

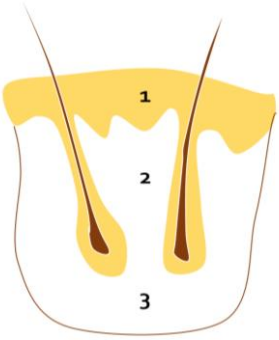


Figure 1. Representation of the layers of the skin.

1.- Epidermis
2.- Dermis
3.- Hypodermis/subcutaneous fat tissue

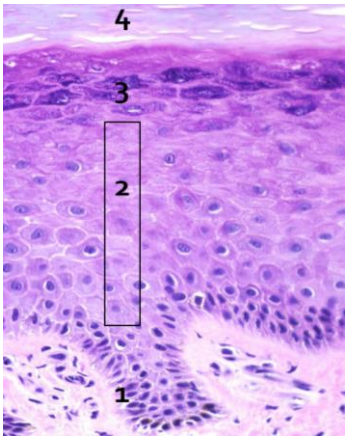


Figure 2.
Histopathological picture representing the epidermis division in levels also called stratums.

1.- Basalis
2.- Spinosum
3.- Granulosum
4.- Corneum

(6)

1.2. - Non-melanoma skin cancer

Incidence and epidemiology

Squamous cell carcinoma (SCC) of the skin is the second most common form of human cancer. Its incidence is quickly rising, and its mortality equals that of melanoma in some regions; therefore, it has become an important health problem.(7)

It is not easy to deal with epidemiology data, given that many national cancer registries (e.g. the US and UK) usually exclude skin cancers, even though some data can be found: skin cancers in the United States resulted in 80,000 deaths a year as of 2010, 49,000 of which were due to melanoma and 31,000 of which were due to non-melanoma skin cancers (NMSC).(8)

Cutaneous squamous cell carcinoma (cSCC) has an incidence of 16 per 100,000 people in Europe (9) and approximately 5% of them metastasize, usually to regional lymph nodes.(10) Lymph node metastasis is critical for survival, with 10-year survival rates below 20%. (11)

Non-melanoma skin cancers in white populations in Europe, the United States, Canada and Australia have had an average annual increase of 3% to 8% since the 1960s. This increase is reported in every single country where the records exist.(12) The exact number of people with basal cell carcinoma (BCC) and SCC is still unknown because these cancers are not specifically reported in most countries' cancer registries.(13)

1.2.1. - Actinic Keratosis

The term actinic keratosis (AK) describes a sun-induced lesion that clinically corresponds to an erythematous lesion covered with scales. There are several classification systems that have been used over the time and that correlate AK's pathological with the clinical features. The aim was to provide accurate information and enable correct decisions to be made regarding treatments and prognosis. These clinical descriptions have a histological diagnosis consistent with AK that is defined as an intraepithelial neoplastic lesion that may progress into iSCC. (14,15)

Clinical presentation

AKs present as erythematous, scaly, sometimes crusted papules and plaques on sun-exposed skin, mainly in the face, scalp, neck and upper extremities. A high percentage of these lesions are clinically indistinguishable from SCC. (16)

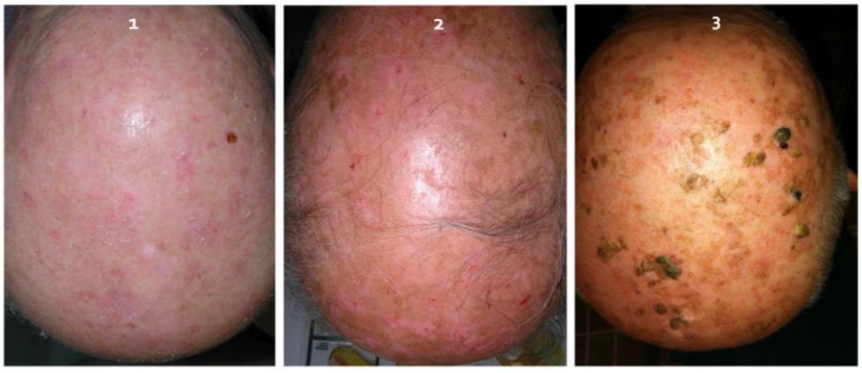


Figure 3.
1.- Slightly erythematous grade 1 AK lesions that are more palpable than visible.
2.- Moderately thick grade 2 AK lesions that are easily palpable and visible.
3.- Thick and hyperkeratotic grade 3 AK lesions that can be difficult to differentiate from the early stages of invasive SCC. Courtesy of Dr Ferrándiz Foraster.

Actinic keratosis can sometimes be pigmented, and lesions are usually less than 1 cm in diameter. The diagnosis of AK is usually made following clinical examination, at the early stages by palpation having a sand paper-like texture. Based on the current histological classification of these lesions, no clear guideline exists to make the clinical distinction between AK and SCC.(14)

No distinct clinical boundaries exist between AKs and SCCs. It has been reported that before AKs progress to invasive SCCs, they may become inflamed and painful.(17) It seems clear from the literature that AK and SCC are indistinguishable in the epidermal layer.(18)

There are some clinical parameters aiming to indicate those AKs at risk of becoming invasive. The established major clinical parameters are induration or inflammation, size more that 1cm in diameter, bleeding, rapid enlargement, erythema and ulceration. These parameters are combined with some minor criteria: pain, palpability, hyperkeratosis and pruritic or pigmented lesions.(19)

Although the diagnosis of AKs is based upon the typical clinical aspects, histological confirmation is necessary when doubts exist. A biopsy, which includes the dermis, can be required when deeper involvement is suspected. A dermoscopy can be helpful, even though other techniques like confocal scanning laser microscopy have reached a high evidence level for the diagnosis. Compared to histology, confocal scanning laser microscopy has reached a sensitivity and specifcity of nearly 98% and could be considered a non-invasive technique in the diagnosis of AK. (20)

Histopathology

Histopathologic analysis prevails as the gold standard for the diagnosis, although a variety of noninvasive optical methods may be useful for supplementing clinical information and assessing treatment efficacy.(21) Actinic keratosis is histologically characterized by atypical keratinocytes in the basal cell layer of the epidermis, which may extend into the entire epidermis in more advanced lesions. This goes with parakeratosis, alternating with hyperkeratosis. Solar elastosis in the dermis is always present and often goes with different degrees of lymphocyte and plasma cell infiltration. Acantholysis with suprabasal clefts can be identified.(14)

Actinic keratosis can be divided into different histological types: hypertrophic, atrophic, bowenoid, pigmented, acantholytic, lichenoid and proliferative, among others.(22)

The intraepidermal involvement by keratinocytic atypia it could be graded as mild (AK I), moderate (AK II) or severe (AK III). In grade I, atypical keratinocytes are found in the basal and suprabasal layer; in grade II, the atypical keratinocytes extend to the lower two-thirds of the epidermis; and in grade III, a full thickness of atypical keratinocytes covering the entire epidermis is found, which was previously called SCC in situ.(14)

Premalignant lesion or something else?

It was suggested that AK could be defined as a 'premalignant' process, owing to the low aggressive potential (23), but more recent literature proposed that AK corresponds to the in situ (intraepithelial) stage of SCC, with a similar genetic profile (14,24), and should therefore be renamed as intraepithelial neoplastic lesion. It was considered for some authors that AK could follow a similar classification system to the one

used in cervix pathology. (25) Many argued that these lesions that were previously labeled AK are actually part of a disease continuum which ends with invasive carcinoma.(14) There were some authors who suggested that AK as a form of intraepidermal keratinocytic neoplasia, describing them as the most common precursor of invasive SCC.(26–28) Other autors considered to use keratinocytic intraepidermal neoplasia (KIN), being more analogous to what happens in cervical pathology with the cervical intraepithelial neoplasia (CIN). In this scenario, one can grade AK as KIN I (focal atypia of basal keratinocytes of lower one third of the epidermis), KIN II (affection of at least the lower two thirds) and KIN III (involvement of the full thickness of the epidermis). It was described that the involvement of adnexal structures can be found in KIN II but specially in KIN III lesions.(29)

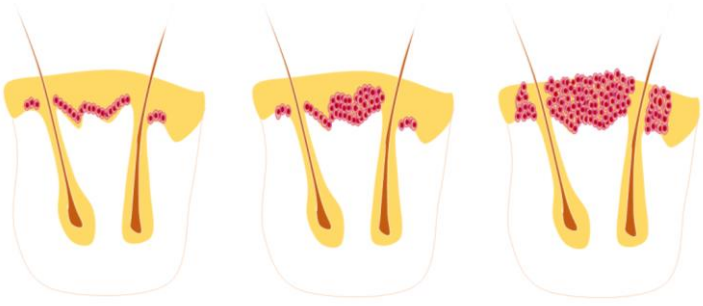


Figure 4. Schematic representation of an AK grade I, grade II and grade III.

Actinic keratoses present atypical keratinocytes, at least, along the basal layer of the epidermis showing enlargement, overlapping, hyperchromatic nuclei, lack of maturation, mitotic figures and dyskeratosis. The dermis itself presents signs of UV damage such as atrophy and solar elastosis.(30)

These new classifications take into consideration that AK is an early stage of cancer and that both AKs and SCCs are continuous processes

characterized by the proliferation of atypical keratinocytes and that both contain atypical keratinocytes with loss of polarity, nuclear pleomorphism, disordered maturation, and increased numbers of mitotic figures.(18)

It was found in one study that included more than 1,000 SCCs on sun-damaged skin that almost 100% of these lesions contained histopathologic changes of AK at the periphery.(31) Actinic keratoses and SCCs are thus frequently co-occurring.

Etiology

Ultraviolet B only penetrates the skin into the upper dermis. Epidermal stem cells and basal keratinocytes are susceptible to UVB-induced DNA damage but not the stem cells and basal keratinocytes that are located in the hair follicle bulge, because they reside in the deep dermis. This generates an umbrella-like appearance in the epidermis.(32) Actinic keratoses are mainly caused by non-ionizing radiation, especially through UV light associated with chronic sun exposure. UVA (320–400 nm) induces photo-oxidative stress and indirectly creates DNA mutations, while UVB (290–320 nm) irradiation directly results in the formation of cyclobutane (thymine) dimer in DNA and RNA. If the repair mechanisms fail, these DNA changes become the initiation of keratinocyte mutations which can develop into AKs.(33)

A mutated p53 tumor suppressor gene plays a key role in the pathogenesis in more than 90% of SCCs. Similar mutations have been found in AKs. Ultraviolet B causes a specific mutation in which cytosine (C) is changed to thymine (T). Proliferation control is lost once this occurs in the p53 gene.(4,34)
Following the two-hit theory of carcinogenesis, early exposure to UVB could be the first mutation, and subsequent UVB exposure in adult life may lead to additional, inactivating mutations on p53. It would then

seem likely that AKs may eventually progress to iSCC if they are left untreated.

In the US, 62% of AKs are found on patients over the age of 65. The annual frequency of AKs is expected to increase as population continues to age.(30,35)

Incidence and ratio of conversion

Cumulative exposure to UV radiation is the primary etiological factor in development of AK, thus increasing with age, lighter skin color, proximity to the equator and outdoor activity.(16,30) The real risk of progression from AK to iSCC is unknown, as the evolution of a particular lesion is unpredictable. It was found in a recent prospective study to be 0.60% at one year and 2.57% at four years (26), while others estimate it to be between 0.025–16%.(36) The risk of iSCC development has moreover been evaluated to be up to 53% per AK lesion and year for patients with a history of NMSC. One should nevertheless also consider that AK has a high rate of clinical regression, sometimes followed by relapses.(27) Using this data, it is suggested that the risk of AK progressing to SCC is approximately 10% in 10 years.(36,37)

There is a special group of high-risk AKs that occur in immunosuppressed patients, mainly organ-transplant patients. It is found that this group has a 250-fold higher risk to develop AKs and a 100-fold higher risk to develop iSCCs.(38,39) While only approximately 10% of immunocompetent patients with AKs progress into iSCC, this rate is up to 40% in immunosuppressed patients.(36,39) Other authors go further, suggesting that the progression between an AK and a subsequent iSCC will take place in approximately two years.(40)

In conclusion, the incidence of AKs is increasing, affecting millions of patients worldwide and becoming the most prevalent carcinoma in situ seen in humans.(41)

Actinic keratosis can develop into iSCC without treatment and has the potential to metastasize and cause death. Based on this clinical behavior, AK can be considered a malignant neoplasm. This progression is analogous to other malignancies, and it is also impossible to determine which AK will progress into iSCC.(14)

Treatment

AK can be treated with different methods comprising lesion- and field-directed therapies, because photodamaged skin frequently carries a significant burden of visible, subclinical and recurrent lesions. This gives physicians the possibility to select tailored treatment.(21)

There are no studies on the frequency and cost of AK treatment in Europe. A study from the US notes that destructive therapies are effective, remaining the standard of care in cost control.(35)

Primarily destructive therapies of individual AKs with surgery, cryosurgery, curettage or lasers will not prevent new cancers from emerging in adjacent areas with subclinical AK or areas of cancer field. Management strategies via the induction of a locally restricted, tumor-specific immune response, the use of phototoxic agents or the induction of apoptosis in dysplastic keratinocytes have the advantage of treating large areas in UV-exposed skin. Topically applied imiquimod, photodynamic therapy and diclofenac 3% gel are non-invasive alternative treatment modalities that are useful to treat larger areas or cancer field.(41)

Invasive procedures

These are ablative procedures that are mainly addressed to treat individual or single lesions but will not prevent new cancers from emerging in adjacent dysplastic tissues or cancerization fields.

Surgery

Excision is not routinely used for AKs and only chosen if iSCC is suspected with the need for a histological diagnosis.

Cryosurgery

A widely used and effective treatment for single AKs. Extensive cryosurgery over large areas (cryopeeling) can be used for treating cancer field.(42) Cryosurgery may be quickly performed and is cost-effective and well tolerated by patients. (43)

Curettage

Shave excision and curettages (removing tissue by scraping or scooping) are often used but can hinder the assessment of invasiveness; therefore, they are not acceptable treatments if SCC is assumed.(41,44)

Non-invasive procedures

These are topical procedures that are addressed to treat both individual lesions and the field.

Photodynamic therapy

Topical photodynamic therapy (PDT) acts through the selective destruction of atypical keratinocytes by means of light activation of a photosensitizer if oxygen is present. It is highly effective for AK.(45)

Topical 5%-fluorouracil (5FU) and imiquimod

5FU is a topical chemotherapeutic antimetabolite, and imiquimod is a member of the class of immune-response modifiers (a toll-like receptor 7- agonist). They stimulate the immune response through the induction,

synthesis and release of cytokines. Cellular immunity is increased as a result of this release of cytokines; therefore, it has an antineoplastic potency.(46,47)

A meta-analysis showed that both imiquimod and 5FU were effective methods for the treatment of AKs located on the face and scalp, with imiquimod seeming to have higher efficacy.(48) However, 5-FU is not as commonly used in clinical practice, because it is associated with more local side effects such as pigmentary changes or scarring.(41)

Imiquimod 5% effectively treats subclinical lesions as an additional benefit.(48) It is also not only effective in the treatment of AK but potentially prevents the development of SCC.(49) Similar to 5%, imiquimod 3.75% was able to highlight subclinical lesions reflected by an increase of AK counts after starting treatment in the majority of the patients. (50)

A study comparing imiquimod, 5FU and cryosurgery in patients with AK showed, respectively, 85%, 96% and 68% initial clinical clearance and 73%, 67% and 32% histological clearance in patients treated. The recurrence rate was significantly lower for imiquimod (after 12 months) than for the other treatments. The clearance rate of initially cleared individual lesions was 73%, 54% and 28% for imiquimod, 5FU and cryosurgery, respectively.(51)

Immune-response modifier resiquimod

Resiquimod is a toll-like receptor 7 and 8 antagonist, which has immunomodulatory effects similar to imiquimod. Resiquimod induces more IL-12 and tumor necrosis factors than imiquimod, targeting the activation of myeloid dendritic cells in addition to plasmacytotoid dendritic cells.(52) It has been suggested in the literature that resiquimod may have better efficacy than imiquimod in this regard.(53)

Diclofenac in hyaluronic acid gel

Diclofenac is a nonsteroidal anti-inflammatory drug that inhibits cyclooxygenase 2 and thus the upregulation of the arachidonic acid cascade and the production of prostaglandins. In addition to having the thoroughly known anti-inflammatory activities, nonsteroidal anti-inflammatory drugs also inhibit neoplastic cell proliferation by inducing apoptosis.(54)

Ingenol mebutate (PEP005)

Ingenol mebutate is a diterpene ester that disrupts the plasma membrane and mitochondria as well as inducing tumor-specific antibodies that lead to inflammation. Both mechanisms end in tumor cell destruction.(55) The current data support the applicability of ingenol mebutate for the short-course treatment of AK.(56)

Retinoids

Retinoids are mainly derivative molecules of vitamin A involved in the regulation of cell proliferation.(57) Topical retinoid treatment has shown some benefit in the prevention and treatment of AKs.(41) Systemic retinoids have been furthermore proposed for their potential to treat multiple AKs. It may be justified in high-risk patients and even as secondary prevention in organ transplant recipients.(58,59)

Prevention

The prevention of AKs plays a key role in AK-management. Patient education, UV-protection, self-examination and early detection of lesions is crucial.(60)

1.2.2. - Proliferative actinic keratosis

Proliferative AK is described as large (> 1cm) and with microulcerations reaching deeper portions of the dermis and therefore resistant to standard therapies and with higher propensity to develop iSCC. (22,61)

One study demonstrated that AK patients with follicular extension (presence of atypical keratinocytes extending into the hair follicle) were 1.8 times more likely to have a previous history of invasive carcinoma than those without follicular extension. It was also demonstrated that patients with follicular extension were 11 times more likely to have a previous history of invasive melanoma than those without follicular extension.(30)

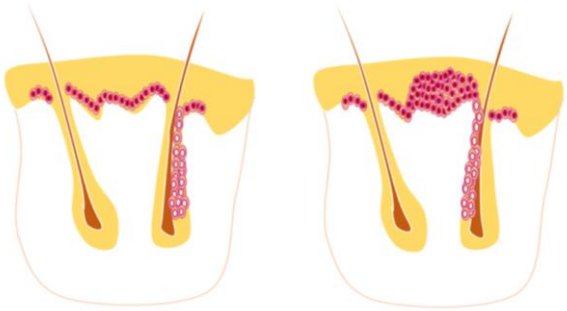


Figure 5. Schematic representation of proliferative AK.

It is well established that proliferative AK is resistant to standard therapies because of the deep migration of abnormal cells along hair follicles and sweat ducts and has a strong propensity to develop iSCC.(61)
It is proposed that patients who present with follicular extension should be treated more aggressively. The prognostic significance of follicular extension in AK can allow physicians to choose more appropriate therapies and provides justification for closer follow up.(30)

It is also relevant that hair follicle stem cells (HFSCs) can act as cancer cells of origin for cSCC and undergo defined cycles of quiescence and activation. It is described that HFSCs are not able to initiate tumors during the quiescent phase of the hair cycle, indicating that the mechanisms keeping HFSCs dormant are dominant to the gain of oncogenes (Ras) or the loss of tumor suppressors (p53). PTEN activity is, furthermore, necessary for quiescence-based tumor suppression, as its deletion mitigates tumor suppression without affecting proliferation, thus demonstrating that stem cell quiescence is a form of tumor suppression in HFSCs, and that PTEN plays a key role maintaining quiescence in the presence of tumorigenic stimuli.(62)

1.2.3. – Cancer field

Cancer field is a way to explain the mechanism by which second primary tumors develop due to a population of cells with early genetic changes. A large number of preneoplastic cells in the proliferating fields are likely to dramatically increase cancer risk.(63)

Tumorigenesis begins long before the growth of a lesion that could be detected clinically and sometimes even before any morphological trace of the pre-malignancy can be recognized. Cancer field is the consequence of the evolution of somatic cells in the body that results in cells carrying some but not all phenotypes required for malignancy. Cancer field is known as the underlying cause of many types of cancer in this sense.(64) This fits well with the fact that AKs rarely develop as solitary lesions; the most common presentation is as multiple lesions due to cancer field.(41)

A normal cell lineage can gain pro-tumorigenic genetic mutations or epimutations before the growth of a malignant lesion. These cells can be positively selected for in the microenvironment of an organ that can be otherwise considered healthy. The mutant lineage can consequently grow to produce large patches, or fields, of cells that are eventually predisposed to progress into a neoplasm. A cancer that emerges from a field will moreover have passed through different phenotypical states, whereas the lineage leading to a sporadic tumor may not have had any phenotypic changes before cancer happens.(64)

It is useful to clarify that one should understand a pre-malignant disease as a morphological and discernible condition, while a cancer field is a gain on some mutations but not all the phenotypic alterations required for malignancy, exhibiting morphological changes or not. Dysplasia implies cells with abnormal and neoplastic morphology or disposition.

Cancerized phenotypes are not always obvious; they are sometimes even ephemeral. A mutation of *TP53* (which encodes p53) in the skin provides a survival advantage (a cancerized phenotype), but this phenotype is evident only in response to ultraviolet exposure.(65)

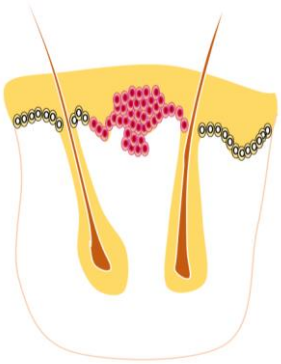


Figure 6. Schematic representation of a cancerization field (sides) including AK with iSCC (center).

It is important to note that a sporadic tumor does not share its tumorigenic mutations with surrounding tissue; for instance, the mucosa around the tumor is morphologically and phenotypically normal. On the contrary, a tumor growing in a cancerized field arises from a malignant cell that initially shared tumorigenic mutations and a concomitant-altered phenotype with the surrounding tissue. The malignancy developed in the field due to additional mutations with possible phenotypic change in an evolving microenvironmental context.(64) Mutational diversity existing within the cancerized field under natural selection will nonetheless cause the most adaptive clone to emerge as the dominant.(66)

The literature also describes the role that stromal cells may play in promoting cancer field. For instance, it is known that epigenetic modification of fibroblasts in the skin are induced by UV exposure, leading to the production of diffusible growth factors, inflammatory cytokines and matrix-remodeling enzymes predisposing to cancer. However, the stromal compartment alone is unable to cause a tumor, even though it can provide selective pressure for particular epithelial genotype-phenotype combinations.(67) It is shown that loss of *NOTCH1* in the epidermal compartment of mice induces spontaneous tumors in the same way, suggesting direct influence from the stroma.(68)

1.2.4. - Squamous cell carcinoma

An SCC is a malignant neoplasm of epidermal keratinocytes. The atypical keratinocytes show a lack of maturation and display eosinophilic and sometimes pale or vacuolated cytoplasm. It is common to find whorls of parakeratosis within aggregations of neoplastic cells, also known as horn pearls. The nuclei are pleomorphic, hyperchromatic and with tendency to be crowded and have an increased number of mitotic figures. Parakeratosis and orthokeratosis can be seen superficially. The cytopathological cellular changes between an AK and an iSCC are indistinguishable. In that sense, it is not possible to tell where AK ends and SCC begins, and it is for this reason that AK is considered an early SCC in situ.(14)

Cutaneous squamous cell carcinomas have the potential to metastasize; they initially do this to regional lymph nodes and then to distant sites. The rate of metastasis in cSCC has been estimated up to 2–5%. Even though these are low percentages compared to other malignancies, the existence of distant metastasis is associated with a grim prognosis and a median survival expectancy of less than two years.(56)

Epidemiology

Cutaneous squamous cell carcinoma is the second most common form of non-melanoma skin cancer, representing 20% of all cutaneous malignancies.(56,69) However, the epidemiological data can be questionable due to the non-systematic record of cases in registries, and the fact that they often mix strictly cutaneous with mucosal SCC. The exact incidence of cSCC is still unknown considering this information. Despite this, some articles suggest that the incidence of cSCC has increased over the past 30 years by 50–200%, with

stabilization trends and sometimes the implications in public health being underestimated.(7,70,71)

In addition, according to Australian registries, the overall incidence rate of cSCC in 2002 was estimated to be 387 cases per 100,000 people. In the US, 2.2 million people were reportedly treated for NMSC in 2006, of which 600000 cases were SCCs. Another, more recent study showed that 3,900–9,000 patients died from cSCC in 2012. It is also published that in the central and southern United States, deaths caused by cSCC were as common as deaths from oropharyngeal carcinoma or melanoma.(7) In Sweden, population-based studies demonstrated that age-standardized incidence rates are rapidly increasing. An increase of approximately 2,000 new SCC cases was found annually in populations of 4.5–9 million inhabitants.(72)

Etiology and pathogenesis

The most important risk factors for cSCC are sun exposure, advanced age and UV-sensitive skin. Considering these factors, the strongest environmental risk factor for cSCC development is cumulative chronic UV radiation exposure, which explains why the incidence increases dramatically with age.(73) It is also known that tumors occur on anatomical areas with chronically UV exposure like the head and neck region and the dorsum of the hands in 90% of cases. For instance, cSCC is more frequent in patients working outdoors and at lower latitudes where the ambient light intensity is higher.(74)

Some articles suggest that cSCC is a tumor even more susceptible to UV radiation than melanoma, especially with chronic exposure.(56) The role of environmental factors is greatly facilitated by genetic elements. For instance, skin phototypes I and II (less pigmented skin) predispose sensitivity to chronic UV radiation exposure and are correlated with a high incidence of cSCCs.(75) All immunosuppressive agents have a

negative impact on this risk. It has been found that iatrogenic immunosuppression in organ transplant recipients is associated with a 65 to 250-fold increased risk for developing cSCC compared with the general population.(76)

The most common mutations found in cSCCs are those that affect the tumor suppressor gene p53.(77) p53 is commonly mutated in AKs and SCCs in situ, indicating that p53 loss occurs prior to the invasive stage. One role of early p53 mutations in SCCs is resistance to apoptosis, allowing to a clonal expansion of keratinocytes. The majority of p53 mutations are localized opposite pyrimidine dimer sites and likely derive from UVB exposure.(78)

Other genetic alterations in cSCCs include aberrant activation of EGFR and Fyn downregulating p53 (79), activating Ras mutations (80) or loss of function mutations in NOTCH receptors.(81)

Clinical presentation

The typical clinical appearance of invasive cSCC is an AK that becomes hyperkeratotic or that acquires an indurated base. These lesions can also sometimes become tender or ulcerated. Even though the majority of cSCCs will arise in the context of AK, the rate of transformation of AKs into invasive cSCC is apparently low.(27,75) In some cases, a cSCC can arise *de novo* or the early keratosis phase is lacking, thus presenting as an asymptomatic small plaque or nodule that enlarges over time.(56)

Cutaneous squamous cell carcinoma mainly occurs in chronically exposed areas, among them the face (especially the lips, ears, nose, cheeks and eyelids) and the dorsum of the hands. The head and neck region are the most frequent site of affection in males, while it is the upper limbs in females. Even though dermoscopic methods can be

helpful in the diagnosis, a biopsy or excision is still the gold standard and is required for a definite diagnosis.(56)

It is important for a differential diagnosis to be made between SCC and a keratoacanthoma, which is a less aggressive lesion that can simulate a cSCC. It presents as a rapidly growing, dome-shaped nodule with a central keratin plug and a crateriform appearance that sometimes resolves spontaneously. It is often easy to recognize histologically by a well-defined, follicular-centered proliferation with abrupt limits and inflammation.(82)

Pathological variants of cSCC

There is a useful cSCC classification system based on histologic subtypes and biological potential derived from studies of their aggressiveness and metastatic behavior. The categories proposed are low (≤2% metastatic and death rate), intermediate (3–10%), high (>10%) and indeterminate (insufficient data to accurately determine) malignant potential.

Variants of SCC classificated into risk-based (malignant potential) categories			
Low	**Intermediate**	**High**	**indeterminate**
SCC arising in AK	Acantholytic	Invasive Bowen´s disease	Clear cell
Verrucous and HPV-related	Lymphoepithelioma-like	Adenosquamous	Signet ring
Spindel cell (with no radiation)		Desmoplastic	Papillary or follicular
Tricholemmal		De novo	Pigmented
		Arising in chronic conditions, Radiation induced or arising from pilar tumors	Arising from adnexal cysts

Figure 7. Adapted from Cassarino DS. (25,83)

There are several factors involving an increased risk of metastasis, such as poorly differentiated lesions, immunosuppression, thickness greater than 4 mm, desmoplasia, horizontal size greater than 2 cm, location in the ears and lips, recurrent tumors and rapid growth.(84)

Diagnostics

The initial diagnosis of cSCC is based on clinical features. A biopsy or excision with histologic confirmation is mandatory in all clinically suspicious lesions in order to know the prognostic values to achieve a correct management.(56) For this, the pathology report should include the histologic subtype, grade of differentiation (well-differentiated, moderately differentiated, poorly differentiated or undifferentiated grade); tumor depth; level of dermal invasion (Clark's level); presence or absence of perineural, lymphatic or vascular invasion; and whether margins are free or involved by tumor cells.(85)

Tumor stage (T-stage) and grading

Staging systems for cSCC (TNM/UICC 2009; AJCC 2010) were considered not optimal and therefore, removed. They have been developed for head and neck tumors, and there was a lack of validation or adequate prognostic discrimination in certain stages with heterogeneous outcome measures. In addition, the Cancer Protocol Templates from the College of American Pathologists are now retired from the net; however, previous systems can be consulted. One of them is the anatomic level following Clark's levels: I, carcinoma in situ; II, carcinoma present in but does not fill and expand papillary dermis; III, carcinoma fills and expands papillary dermis; IV, carcinoma invades reticular dermis; and V, carcinoma invades subcutaneum.

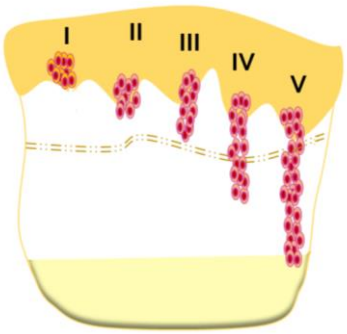


Figure 8. Schematic representation of Clark´s levels.

Another method is to consult previous classifications in order to obtain information about how to grade a tumor; for instance, consulting the College of American Pathologists;

- Gx: Cannot be assessed.
- G1: Well-differentiated: Tumors characterized by squamous epithelium that frequently show easily recognizable and often abundant keratinization. Intercellular bridges are readily apparent. There is minimal pleomorphism, and mitotic figures are mainly basally located.
- G2: Moderately differentiated: Tumors characterized by more structural disorganization in which squamous epithelial derivation is less obvious. Nuclear and cytoplasmic pleomorphism is more pronounced, and mitotic figures may be numerous. Keratin formation is typically limited to keratin pearls, horn cysts, and scattered individually keratinized cells.
- G3: Poorly differentiated: Tumors in which is difficult to establish the squamous differentiation, usually by identification of rare intercellular bridges or small foci of keratinization.
- G4: Undifferentiated: Used to denote anaplastic or undifferentiated tumors.

According to the previous AJCC 7th edition, cSCCs from head and neck were classified (staging system) as (86);

TX Primary tumor cannot be assessed
To No evidence of primary tumor
Tis Carcinoma in situ
T1 Tumor ≤ 2 cm in greatest dimension with < 2 high-risk features
T2 Tumor > 2 cm in greatest dimension with or without one additional high-risk feature, or any size with ≥ 2 high-risk features
T3 Tumor with invasion of maxilla, mandible, orbit, or temporal bone
T4 Tumor with invasion of skeleton (axial or appendicular) or perineural invasion of skull base.

Recently has been printed the 2018 World Helath Organitzation Classification of Tumours corresponding to the skin tumors where the SCC of the head and neck are classified as;

TX Primary tumor cannot be identified.
To No evidence of primary tumor
Tis Carcinoma in situ
T1 Tumor ≤ 2 cm in greatest dimension
T2 Tumor > 2 cm and ≤ 4cm in greatest dimension.
T3 Tumor >4cm in greatest dimension or minor bone erosion or perineural invasion or deep invasion
T4a Tumor with gross cortical bone/maroow invasion
T4b Tumor with skull base or axial skeleton invasion including foraminal involvement and/or vertebral foramen involvement to the epidural space.

The pT categories correspond to the clinical T categories. This classification exclude several locations like eyelid, perianal, vulva and penis. (15)

Treatment

The gold standard for the treatment of cSCC is complete surgical excision with histopathological control of excision margins. Surgical removal enables histologic confirmation of the diagnosis and crucial information about surgical margins. Cure rates are estimated around 95%.(87)

Some patients present cSCC surrounded by areas with multiple AKs or large areas of cancer field. In those cases, a number of blind destructive modalities can be applied like cryotherapy, curettage or photodynamic therapy and others like topical agents (imiquimod 5% and 3.75%; 5-fluorouracil 0.5%, 1% and 5%; diclofenac 2.75%; ingenol mebutate 0.05% and 0.015%) in order to prevent second primary tumors arising from other AKs or from the cancer field.(88)

Radiotherapy represents an alternative to surgery and could be considered a primary or adjuvant treatment for inoperable cSCC.(89)

Stage IV cSCC can be responsive to various chemotherapeutics, but there are no established protocols. Targeted therapies using EGFR inhibitors such as cetuximab are currently approved for the treatment of head and neck SCC in the metastatic stage. The activation of EGFR has been observed in cSCC and is associated with a worse prognostic outcome.(90)

Prognosis

The prognosis for the vast majority of patients who suffer cSCC is excellent, with an overall five-year cure rate greater than 90%. These numbers are much better than other SCCs of the head and neck area. There is a study including more than 900 patients with cSCC that were followed for 10 years that demonstrates a 4.6% rate of recurrence, 3.7% for nodal disease and 2.1% of disease-specific death.(91)

The metastatic risk for cSCCs is low in the majority of the cases and never exceeded 2–5% over a five-year follow up period.(92)

1.3. – Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a crucial process for organizing the cell layers during embryogenesis in wound healing and also in cancer progression.(11,93)

It is defined by a functional loss of E-cadherin, among many others. The epithelial tumor cells acquire traits of motile mesenchymal cells and therefore increased capacity of invasion and vascular dissemination. This phenomenon is called epithelial-to-mesenchymal transition. The process can be reversed and is common once the tumor cells have implanted in the metastatic organ. This phenomenon is called mesenchymal-to-epithelial transition.(94)

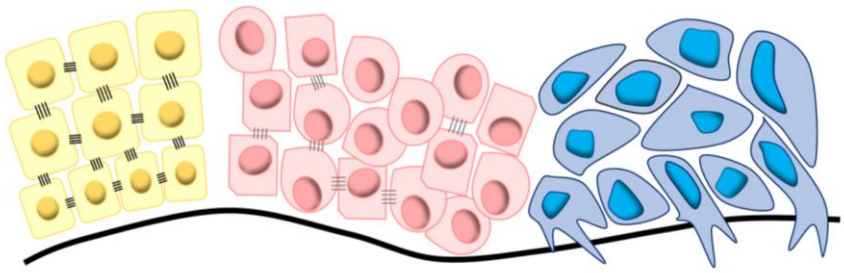


Figure 9. Schematic representation of epithelial (left) and mesenchymal (right) profile.

The most remarkable event in this process happens at the beginning where an epithelial cells adquire a mesenchymal fenotipe that can migrate from the epithelium where it originated. There are many molecular processes involved in making this possible. These molecular processes include the expression of specific cell-surface proteins, activation of transcription factors, reorganization of cytoskeletal proteins, production of degrading enzymes and changes in the

expression of microRNAs. All of these molecular steps can be used as biomarkers, demonstrating the passage of a cell through an EMT.(95)

Three different biological subtypes of EMT are described.(96)
Type 1 is associated with zygote implantation, placenta formation and embryogenesis (97), while type 2 is associated with tissue repair and fibrosis.(98,99) Type 3 occurs in neoplastic cells with previous genetic anomalies that favor clonal localized growth. Once the EMT machinery comes into play, tumor cells acquire new capabilities like invasion and therefore metastatic power. This is a heterogeneous and multi-step process, because not all cells undergo EMT; therefore, they can retain many epithelial traits, while others acquire a true mesenchymal profile. It remains unclear which and where the signal comes from to induce such EMT in tumor cells.(95)

The activation of an EMT program is proposed in some articles as the critical point for the acquisition of malignancy by epithelial cancer cells. These are the cells that are characteristically seen in the invasive front and are eventually more susceptible to metastasizing.(100)

Downregulated E-cadherin expression is seen during this process due to the action of several EMT-associated transcription repressors (such as members of the Snail, Twist and Zeb families). The gain of mesenchymal markers, like vimentin, is promoted at the same time. Podoplanin is also a protein related to contractile properties that is associated with the mesenchymal phenotype.(11,100)

2. - Main Investigative Tools

2.1. - Immunohistochemical biomarkers

A biological marker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological and pathogenic processes or pharmacologic responses to a therapeutic intervention and can be prognostic as well as predictive of therapeutic response.(101,102)

Immunostains are a common laboratory test that uses antibodies to test for certain antigens in a sample of tissue. The antibodies are usually linked to an enzyme or a fluorescent dye. When the antibodies bind to the antigen, the enzyme is activated and can be seen under a microscope. Immunohistochemistry (IHC) is routinely used for diagnostic purposes, especially to differentiate types of cancer and their possible origins.(103)

Although the cure rate for cSCC is high, the wide spectrum of presentations that this kind of tumor can have hinders an early diagnosis, and some types are aggressive and associated with poor prognosis. It is therefore important to find molecular or IHC markers to diagnose such aggressive subsets and deliver more optimal therapy.(104)

The following markers are used in this thesis:

p63

Tumor protein (TP) 63 was discovered as TP53's homolog many years ago and has become crucial as a regulator of skin development, proliferation and stem cell maintenance. Tumor protein 53 is the most mutated gene in human cancer, while TP63 is less ubiquitous. All TP53's family members, including TP63, function as transcription factors to regulate cell cycle arrest, stem cell renewal, apoptosis, senescence or autophagy. They are also involved in metabolism through the activation and repression of downstream target genes or proteins.(105)

Tumor protein 63 is not often mutated in cancer, even though it can be overexpressed in multiple human epithelial cancers. However, it is frequently mutated in heritable skin disorders like in ectodermal dysplasia.(106)

Tumor protein 63's proteins exercise their functions through transcriptional regulation by binding to specific gene targets; many of them are involved in the control of the skin's epithelial architecture and aging. They are also involved in its cellular response to UV radiation. With all of this, TP63 contributes to the pathogenesis of several genetic diseases of the skin and epithelial tissues.(105) It is also known that interactions between NOTCH and TP63 are involved in the balance between keratinocyte self-renewal and differentiation.(107)

p53

p53 is a DNA binding protein and also a transcription factor that controls the expression of a vast number of genes. It has been called the guardian of the genome due to its ability to block cell proliferation when DNA is damaged.(108,109)

p53 is the most commonly mutated gene in all types of cancer. A mutation in the p53 gene increases protein stability in some way; therefore, strong IHC expression indicates that it is mutated, although not every single one of all mutations can be detected using IHC.(110,111)

Mutations are always an early event in carcinogenesis and in this case start at the cancer field and AK, knowing that TP53 is frequently mutated in SCC. Particularly, neovascularization in early stages and proliferative activity in the invasive stage are both associated with p53 immunoexpression.(112)

Furthermore, p53 performs a wide variety of tumor surveillance functions, correcting DNA damage caused, for instance, by external physical agents like UV radiation.(113)

Ki67

Ki67 is a nuclear protein that is expressed in all active phases of the cell cycle except in resting cells. It is currently standard to use Ki67 as an index of cellular proliferation.(114,115)

Ki67 has thus been incorporated into plenty of clinical protocols as a prognostic marker for several types of cancer where an association between high proliferation rates and poor prognoses exists.(116)

p16

p16 is an established marker of cervical dysplasia and carcinoma arising from high-risk human papillomavirus (HPV) infection. Increased p16 expression can be found in squamous neoplasms from other sites—in

head and neck carcinomas, for instance, as well as in carcinomas arising from the oropharyngeal tract.(117)

Increased p16 expression is found in HPV-associated squamous lesions. Using IHC techniques, one can corroborate those cases that exhibit strong diffuse nuclear positivity with high-grade lesions; therefore, a close correlation exists between the stain and the degree of dysplasia.(118–120) This increased p16 expression in the infected cells comes from the neutralization of cellular p53 and pRb tumor suppressor proteins by HPV E6 and E7 oncogenes.(121,122)

Given that low-risk HPV subtypes do not exhibit this p16 positivity, as they produce much less E6 and E7 viral oncogenes, p16 expression is commonly employed as a surrogate marker of high-risk HPV infection.(117,123).

β-catenin and E-cadherin

This is a family of transmembranous glycoproteins called cadherins that mediate calcium-dependent intercellular adhesion. At least 10 different subtypes exist; among them, E-cadherin is especially expressed in epithelia and plays a key role in the maintenance of epithelial integrity.(124)

E-cadherin is the principal component of the adhesion molecules, because it anchors intercellular junctions. The cytoplasmic domain of E-cadherin interacts with groups of cytoplasmic protein called catenins, among them β-catenin. Both contribute to the formation of adherens junctions in association with the actin cytoskeleton.(125)(126)

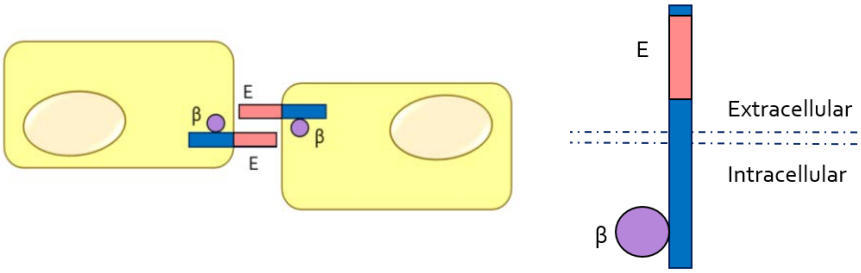


Figure 10. Schematic representation of E-cadherin and and β-catenin.

Given that tumor cells exhibit reduced intercellular adhesion, it is proposed that this cadherin-catenin complex can play a critical role in the pathogenesis of carcinomas.(124,125,127)

There are several studies that demonstrate an abnormal or reduced expression of one or both E-cadherin and β-catenin in various types of carcinomas.(124) The reduction or absence of E-cadherin was related to the loss of differentiation, invasion and the metastatic behavior of tumor cells in some cases.(128,129)

Vimentin

Vimentin is a type III intermediate filament and is responsible for the cytoplasmic architecture.(130) Vimentin is strongly related to the EMT as a marker. In that sense, an overexpression of vimentin in cancer cells correlates with cancer progression. Apart from the intracellular functions, there is a second possibility or function: the recruitment of vimentin to the cell surface. This surface recruitment is identified as a participant in cell adhesion, migration and cellular signaling.(131)

Its role related to EMT is to enhance migration and invasiveness; therefore, vimentin overexpression can be related to aggressiveness for cancers with epithelial origins.(131,132)

D2-40 (Podoplanin)

Podoplanin (PDPN) is a transmembrane mucin-like protein. There are several articles that relate PDPN to tumor cell invasion. It is published that PDPN can be a chemotherapeutic target for primary or metastatic cancers—oral SCC in particular.(133)

Podoplanin is found at the invasive front of many tumors, and this is consistent with its role in promoting invasion.(134) The expression of PDPN is also demonstrated to be upregulated in several human SCCs, being involved in enhanced cell migration and invasion.(135)

PTEN

Phosphatase and tensin homolog (PTEN) is found mutated or deleted in many tumors. PTEN/PI3K pathway activation is suggested to be involved in the metastasis and poor prognosis in some tumors (136) but has also been related to anti-cancer targeted therapies.(137)

Immunohistochemistry can be an effective method to demonstrate PTEN loss. Nevertheless, some authors have found variability and poor reproducibility with different antibodies and techniques.(136)

Stem cells usually exhibit a high proliferative capacity but remain quiescent in comparison to their descendant progenitor cells. Hair follicles can be found either in anagen, where the follicle is completely formed, or in telogen, where the follicle is in a quiescent, or resting, state. Hair follicle stem cells rarely divide during either telogen or full

anagen but undergo proliferation at the start of anagen state. Hair follicle stem cells by themselves were found to act as SCC cancer cells of origin using inducible, cell-type specific, genetically defined mouse models. In these experiments, it was found that tumorgenesis only begins when HFSCs are released from quiescence during the transition between telogen to anagen. Moreover, it was demonstrated that PTEN activity was necessary for quiescence-based tumor suppression, because its deletion alleviates tumor suppression without affecting proliferation. Therefore, quiescence is a form of tumor suppression in HFSCs, and PTEN plays a role in maintaining this quiescence when a tumorgenic trigger is present.(62,138)

MMP (MMP1 and MMP3)

Matrix metalloproteinases (MMP) are proteases involved in the modulation of the local environment and are also known as the degradome. There is an association between them and the invasive potential of the tumor cells.(139,140)

Matrix metalloproteinases are differentially expressed in cSCC compared with normal skin samples. They are specifically zinc-dependent endopeptidases that act on the extracellular matrix by degrading it and therefore facilitating the migration of the tumor cells.(104) Besides their role in the invasiveness, they are also associated with the early steps of carcinogenesis such as angiogenesis and apoptosis.(141)

CD31

This is a cell adhesion molecule also known as a platelet endothelial cell adhesion molecule. It is often used by pathologists as a specific endothelial marker and can be used to measure microvessel density regarding tumoral angiogenesis. It has been significantly associated with dissemination and therefore provides information about prognosis. Many techniques have been developed to study and quantify angiogenesis. There is consensus that the assessment of angiogenesis with the Chalkley method associated to CD31 or CD34 stainings is a safe choice.(142–146)

2.2. - Tissue microarray investigation

A tissue microarray (TMA) is a paraffin block containing multiple donor tissue cores. The cores, usually 0.6–2 mm in diameter, are taken from different formalin-fixed, paraffin-embedded parts of a tumor or from different tumors or tissues. Sections are cut from the TMA block and subjected to examination, thus enabling high-throughput simultaneous in-situ detection of DNA or protein expression using only a fraction of antibody and tissue material compared to analyzing full-face tissue sections.(147) Since its introduction in 1998, this technique has become an important tool in biomarker research.(148) One caveat concerning this technique is that because many tumors are heterogeneous, very small tissue samples may not always reflect the biological properties of the entire tumor.(149) This can, at least in part, be compensated for by using more than one tissue core from each tumor, ideally from different areas of the tumor, and by verification of the TMA results by analysis of larger tissue specimens before clinical application.(147) Even with the use of full-face sections, sampling bias is not excluded, as these also represent only a limited fraction of the tumor. An advantage of the TMA approach is the great number of tumors that can be studied simultaneously, conceivably might conceivably compensate for false-negative or false-positive tissue cores.(150)

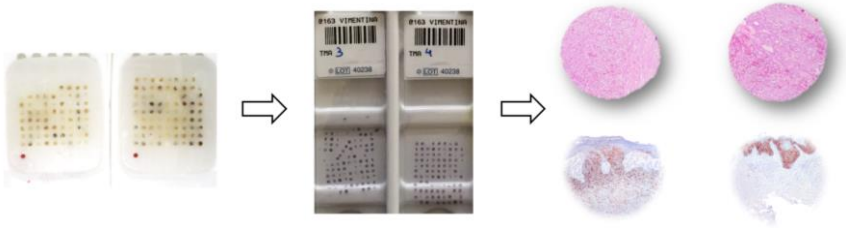


Figure 11. Schematic steps illustrating the construction of a TMA.

2.3. - The microRNA CISH-technique

In situ hybridization (ISH) technology allows for the direct quantification of gene copy number per nucleus by the use of a DNA probe labeled with a fluorescent, chromogenic or silver detection system—fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) or silver in situ hybridization (SISH), respectively—complementary to the target DNA sequence.(151)

miRNAs (micro-RNA) are small, non-coding RNAs, evolutionally conserved, working as post-transcriptional regulators of gene expression. More than 1,200 miRNAs have been identified in the whole human genome, which regulate the codification of more than 30% of genes. This explains why miRNAs play a critical role in every biological process, including cell proliferation, differentiation and apoptosis.(152)

In situ hybridization-based miRNA detection can provide assessments of the physiologic function of miRNA at spatial location and the single-cell level.(153) miRNA ISH has also some disadvantages, however, especially a strong background and absence of detecting signal. Both can make the assessment quite difficult.(152) Tissue microarrays are widely used in molecular pathogenesis cancer research.(147) With TMAs, the expression of multiple targets on the RNA or protein level can be examined at once. Unlike mRNAs (messenger RNAs), miRNAs are relatively stable and tolerate molecular cross-linking caused by formalin used in the fixation of routine surgical samples. This makes TMAs a powerful tool in miRNA research.(154,155) In situ hybridization techniques also provide crucial information about the spatial localization of miRNAs at a cellular level and therefore inform on their physiologic function.(153)

miRNA expression is highly tissue-specific, describing distinct profiles for different cancer types.(156,157) In that sense, miRNAs show a heterogeneous functioning with several types of aberrant expressions. Some miRNAs have shown to be upregulated, acting as oncogenes, whereas others have been found downregulated, acting as tumor suppressors.(158,159) For instance, miRNA-21 is one of the most studied miRNAs, and it is overexpressed in a significant number of solid tumors.(160)

The deregulation of miRNAs has also been directly associated with metabolic diseases (161), cardiovascular disease (162) and even neurodegenerative disorders. (163)

One of the strengths of studying miRNAs is that emerging evidence suggests that they will soon be used as biomarkers and molecular targets for novel therapies.(152)

miRNA-31 and miRNA-21

miRNA-31 is found upregulated in cSCC, acting as an oncogene facilitating cell proliferation and invasion, and it is proven that this upregulation appears in mice skin following acute UV exposure.(164,165)

miRNA-21 was also found upregulated in cSCC, as in other cancer types, while normal levels where found in normal skin samples.(166,167)

3. - Aims of the Thesis

This thesis presents a range of studies with the shared aim to understand the progression pathways of SCC associated with actinic damage.

The specific aims of the separate studies are:

I. To demonstrate that invasive squamous cell carcinomas can be originated from actinic keratosis with transformation limited to the basal layer of the epidermis. This pathway is known as differentiated in the oropharynx and anogenital area, where it had been already described.

II. To evaluate the percentage of invasive squamous cell carcinomas originated from actinic keratosis following the differentiated pathway in contraposition to those that followed the classical pathway, requiring complete or almost complete transformation of the epidermis before being able to invade.

III. To confirm the results of the initial study performed in cases from the Hospital Universitari Germans Trias i Pujol (Spain), in a series of cases from the Hospital of Lund (Sweden). To perform this study, the presence actinic elastosis was used as a demonstration of solar damage as etiological factor.

IV. To build several tissue microarray to investigate the mechanisms underlying the classical and different pathways. For each tumor, the tissue microarray had to contain (whenever

it was possible) up to 3 cores representative from (1) the superficial portion (2) the advancing edge corresponding to the deep portion of the tumor (3) areas of follicular extension of the tumor and (4) non-tumoral epidermis at the edges of the neoplasia.

V. To investigate the differences in the expression of p53 and p16 in both pathways, known to be differentially expressed when it comes to anogenital tumors, using the tissue microarray.

VI. To search for differences in the expression of molecules related to epithelial to mesenchymal transition in both pathways (Vimentin, podoplanin, e-cadherin, beta-catenin), using the tissue microarray.

VII. To search for differences in the proliferation in both pathways using the proliferative marker Ki67 in the tissue microarray.

VIII. To search for differences in the miRNA-31 and miRNA21 in both pathways by in situ hybridization in the tissue microarray.

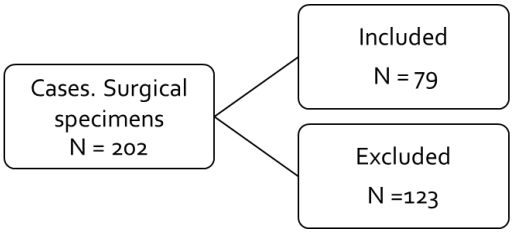
IX. To investigate the role of follicular extension of actinic keratosis in the development and aggressivity of invasive squamous cell carcinomas.

4. - The Present Investigation

4.1. - Material and methods

4.1.1. - Study 1

Patients



Main inclusion criteria	Main exclusion criteria
Sun exposed	Curettages or fragmented biopsies
>3mm	Re-excision or large ulceration
iSCC	Keratoacanthomas

Figure 12. Patients and criteria in Study 1.

Cases of cutaneous iSCC from the files Hospital Universitari Germans Trias i Pujol's pathology department, corresponding to three consecutive years, were reviewed. Of these, 503 cases fulfilled the criteria established for inclusion in the study, namely sun-exposed skin biopsies measuring over 3 mm and containing invasive tumors smaller than 25 mm in diameter. Some of these biopsy specimens corresponded to fragmented curettages, and the features of the overlying epidermis could not be evaluated. Lesions with extensive epidermal ulceration or detachment (more than 50% of the epidermis overlying the tumor), re-excisions with superficial scars and iSCC presumably originating from keratoacanthomas were also excluded. Finally, 196 cases with adequate sections were selected for further evaluation. They were assessed on four main points: the type of AK over the iSCC, the type of AK on the edge of the iSCC, the presence of ulceration over the iSCC and the extension along the adnexal epithelium. The thickness of the epidermal proliferation of atypical keratinocytes overlying the tumor and at its edges was studied independently by three pathologists (Xavier Sáenz, Cristina Carrato and Maite Fernández Figueras). For cases with different evaluations, the final score was agreed after study under a multiheaded microscope.

AK I was assigned to lesions with atypical features (keratinocytes with nuclear pleomorphism and hyperchromasia, increased mitotic rate, crowding and overlapping) limited to the epidermal basal layer or lower third, AK II to lesions with atypical features involving the epidermal lower two thirds, and AK III to lesions with atypical features from the base to the upper third of the epidermis. In cases where several degrees were present, the highest degree was scored, even though it was not the most extensive at times. All cases containing pagetoid areas corresponded to AK III lesions and were classified as such.

We recorded the extent of the proliferating atypical keratinocytes' migration down hair follicles and sweat ducts. Such migration of atypical keratinocytes with nuclear pleomorphism, hyperchromasia, increased mitotic rate, crowding and overlapping is considered to be the hallmark of so-called proliferative AK. Ulceration, mean width of iSCC areas, anatomic level of infiltration and maximum depth of infiltration were also evaluated.

4.1.2. - The Lund project

There are several factors that contribute to the development of AK such as geographical factors (like altitude and latitude), increased vacational and recreational sun exposure, a history of severe sunburns in childhood and a sensitive skin phototype.(168)

Systematic studies of the prevalence of both the classical and differentiated pathways in iSCC had never been performed before this thesis project was initiated. We believed it therefore valuable to reproduce the same scheme made in first study.

During my stay as prespecialist senior resident in the Department of Pathology at Lund University Hospital, we worked and extended the study of the origin of these tumors. With that aim, we revised 202 extra cases of iSCC in sun-exposed regions from the department archives of (Lund University Hospital). All cases were from 2015. Seventy-nine met the inclusion criteria previously established following those already decided in the study 1. We systematically noted the patient's age and gender and the tumor's level of anatomical infiltration and histological grade, presence of extension of AK through the adnexal structures, cutaneous ulceration on the surface and hypertrophic features.

We included a study about solar damage based in the percentage of solar dermal elastosis, since Sweden is not a country with intense and chronic sun exposure like the south of Europe. We graded according to the average of dermal affection (<33%, 33-66% and >66%).

4.1.3. - Study 2

All cases of cutaneous iSCC from the Hospital Universitari Germans Trias i Pujol pathology department's files from 2005 to 2007 were reviewed. Eighty of these specimens contained enough tissue to allow the TMAs creations to be selected. The degree of dysplasia in the epidermis overlying the tumor was evaluated by the same three pathologists as in study 1.

Cases of iSCC showing recognizable epidermis on their surface with dysplasia limited to the epidermal basal layer or the lower third of the epidermis were considered to have originated from AK I and thus through the differentiated pathway (DP). Invasive tumors with unrecognizable epidermis, with dysplasia involving the epidermal lower two thirds (AK II) or with dysplasia from the basal layer to the upper third of the epidermis (AK III), were conversely considered to have originated through the classical pathway (CP).

When several degrees of dysplasia were present, the highest degree was scored. Lesions with extensive ulceration were eliminated from the study. Tissue use was approved by the Biobank (Germans Trias i Pujol foundation for research) and the research ethics committee of the Hospital Universitari Germans Trias I Pujol (No. PI-15-044).

Six different tumor areas from each case were selected from hematoxylin and eosin (H&E)-stained sections; three of them corresponded to the superficial portion of the tumor, and the other three were from the invasion front. Three additional areas from the adjacent nontumoral epidermis were selected. Cylindrical cores from these areas, each one measuring 0.6 mm in diameter, were obtained from every donor block using a tissue microarray workstation MTA-1 (Beecher Instruments, Silver Spring, MD, USA). The samples obtained from each case were inserted into separate areas or different receptor paraffin blocks. When the donor blocks contained only a thin layer of tissue, adjacent areas were sampled. Positive and negative internal controls were used for each antibody.

<table>
<tr><td colspan="2" rowspan="2">AK 30 cases
15 AK1/15 AK3</td><td>Lesion</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA1</td></tr>
<tr><td>Cancer Field</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA2</td></tr>
<tr><td rowspan="8">iSCC
60 cases</td><td rowspan="3">30 AK I
(6prolif)</td><td>Surface</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA3</td></tr>
<tr><td>Cancer Field</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA4</td></tr>
<tr><td>Invasive front</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA5</td></tr>
<tr><td rowspan="3">30 AK III
(6prolif)</td><td>Cancer Field</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA6</td></tr>
<tr><td>Surface</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA7</td></tr>
<tr><td>Cancer Field</td><td>30 samples (3 cores)</td><td>90 cores</td><td>TMA8</td></tr>
<tr><td colspan="2">Areas of proliferative growth</td><td>12 samples (3 cores)</td><td>36 cores</td><td>Free
spaces</td></tr>
</table>

Figure 13. Schematic representation of our TMA.

I Immunohistochemical stainings were performed on 4-µm sections using the antibodies. Results were evaluated by three pathologists. Some examples of each staining were initially examined simultaneously

under a multiheaded microscope to decide the most suitable scoring method for each antibody according to its staining characteristics. This initial study was also intended to unify the investigators' interpretation criteria. Later, TMAs were studied independently. Individual disagreements were usually solved by rechecking the slides. Other disagreements were infrequent and discussed until a consensus was reached.

Antibody	Clone	Dilution	Source
B-catenin (E-5)	SC-7963	1 : 350	Santa Cruz Biotechnology, Dallas, TX, USA
E-cadherin	NCH-38	1 : 100	Dako, Glostrup, Denmark
Ki67	MIB-1	1 : 600	Dako, Glostrup, Denmark
Podoplanin	D2-40	1 : 50	Dako, Glostrup, Denmark
P16	E6H4	Prediluted	Ventana (CINtec Histology kit,), Basel, Switzerland
p53	DO-7	1 : 500	Novocastra, Newcastle Upon Tyne, UK
Vimentin	V9	1 : 3000	Dako, Glostrup, Denmark

Figure 14. Antibodies, dilutions and detection methods used in the study.

The scoring method for Ki67 was the evaluation of the percentage of positive cells. For vimentin, β-catenin, p53 and p16, the percentage of positive cells was divided into five scores (0: <5%; 1: 5–25%; 2: 26–50%; 3: 51–75%; and 4: 76–100%). The intensity of the expression was independently evaluated for β-catenin (1: weak; 2: intense) and vimentin (1: weak; 2: moderate; 3: intense) in addition. A cut-off value for high expression of p16, considered to correlate with HPV status in oropharyngeal iSCC, was also applied. Cases above the threshold of 75% p16 staining or, alternatively, 50% staining combined with 25% confluent areas, were classified as tumors with high expression. Those that did not reach this level were considered tumors with low expression of p16. The membranous and often discontinuous staining of E-cadherin and D2-40 hampers a reproducible evaluation of the percentage of positive cells. The intensity and percentage of positive tumor area are usually evaluated in these IHC stainings instead. E-cadherin and D2-40 expression were graded according to the

percentage of stained tumor area as 1: 5–33%; 2: 34–66%; and 3: 67–100%. The intensity of the expression was independently scored as 1: weak; 2: moderate; and 3: intense

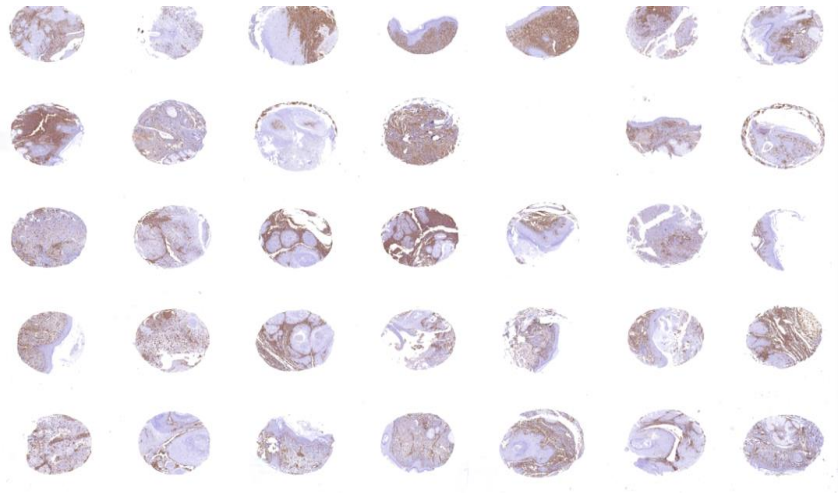


Figure 15. TMA stained by vimentin immunstaining.

For statistical purposes, the average Ki67, p16, p53 and E-cadherin scores of the three cylinders representative of the superficial portion and the three cylinders representative of the invasion front were separately assessed in each case, and their mean value was calculated. For vimentin, D2-40 and β-catenin, the same procedure was performed independently for extension and staining intensity. High and low p16 expression, above or below the HPV-related cut-off for oropharyngeal iSCC, was also assessed.

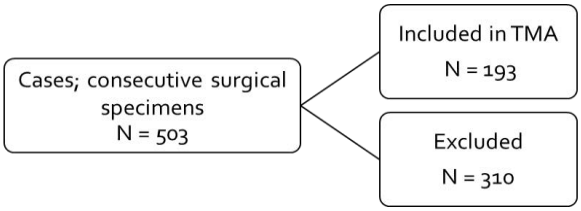
Paraffin blocks with TMAs were constructed in this thesis using 1-mm cores of non-necrotic tissue from primary iSCC tumors. Triplicate cores were, whenever possible, obtained from the blocks.

4.1.4. - miRNA investigation

In this study, miRNA-21 and miRNA-31 ISH was performed on the TMA blocks described above by using LNA-based (Locked Nucleic Acid) in situ hybridization. 4 LNA probes were included, all double FAM (carboxyfluorescein)–labeled. The scramble probe was a universal negative control probe, and miRNA126 was included as positive control - staining endothelial cells. miRNA126 probe showed positive reaction in all TMAs and in virtually all cores evaluated. Variation in staining intensity was evident and may be related to both technical and biological variation. Negative control probe showed little or no background staining. miRNA21 and miRNA31 probe showed positive reaction in all TMAs and in virtually all cores evaluated. Variation in staining pattern and intensity was evident. It was clear that the TMA sections were not always homogenously stained, but the inclusion of duplicates allowed a firm conclusion of the staining patterns.

4.1.5. - Study 3

The study was performed using the same samples and excluding all biopsy specimens smaller than 3 mm and larger than 2.5 cm in size and those specimens with poor architectural preservation (i.e. superficial or fragmented specimens and tumors with extensive ulceration). Cases of Bowen's disease, HPV-associated iSCC and infundibulocystic iSCC were also excluded.(169–171)



Main inclusion criteria	Main exclusion criteria
Sun exposed	Curettages or fragmented biopsies
>3mm	Lesions from the labial mucosa
iSCC <2.5 cm	Keratoacanthomas
Architectural preservation	Infundibulocystic SCC

Figure 16. Patients and criteria in Study 3.

All H&E sections were studied by three pathologists (Xavier Sáenz, Maite Fernánadez and Pilar Vargas) searching for follicular extension of atypical keratinocytes along the follicular basalis and near the iSCC.

The depth reached by the atypical keratinocytes was measured in millimeters from the epidermal surface (Breslow thickness) and also associated with the level of the follicular unit, using the sebaceous gland as a landmark. Follicular extension was classified as "infundibular" when atypical keratinocytes were located above the sebaceous gland, "isthmic" when atypical keratinocytes involved the sebaceous duct and sebaceous gland and "subisthmic" when atypical keratinocytes were present below the sebaceous gland. Involvement of the eccrine sweat glands was also analyzed. As one additional assessment, the tumors were classified based upon the location and extent of the atypical keratinocytes in the overlying interfollicular tumor. Nomenclature that

has been applied to epithelial tumorigenesis in the skin and other anatomic sites (anogenital and oral) was employed.(172–174)

Cases in which the AK consisted of atypical keratinocytes confined to the lower one third of the epidermis near the basalis were classified as having originated through the DP. Cases in which the AK showed near full thickness atypia were classified as having originated through the CP.

4.2. - Statistical analyses

I. Statistical analysis was performed using SPSS (V11.5) software (SPSS Inc., Chicago, IL, USA), with two-sided tests.

II. Statistical analysis was performed using SPSS (V11.5) software (SPSS Inc., Chicago, IL, USA), with two-sided tests. The statistical significance of differences in IHC expression levels of the different markers was evaluated using the Mann–Whitney test. Fisher's exact test was used to determine the statistical significance of differences between proportions. The Spearman's Rho and Pearson's r were used to test the correlation between the expression levels of the different markers. Statistical significance was set at $P < 0.05$.

III. Statistical analysis was performed using SPSS (V11.5) software (SPSS Inc., Chicago, Il, USA), with two-sided tests. The statistical significance of differences in depth of follicular invasion was evaluated using the Mann–Whitney test and Kruskal–Wallis test, as required. Fisher's exact test was used to determine the statistical significance of differences between proportions. Statistical significance was set at $P < 0.05$.

Statistical methods were chosen according to the properties of the material and in accordance with common practice in medical statistics.

5. - Summary of Results and Discussion

5.1. - Study 1

5.1.1. - About differentiated and classical pathways

Biopsy specimens were obtained from 196 patients, 79 women and 117 men, with a mean age of 77.3 years [median, 79; standard deviation (SD), 10.1; range, 51–101]. Women were older than men [80.8 years (SD 9.7) vs. 74.8 (SD 9.7); P < 0.0001]. Most lesions (108 out of 196) were located on the face. Facial lesions accounted for 26.0% of cases in women and 29.1% in men. There were differences in distribution according to sex in other locations: lesions in men were more prevalent on the scalp, trunk-neck and ears (10.7% vs. 2.0%, 5.6% vs. 1.0%, and 6.6% vs. 0%, respectively) and less prevalent on the upper and lower limbs (3.1% vs. 5.1% and 1.0% vs. 3.1%, respectively; chi-squared test, P < 0.0001).The location was unknown in seven men and six women.

The mean diameter of the selected biopsy specimens was 19.30 mm [median, 17 mm; SD 13.01 mm; interquartile range (IQR), 10.00–27.50 mm]. The mean width of the iSCC areas was 9.05 mm (median, 7 mm; SD 7.17 mm; IQR, 3.50–13.00 mm). The anatomic level of infiltration was II in two cases (1.0%), III in 14 cases (7.1%), IV in 127 cases (64.8%), and V in 53 cases (27.0%). The mean maximum depth of infiltration was 3.83 mm (median, 3.00 mm; SD 2.85 mm; IQR 2.00–5.00 mm).

The transformation of AK into iSCC has been proposed to occur following progressive stages of keratinocytic intraepidermal neoplasia (KIN or AK). Lesions showing atypical keratinocytes with nuclear pleomorphism and hyperchromasia, increased mitotic rate, and crowding and overlapping involving the epidermis lower third (KIN I/AK I) would thus progress to lesions with proliferation of atypical keratinocytes in the lower two thirds of the epidermis (KIN II/AK II) and subsequently to lesions with full thickness epidermalneoplasia (KIN III/AK III).(175,176) This classification is analogous to the progressive stages of HPV-associated CIN and vulvar intraepithelial neoplasia. This model, which requires complete dedifferentiation of the epidermis, is the CP.(37,177)

Through analogy to CIN, lesions with KIN I/AK I features have been considered to be low risk, since they would require progression to more advanced grades before acquiring dermal-infiltrating capacity. Lesions with KIN II/AK II and KIN III/AK III features are conversely considered to be high risk, since they would correspond to the final steps prior to invasion and require immediate treatment. A later classification introduced the denomination AK I, AK II and AK III (14) for the same three histopathological stages. The clinicopathological criteria were better defined in this classification, and the importance of considering all AKs as potential precursors of iSCC was stressed independently of AK clinicopathological stage.(14,178)
It is common to find cases where iSCC seems to arise directly from proliferating atypical basaloid cells limited to the lower third of the epidermis (KIN I/AK I), whereas the mid and upper epidermal layers remain virtually intact. This transformation model resembles the DP of progression that has been described for iSCC of the vulva, oral cavity and pharynx, among other locations.(177,179)

In some studies, the behavior of tumors developed through the DP has been found to be more aggressive than that of lesions following the CP.(177,180)

To the best of our knowledge, systematic studies of the prevalence of both pathways in iSCC have never been performed. With this goal in mind, we and others (181) considered that the best approach to identify the type of precursor lesion was to evaluate the characteristics of the epidermis adjacent to and overlying the infiltrative tumor as well as the presence of ulceration, which could affect representativeness.

The number of cases and percentages of AK I, AK II and AK III overlying iSCC were 125 (63.8%), 35 (17.9%) and 36 (18.4%), respectively. Foci of ulceration were present in 62 cases (31.6%), of which 40 corresponded to iSCCs with AK I in the overlying epidermis (32%), 10 to iSCCs with AK II (28.6%) and 12 to iSCCs with AK III (33.3%) (P = 0.9014, chi-squared test).

With regard to the epidermis adjacent to iSCC, it showed AK I (77.9% of evaluable cases) in 141 cases, AK II in 12 cases (6.6%) and AK III in 15 cases (8.3%); it could not be evaluated in 8.1% of total. The epidermis adjacent to iSCC was normal in 13 cases (7.2%). The corresponding values in the adjacent epidermis were 42/141 (29.8%), 2/12 (16.7%) and 2/15 (13.3%) (chi-square, P = 0.3161).

Patient sex and lesion location, diameter, depth, anatomic level and adnexal involvement (proliferative AKs) had no significant correlation to the thickness of atypical keratinocyte proliferation in the epidermis overlying or adjacent to iSCCs.

	Over iSCC	Edge of iSCC
AK I	63.8%	77.9%
AK II	17.9%	6.6%
AK III	18.4%	8.3%

Figure 17. Results regarding the precursor lesions in iSCC.

Actinic keratosis with atypical basal cells was found to be the most common lesion associated with iSCC of the skin.

Three features constituted the minimum common denominator of all AK cases. The first and most important was the presence of keratinocytic atypia with nuclear pleomorphism, hyperchromasia and a high nucleo-cytoplasmic ratio. Atypical keratinocytes were randomly arranged and showed crowding and overlapping in at least the epidermal basal layer, although these findings may extend into the upper epidermis and result in a Bowenoid appearance. In some cases, there was an increased mitotic rate with atypical and suprabasal mitoses. The second steady feature of AK was the presence of a thick horny layer, either orthokeratotic and compact or parakeratotic in nature. Hyperkeratosis was only absent in biopsies with extensive ulceration, trauma or local treatment effect. The third and last of the capital features of AK was actinic elastosis, present to a greater or lesser extent in all cases, although it was occasionally replaced by fibrotic collagen after repetitive epidermal detachment, ulceration or local treatments leading to superficial dermis scar formation.

The presence of elastosis is considered to be a collateral effect of sun exposure, although recent studies have raised the possibility that this form of stromal atrophy may have a pathogenic role in the cancer field by facilitating epidermal transformation.(67)

In addition to these three consistent microscopic findings, there were other features that are extremely variable. The epidermis may have shown either marked atrophy with barely a couple of keratinocytic layers or extreme hypertrophy with findings difficult to tell apart from iSCC with expansive growth. Thin cords of keratinocytes may have been attached to the epidermal base, forming anastomosing structures reminiscent of reticulated seborrheic keratosis or solar lentigines. Some lesions showed conspicuous melanin pigmentation, whereas others contained areas of extensive acantholysis or epidermolytic hyperkeratosis. Bases showed chronic inflammation ranging from just scattered lymphoid cells to dense, band-like lymphocytic infiltrates with lichenoid features.

Classifying AK according to the aforesaid morphological features may well contribute to microscopic identification of AK variants and their clinical correlation, but it is devoid of any interest from a biological viewpoint.(182) In pursuit of a more rational classification, allowing distinction between cases with low and high risk of iSCC transformation, Yantsos, under the direction of Cockerell in 1999, proposed a three-tiered grading system, analogous to that used for HPV-associated CIN I, CIN II, CIN III; AK was called KIN and graded as KIN I, KIN II or KIN III according to clinicopathological criteria.(175) Histologically, atypical keratinocytes involve the epidermal lower third in KIN I lesions, the epidermal lower two thirds in KIN II lesions, and the full thickness of the epidermis in KIN III lesions. The adnexal structures were said to be systematically involved in KIN III and occasionally in KIN II lesions. The latter could thus be subdivided into KIN IIa and KIN IIb depending on the absence or presence of at least one of the following

features: adnexal involvement, acantholysis and prominent acanthosis with buds of keratinocytes in the papillary dermis.

Clinical features such as erythema, scaling and induration would be progressively more apparent from KIN I to KIN III lesions. KIN I would be associated with an area of barely perceptible, slightly palpable, thin-scaling erythema, whereas KIN III would show an obviously keratotic, indurated appearance.

Cockerell further refined this classification in 2003 and stressed the progressive nature of the three stages from KIN I to KIN III, the latter being considered synonymous with in situ SCC or Bowen disease.(176) This initial classification attempt was criticized for several reasons.(18,181) It was considered too vague, allowing the inclusion of cases of seborrheic keratosis under the KIN denomination. In addition, a clinical diagnosis of these common entities based on clinicopathological correlation was not a realistic goal. Another criticism of this classification is that it equaled full-thickness neoplasia (KIN III) with Bowen disease and AK, while they actually are two distinct variants of intraepidermal SCC with different pathogeneses and clinicopathological features.

Finally, the hypothesis that iSCC may only develop from KIN III or at least KIN II, always requiring prior progression from KIN I to KIN III, is not widely accepted. In a well-argued letter, Scurry (181) claimed that areas of KIN III should be found at the edges of iSCC for this proposal to be true, as in cervical neoplasia, which typically shows CIN III epithelial changes interspersed with foci of iSCC. In contrast, KIN I was the most frequent finding at iSCC edges in Scurry's experience, and it is quite difficult to believe that KIN III changes had been obliterated by invasive tumors in all these cases.

In 2007, Rowert-Huber recommended a similar but better defined clinicopathological classification system in which AK progression stages were known as "early in situ SCC type AK I, type AK II and type AK III".(14) This classification's aim was to emphasize that all these lesions are in situ SCC since the beginning but at different phases of progression. When several grades were present in the same lesion, the highest grade defined the stage. The hypothesis of progression through three sequential steps was not further mentioned, and it was stated that it is not possible to predict which lesion will become invasive, concluding, as a result, that all AK lesions must be treated. A clinical correlate of this classification was also developed, Grade 1 being a slightly palpable AK lesion (better felt than seen), Grade 2 a moderately thick AK lesion (easily felt and seen) and Grade 3 a very thick, hyperkeratotic or obvious AK lesion.

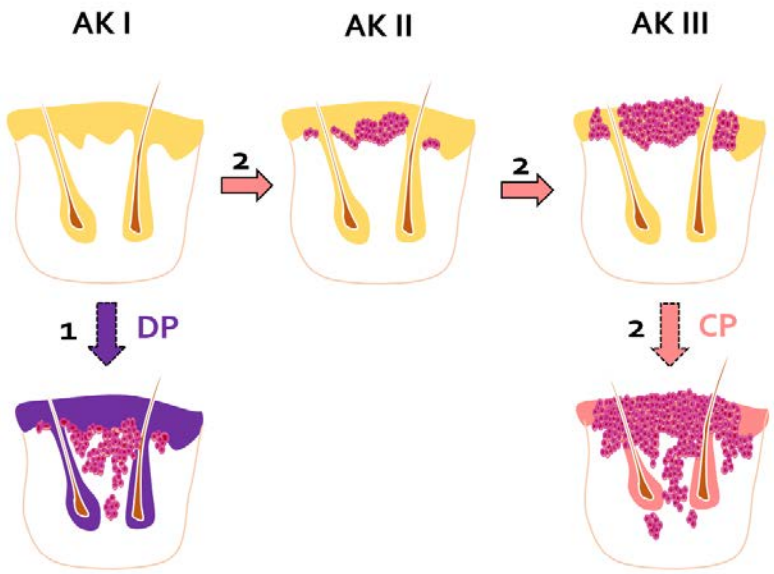


Figure 18. Schematic view of the progressive pathways;
1 corresponds to the differentiated pathway and **2** to the CP.

One year later, Stockfleth and his co-authors from the European Skin Academy European Consensus published a treatment algorithm for AK (178), insisting on the idea that although biopsy performance may be advisable in some situations, all AKs have to be regarded as intraepidermal SCCs, without it being possible to determine which cases have the potential to progress to invasive SCC. This point of view is not widely accepted, and AK has been recently defined as pre-malignant or precancerous by some authors for whom only KIN III/AK III lesions may be construed as in situ SCC.(183,184)

For many of the authors making use of the new in vivo image techniques, AK histological grading is clinically relevant, implying that it is indicative of progression stage.(185) The contrasting points of view between those who consider AK as in situ carcinoma and those who prefer to classify AK as a pre-malignant process have generated important debates.(18,186) The relevance of this controversy has nevertheless faded due to the general agreement that most iSCCs arise from AKs. Neither the major pathways AK follows in its transformation to iSCC nor their prevalence have been further investigated.

The presence of ulceration was a relative limitation of our study. Ulceration might have led to loss of AK II and AK III areas, and these cases would have been mistakenly assigned to the AK I group. Ulceration was nevertheless found in similar percentages (ranging from 28.6% to 33.3%) in all AK groups. Unmistakable, non-ulcerated AK I was furthermore present on the surface of a significant proportion of iSCC cases (85 of 196; 43.4%).

Ours is the first study aimed at investigating the prevalence of the CP and DP transformation from AK to iSCC. Our results partly support the hypothesis of sequential progression (CP) from AK to iSCC, since we have identified AK III as an iSCC precursor in a significant number of cases. Specifically, iSCC cases with AK II and AK III in the overlying epidermis jointly account for one third of the tumors (36.3%). This model of progression, analogous to HPV-associated intraepithelial neoplasia, is therefore an important mechanism of transformation in cutaneous iSCC. More importantly, our study provides significant evidence in favor of the existence of DP in cutaneous iSCC.

Given that most of the biopsy specimens showed AK I on their surfaces (125/196, 63.8%), at their edges (141/181, 77.9%), or both (105/181, 58.0%) we conclude that a direct transformation from AK I to iSCC (DP) is the most prevalent mechanism of transformation

5.1.2. - About follicular extension (proliferative actinic keratosis).

The presence of atypical keratinocytes migrating downwards along hair follicles and sweat ducts would define a subtype of AK with "proliferative" characteristics. The importance of this subtype resides in its resistance to treatment because of the deep location of abnormal cells. This variant of AK is additionally difficult to recognize by in vivo imaging techniques such as reflectance confocal microscopy, which only reaches the upper dermis (approximately 200 lm in depth).(187)

Previous studies have considered that proliferative AKs appear only in AK II and AK III stages (175,176), but in our study this phenomenon was present in lesions with overlying AK I (31.2%), AK II (22.9%) and AK III (13.9%) (chi-square P = 0.1007). The corresponding percentages in the adjacent epidermis were 37.7%, 25% and 11.1% (chi-square P = 0.3161).

	AK I	AK II	AK III
Adnexal involvement by atypical cells (proliferative AK)	75%	15.4%	9.6%
Absence of adnexal involvement by atypical cells	59.7%	7.7%	21.5%

Figure 19. Adnexal involvement by atypical cells (proliferative AK) according to the thickness of atypical changes in the epidermis overlying iSCCs.

Adnexal involvement by atypical keratinocytes (proliferative AK) was more frequent in iSCCs with overlying AK I (39/125, 31.2%) than in iSCCs with AK II (8/35, 22.9%) or AK III (5/36, 13.9%), but the proportions were not significantly different (chi-square P = 0.1007).

Most cases (39/52, or 75%) of proliferative AK actually arose on lesions with overlying AK I in our series; thus, tumor advancement along adnexal structures (proliferative AK) might be one of the leading modes of iSCC development from AK I lesions.

5.2. - The Lund project

Biopsy specimens were obtained from 79 patients, 35 women and 44 men, with a mean age of 77.3 years. The number of cases and percentages of AK I, AK II and AK III overlying iSCC were 54 (68.4%), 12 (15.2%) and 13 (16.5%), respectively. Foci of ulceration were present in 31 cases (39.3%). With regard to the epidermis adjacent to iSCC (edges), it showed AK I in 59 cases (74.7%), AK II in 12 cases (15.2%) and AK III in five cases (6.3%). The epidermis adjacent to iSCC was normal in three cases (3.8%). Adnexal involvement by atypical keratinocytes (proliferative AK) was again more frequent in iSCCs with overlying AK I (54.5%) than in iSCCs with AK II (24%) or AK III (21%). Proliferative features were found in 41.8% of the cases in total.

Actinic damage was assessed according to the average of dermal affection in three different grades: grade I (<33%), grade II (33-66%) and grade III (>66%). Actinic damage grade I was found in 19 cases (24%), grade II in 25 cases (31.6%) and grade III in 35 cases (44.3%).

	Barcelona		**Lund**	
	Over iSCC	*Edge of iSCC*	Over iSCC	Edge of iSCC
AK I	*63.8%*	*77.9%*	68.4%	74.7%
AK II	*17.9%*	*6.6%*	15.2%	15.2%
AK III	*18.4%*	*8.3%*	16.5%	6.3%

Figure 20. Comparative results between study 1 (Barcelona) and 2 (Lund).

Our results support what was found in our first study and therefore consolidate the DP as the main pathway in the genesis of the cSCC.

The results have been presented in session in the context of the scheduled, regular education seminars in the Department of Pathology (Lund University Hospital).

5.3. - Study 2

We selected 80 consecutive specimens out of all reviewed cases that contained enough tissue to allow the construction of several TMAs. Fifty-three of the 80 cases corresponded to the DP and 27 to the CP.

The development of iSCC on sun-damaged skin is a multi-step process with a high mutation burden that causes alterations in all major signaling cancer pathways.(188) Epigenetic events associated with age, actinic damage, tobacco and other factors, as well as decreased immune surveillance, may further contribute to the development of cutaneous iSCC.(189) Despite this complex network of activating stimuli, most cutaneous iSCCs exhibit relatively indolent behavior. Moreover, its intraepidermal precursor, AK, does not consistently achieve the stage of invasive neoplasia. The prognosis of cutaneous iSCC nonetheless changes completely in about 2–4% of cases when metastases develop, reaching a mortality rate higher than 70%.(189) The risk of metastases is higher in immunosuppressed patients (190–192) as well as in tumors with high-risk features (193), but there are few clues so far to adequately stratify this hazard. These striking behavioral differences are a challenge to understanding the underlying causes essential to recognizing the most aggressive variants and search targets for specific treatment of cutaneous iSCC.

The two main pathways of progression from AK into iSCC – CP and DP (194) – are also present in the anogenital mucosa, where the tumors originating through the CP are related to high-risk mucosal HPV. Viral proteins E6 and E7 are responsible for p53 and pRB inactivation.(173,195) The decrease of pRB, a negative regulator of the cyclin-dependent kinase inhibitor p16, facilitates the upregulation of p16, which is used as a surrogate marker for tumor-associated HPV infection. Inversely, absent p16 expression and positive immunostaining for p53 characterize iSCC originating through the DP.(195) We did not find any significant differences between these two markers in our series. The percentage of cases with high p16 expression was furthermore higher in the DP than the CP, opposite to what could have been expected, although this difference was not significant. These results suggest that HPV-related mechanisms cannot explain the existence of two pathways in cutaneous iSCC.

A hallmark of the DP is the ability of transformed basal squamous cells to cross the basal membrane and invade the stroma. Activation of the EMT program by basal epidermal cells has a key role in dermal invasion. Attached squamous epithelial cells acquire a mesenchymal-like phenotype and become released from cell to cell junctions, enabling their motility. Epithelial–mesenchymal transition plays a critical role in physiological processes like embryogenesis and tissue reparation, but a complete or partial EMT may play a major role in tumor invasion and metastases.(196–199) The minimum common denominators of EMT are the acquisition of the mesenchymal intermediate filament vimentin and disruption of the E-cadherin/β-catenin complex, which plays an important role in epithelial adhesion. The acquisition of a mesenchymal phenotype is recognized immunohistochemically through the expression of vimentin and loss of E-cadherin and β-catenin staining.(196–198,200–204)
We have used these pivotal markers to demonstrate that EMT program activation is significantly higher in cases of iSCC originated from the DP

than in those arising through the CP. Alternative mechanisms involved in the invasiveness of iSCC developed through CP will require additional investigations. The importance of EMT in the progression from AK into iSCC has been well established by several studies. Compared to AK and normal skin, iSCC presents increased vimentin expression and a loss of E-cadherin. Toll et al. demonstrated that partial EMT of iSCC is associated with increased metastatic risk.(202) These results have been recently confirmed by Hesse et al., who demonstrated E-cadherin downregulation in poorly differentiated iSCC and lymph node metastases, suggesting partial EMT. The same group found a significant upregulation of D2-40 in metastatic and poorly differentiated iSCC, which was a statistically independent prognostic factor for disease-free survival.(200)

A relation between vimentin expression, enhanced migration activity, and metastatic capacity has also been demonstrated in oral iSCC.(203) In a nude mice model with human squamous xenograft tumors, cyclosporine-induced immunosuppression was associated with augmented EMT and significantly increased malignant potential, suggesting an important contribution by EMT to the aggressive behavior of iSCC in immunosuppressed patients.(204)

Finally, Ki67 is a nuclear antigen marker of proliferating cells (G1, S, G2 and M cycle phases) that is absent in quiescence (G0). The expression of Ki67 thus usually correlates with the mitotic count, and it is used as a surrogate marker for proliferation rate analysis and for diagnosis, classification and prognosis in several neoplasms.(205) We have found significantly higher scores in the DP than in the CP. This increased proliferation explains the progression of AK from a lesion with basal atypia into a bowenoid neoplasia with complete replacement of the epidermis by atypical cells and frequent mitoses in all epithelial layers.

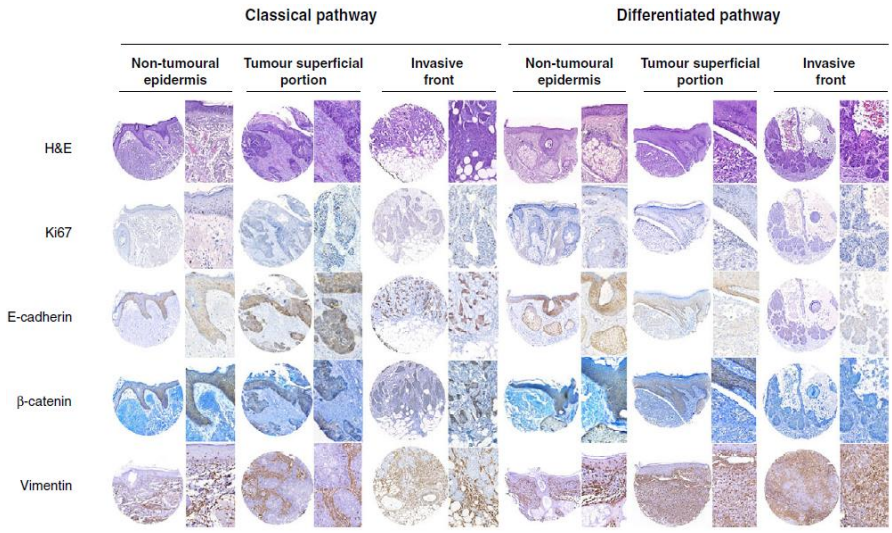


Figure 21. Representative images of the markers' staining patterns with significantly different expression in invasive SCC cases originating through the classical and differentiated pathways.

The values presented are those corresponding to the mean of superficial and invasive front for Ki67, p16 and p53 and the addition of extension and intensity for vimentin, E-cadherin, β-catenin and D2-40. No significant differences were found either for extension or extension plus intensity scores when the superficial portion and invasive front of the lesions were analyzed separately.

Invasive SCC originating from DP had lower proliferative activity (percentage of cells expressing Ki67) than CP ($P = 0.003$). They also had significantly lower expressions of vimentin ($P < 0.001$), E-cadherin ($P < 0.001$) and membranous β-catenin ($P < 0.001$) than CP. No nuclear expression of β-catenin was observed. There were no significant differences regarding the expression of p53 and p16. In addition, 17.4% of cases originated through CP, and 21.1% of the DP cases showed high p16 expression above the cut-off that identifies cases presumably associated with HPV in oropharyngeal iSCC. These differences were not

significant (P-value: 0.6569). There was a significant correlation between the expression of E-cadherin and membranous β-catenin (Pearson's r = 0.386, Spearman's Rho = 0.439, P < 0.001). No significant differences between pathways were found in D2-40 expression, and no other significant correlations were found.

Differences in Ki67 scores between both pathways have also been described in vulvar SCC, in which the CP often shows diffuse Ki67 expression. In those lesions originated from the DP, Ki67 positive staining was limited to the periphery of the larger nests and even absent at the invasive front.(206)

	Differentiated pathway	Classical pathway	*P*
Ki67 (mean, SD)	30.1, 25.5	45.9, 29.4	*P* <0.001
p53 (mean, SD)	2.4, 1.6	2.4, 1.6	Not significant
p16 (mean, SD)	1.0, 1.4	1.5, 1.7	Not significant
Vimentin (extension+intensity) (mean, SD)	2.8, 2.4	1.1, 1.8	*P* <0.001
E-cadherin (extension+intensity) (mean, SD)	3.4, 1.2	4.6, 1.7	*P* <0.001
β-catenin (extension+intensity) (mean, SD)	3.9, 1.5	5.1, 1.8	*P* <0.001
D2-40 (extension+intensity) (mean, SD)	3.59, 1.52	3.61, 2.10	Not significant

Figure 22. Statistical analysis of the results demonstrating significant differences in Ki67 and β-catenin and E-cadherin that were lost in the DP. There were no significant differences regarding the expressions of p53, p16 and D2-40.

Levels of CD31 were significantly higher in tumors arising from the DPpathway (p:0,0041).

Matrix metalloproteinases 1 and 3 were also significantly higher in tumors arising from the DP (p:0,0026 and p:0,049 respectively).

With this data (that will be submitted for publication soon), one can link the tumors arising from a DP with higher microvessel density, which provides essential nutrients that enable the tumor to grow, and higher

levels of MMP that carry out "path-clearing" for cancer cells and therefore allow more aggressive behavior.

5.4. - Tissue microarray investigation on miRNA

Sixty-five cases of cSCC arising from the CP and 123 cases arising from the DP were analyzed using the same TMA previously described. Preliminary analyses found significant differences regarding the intensity and extension among the two pathways in miRNA-31. miRNA-31 showed more intensity and extension (p:0,0002) in those tumors arising from the DPBy contrast, no significant differences were found among the two pathways regarding the expression of miRNA-21 (p:0,0803). The complete results will be submitted for publication soon.

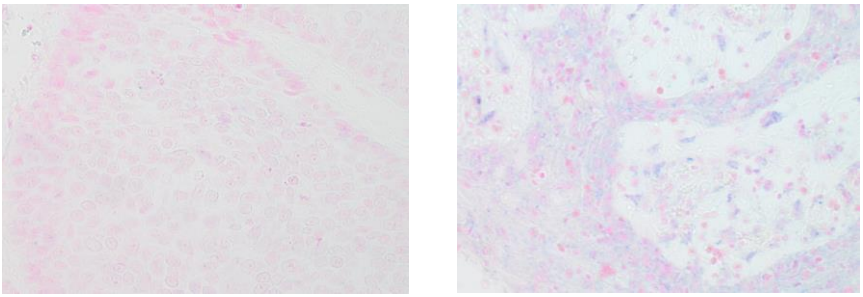


Fig 23. Examples of different miRNA expression in cSCC. Low miRNA expression on the right, high miRNA expression on the left (blue dot-like pattern).

5.5. - Study 3

We identified 503 cases of iSCC diagnosed over a period of the years, and 193 of them fulfilled the same criteria used in the first study. In addition, cases that could correpond to infundibulocistic squamous cell carcinoma were also eliminated from the study.

Follicular extension of atypical keratinocytes in an AK was identified in 50 cases (25.9%). Of the 50 cases with follicular extension, 12 cases reached the infundibular level, 33 reached the isthmic level and five reached the subisthmic level. Invasive squamous cell carcinoma originated from the follicular basalis in three of 12 cases with infundibular involvement (25%), 21 of 33 cases with isthmic involvement (63.6%) and five of five cases with subisthmic involvement (100%; $P < 0.01$). The median tumor thickness of iSCC developing from the follicular basalis was higher than in iSCC arising from the epidermis (1.75 mm vs. 0.9 mm, $P < 0.001$).

In an assessment of the characteristics of the tumor surface, 122 cases were classified as having originated from the DP, and 71 cases with near full-thickness atypia were considered to have originated through the CP. The proportion of those corresponding to the DPpathway (36/122, or 29.5%) was higher than that corresponding to the CP (14/71, or 19.7%), but the difference was not statistically significant. No significant differences in depth of follicular involvement were found between iSCC originating through the DP and CP.

For eccrine sweat gland involvement, atypical keratinocytes were limited to the acrosyringia in 39 cases, but the atypical keratinocytes never extended to the dermal level of the duct, and no iSCC appeared to originate from the sweat gland epithelium.

This study demonstrates that the depth of follicular extension of atypical keratinocytes in an AK directly correlates with the depth of invasion by an associated iSCC. The study complements a prior study which showed only superficial follicular extension in Bowenoid AKs.(207) The findings taken together strongly suggest that deep iSCC often arises from the follicular basalis.

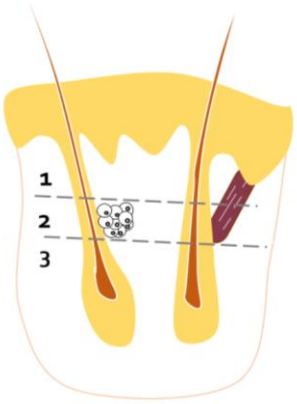


Figure 24. The three follicular levels (infundibular, isthmic and subisthmic)

This study has direct therapeutic implications, providing an explanation for the recurrence and progression of some AKs when superficially destructive treatment modalities such as cryotherapy are followed, as these are unlikely to reach the deeper levels of the follicular epithelium. If the histopathological assessment carefully notes the presence and the depth of follicular extension of an AK, then more aggressive treatment modalities such as curettage, excision or the use of photosensitizers may be employed. Such treatment could limit the incidence of recurrence and the development of iSCC.

	Atypical cells in the hair follicles	iSCC originated from the follicular basalis
1.-Infundibular	12 cases	3 cases (**25%**)
2.- Isthmic	33 cases	21 cases (**63.6%**)
3.- Subisthmic	5 cases	5 cases (**100%**)

Figure 25. Summary of the results.

The relationship between the atypical keratinocytes of the AK and the malignant keratinocytes of the iSCC with follicular stem cells in the bulge is an additional point of interest. Studies in nude mice have demonstrated that malignant squamous cells may take advantage of the follicular cycle machinery to overcome negative regulatory signals and induce growth. Disruption of an anagen-phase follicle by follicular extension could induce cycling into the catagen phase. When the follicle then cycles back into anagen phase, HFSCs are released from PTEN-induced quiescence, triggering an intense epithelial proliferation during the early anagen phase.(62)

Mutated keratinocytes may also respond and proliferate, leading to iSCC.(208,209) Even though the results of these experimental animal studies may be difficult to translate to humans, our work supports the idea that the hair follicle may contribute significantly to the development of deep iSCC. Initiation of the dermal invasion at a deeper level leads to a more aggressive iSCC than iSCC arising from the epidermis. Of particular interest, this study suggests that the sweat gland does not play a major role in the development of iSCC in the presence of an AK. The sweat glands were only involved in their most superficial portion in this study, and iSCC was never identified in their proximity. These observations reinforce the concept that only the hair follicle, among adnexal structures, plays a significant role in the genesis of iSCC.

6. - Strengths and Limitations

In addition to the strengths and limitations already exposed in results and discussion above, a few further aspects can be highlighted.

Cases with different evaluations or that showed controversial assessment were reevaluated for a final score under a multiheaded microscope by three different pathologists. Strong disagreements were extremely infrequent but discussed until a consensus was reached. The immunostainings were examined under a multiheaded microscope to decide on the most suitable scoring method for each antibody according to its staining characteristics. This was also an effort to unify the investigators interpretation criteria. The use of well-validated antibodies is crucial for yielding correct results. Therefore, a further strength is that all antibodies used have either previously been demonstrated to be specific or have been validated within this thesis. The establishment of the prevalence of the CP and DP of transformation from AK to iSCC and providing data in favor of the existence of a DP in cutaneous iSCC being one the most prevalent mechanisms of transformation is supported in two consecutive studies.

There were several limiting factors in our study. All these studies have been performed in a limited number of cases, although we considered them representative, larger series and confirmation by other researchers would be conveninet to confirm our results. The presence of ulceration was a relative limitation of our study; for instance, ulceration might have led to a loss of AK II/AK III areas, and these cases would have been mistakenly assigned to the AK I group. Unmistakable non-ulcerated AK I was nevertheless present on the surface of a

significant proportion of iSCC cases. However cases with extensive ulceration were removed from the series. Even though to exclude keratoacanthomas is easy in general, a few cases needed to be revised to attain a consensus. In this type of study, with quantitative conclusions coming from histological and IHC observations, a risk of a bias in the evaluation is an unavoidable limitation. Tumor heterogenicity can be masked by using TMA where very small sample tissues are used and may not always reflect the biological properties of the entire tumor. This can be partially compensated for by using multiple tissue cores from different areas of each tumor and by achieving triplicate cores from the blocks. Moreover, the high number of tumors that can be studied simultaneously might compensate for false-negative or false-positive tissue cores. Many tests have been performed on the limited material, which confers a risk for type I statistical errors. However, given that the studies in this thesis are more exploratory than confirmatory and that the results were interpreted with caution, the value of the studies would decrease if the number of analyses was decimated too much, since that would lead to an increased risk for type II errors. The study of miRNA using CISH-techniques presented background-like artifacts mixed with a partial absence of detecting signals. Nonetheless, the majority of the samples showed a weak staining that make the assessment complicated. All cases were obtained from the archives of the Hospital Universitari Germans Trias i Pujol and probably a large majority come from Mediterranean patients. Trying to overcome this limitation, the first study demonstrating a higher prevalence of iSCC originated from the differentiated pathway was reproduced in a series of Swedish patients, obtaining similar results. However, it would be interesting to know whether this distribution and the other results are reproduced in other racial groups.

7. - Conclusions

I. Invasive squamous cell carcinomas can be originated from actinic keratosis with transformation limited to the basal layer of the epidermis following the differentiated pathway, similarly to what has been previously found in the oropharynx and anogenital area.

II. The percentage of invasive squamous cell carcinomas originated from actinic keratosis following the differentiated pathway is higher than following the classical pathway. This is demonstrated by the presence of remains of actinic keratosis with atypia limited to the basal layer above 63.6% of the invasive squamous cell carcinomas. Conversely, only 36.3% of cases presented complete or almost complete transformation of the epidermis above the invasive squamous cell carcinoma.

III. The results of the initial study performed in cases from the Hospital Universitari Germans Trias i Pujol (Spain) was confirmed in a series of cases from the Hospital of Lund (Sweden) were 68.4% of the invasive squamous cell carcinomas showed actinic keratosis with atypia limited to the basal layer above. All cases presented actinic elastosis as a demonstration of solar damage as etiological factor.

IV. Eight tissue microarrays representative of 80 consecutive invasive squamous cell carcinomas were built containing cores representative of different portions of the tumor as planned. They corresponded to 53 tumors originated from the

differentiated pathway and 27 tumors originated from the classical pathway.

V. No significant differences in the expression of p53 and p16 were found between both pathways, suggesting that the mechanisms underlying both pathways differ from those in the anogenital tumors.

VI. No significant differences in the expression of Podoplanin were found between both pathways, indicating that it does not play a differential role in both pathways.

VII. The epithelial to mesenchymal transition shift was significantly higher in the differentiated than in the classical pathway (lower expression of vimentin ($P < 0.001$), E-cadherin ($P < 0.001$) and membranous b-catenin ($P < 0.001$). This result indicates that in the differentiated pathway, basal keratinocytes take advantage of epithelial to mesenchymal transition to invade.

VIII. The proliferation indexes where significantly higher in tumors originated from the classical pathway than from the differentiated pathway (46% vs 30%, $P = 0.003$). This elevated proliferation in the absence of invasive capacity constribjutes to explain the intraepidermal extension of the tumor.

IX. The depth of follicular involvement by actinic keratosis correlates with the risk of developing invasive squamous cell carcinoma from the wall of the hair follicle and with the depth of the iSCC.

X. The expression of miR31 was higher in the differentiated pathway, than in the classical pathway. This upregulation can

contribute to explain the early invasive capacity of AK with transformation limited to the basal layer.

XI. No significant differences in miRNA21 expression were found between both pathways.

This thesis contributes to comprehending the methods of iSCC transformation associated with actinic damage and the alterations that occur in the cancer field by identifying molecules related to proliferation, regulation and stimulation of the cell cycle. This will allow better understanding of the disease, the modification of treatment plans and the development of new treatments.

8.-Disclosure: Conflict of Interests and Relationships with Industry

This thesis is based on a series of studies partially supported by grants received from LeoPharma and Almirall, but no one has been directly employed.

There were no non-financial conflicts of interest.

The research results were not influenced by external factors or misconduct such as the trade of financial incentives for positive results.

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Paper I

Paper II

Paper III