

TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 1000

MEDICA - ODONTOLOGICA

**MOLECULAR MARKERS FOR
PROGRESSION OF SQUAMOUS CELL
CARCINOMA OF THE SKIN**

by

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2011

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ISBN 978-951-29-4859-8 (PRINT)
ISBN 978-951-29-4860-4 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2011

To my family

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Molecular markers for progression of squamous cell carcinoma of the skin.

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Painosalama OY, Turku, 2011

ABSTRACT

Incidence of nonmelanoma skin cancer (NMSC) is increasing. Ultraviolet (UV) – light is a major risk factor for the development of cutaneous SCC. Cutaneous SCCs that develop to chronic ulcers are known to progress and metastasize more easily than UV-induced SCCs. Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes which are suggested to have a role in cancer growth and invasion.

The molecular background for progression of cutaneous SCC was examined by immunohistochemistry (IHC) using tissue samples of recessive dystrophic epidermolysis bullosa (RDEB) –associated SCC, sporadic UV-induced SCC, and SCC precursors. IHC studies using tissue microarray (TMA) technique revealed overexpression of MMP-7 and MMP-13 in SCC tumor cells. MMP-7 expression was enhanced especially in the SCC tumor cells of the RDEB –associated SCCs. Studies with SCC cell lines showed that tumor cell derived MMP-7 activated heparin binding epidermal growth factor –like growth factor (HB-EGF) which enhanced the growth of SCC tumor cells.

Further, it was shown that type VII collagen (COL7) is expressed in sporadic SCC tumor cells. Interestingly, it was shown that SCC –associated MMP-13 is capable of cleaving COL7 *in vitro*. COL7 cleavage may have a role in the progression of cutaneous SCC.

Studies on serine proteinase inhibitor gene family using SCC tumor cell gene array, quantitative real-time PCR, SCC cell lines, normal human epidermal keratinocytes and IHC of TMA samples showed that serine proteinase inhibitor clade A, member 1 (serpinA1, alpha-1-antitrypsin) is expressed and produced by human SCC tumor cells but not by normal keratinocytes. Moreover, serpinA1 expression was shown to correlate with the progression of cutaneous SCC using transformed HaCaT-cell lines and mouse chemically induced skin SCC model. SerpinA1 may serve as a novel biomarker for the progression of cutaneous SCC.

This study elucidated putative mechanisms of the progression of cutaneous SCC and revealed novel biomarker candidates for the progression of SCC of the skin.

Keywords: *squamous cell carcinoma of the skin, recessive dystrophic epidermolysis bullosa, matrix metalloproteinase, serpinA1, alpha-1-antitrypsin, type VII collagen*

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Ihon okasolusyövän etenemisen merkkitekijät.

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Painosalama OY, Turku, 2011

LYHENNELMÄ

Ei-melanoottisten ihosyöpien ilmaantuvuus on kasvussa. Auringon ultravioletti (UV) –säteily on tärkeimpiä ihon okasolusyövän kehittymisen riskitekijöitä. Ihon kroonisiin haavoihin kehittyvät okasolusyövät ovat luonteeltaan herkästi leviäviä ja ne lähettävät etäpesäkkeitä UV-valon aiheuttamia okasolusyöpiä useammin. Matriksin metalloproteiinaasit (MMP) ovat ryhmä soluväliainetta pilkkovia entsyymejä, joiden on arveltu edesauttavan syövän kasvua ja leviämistä.

Ihon okasolusyövän etenemiseen liittyviä tekijöitä tutkittiin kudosten immunovärjäysmenetelmällä käyttäen hyväksi resessiivistä dystrofista epidermolysis bullosa (RDEB) –tautia sairastavien potilaiden okasolusyöpäkudosnäytteitä sekä UV-valon aiheuttamia okasolusyöpiä ja niiden esiasteita. Merkkiaineiden tutkiminen kudossirutekniikalla osoitti, että MMP-7 ja MMP-13 ilmentyminen oli korostunutta ihon okasolusyöpäsoluissa. MMP-7 ilmentyminen oli erityisen voimakasta RDEB:aan liittyvissä okasolusyöpäsoluissa. Syöpäsolulinjoilla osoitettiin, että MMP-7 pystyy aktivoimaan erästä syöpäsolun pinnan kasvutekijää (hepariinia sitova epidermaalisen kasvutekijän kaltainen kasvutekijä, HB-EGF), jonka aktivoituminen lisäsi syöpäsolujen kasvua.

Tässä työssä osoitettiin lisäksi, että tyypin VII kollageenia (COL7) tuotetaan UV-valon aiheuttamissa okasolusyöpäsoluissa. Uusi mielenkiintoinen havainto oli, että okasolusyöpiin liittyvä MMP-13 pystyy pilkkomaan COL7:a. COL7:n pilkkoutuminen voi edesauttaa okasolusyövän etenemistä.

Seriiniproteaasin estäjien geeniperheen tutkiminen syöpäsolujen geenilastuanalyyseillä, kvantitatiivisella käänteiskopioija-PCR menetelmällä, syöpäsolulinjoilla, normaaleilla ihon okasoluilla sekä kudossirutekniikalla osoitti, että seriiniproteaasin estäjä A1:tä (serpinA1, alfa-1-antitrypsiini) ilmennetään ja tuotetaan okasolusyöpäsoluissa, mutta ei normaaleissa ihon okasoluissa. Lisäksi transformoiduilla HaCaT-solulinjoilla ja hiiren ihoon kemiallisesti aiheutetulla okasolusyöpämallilla osoitettiin, että serpinA1:n ilmentyminen korreloi okasolusyövän etenemiseen. SerpinA1:tä voidaan mahdollisesti hyödyntää ihon okasolusyövän etenemisen merkkiaineena.

Tässä tutkimuksessa saatiin uutta tietoa mahdollisista ihon okasolusyövän etenemisen mekanismeista sekä löydettiin uusia mahdollisia ihon okasolusyövän etenemisen merkkitekijöitä.

Avainsanat: *Ihon okasolusyöpä, resessiivinen dystrofinen epidermolysis bullosa, matriksin metalloproteiinaasi, seriiniproteaasin estäjä A1, alfa-1-antitrypsiini, tyypin VII kollageeni*

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ABBREVIATIONS

AAT	Alpha-1-antitrypsin (synonym for serpinA1)
ADAM-17	A disintegrin and metalloproteinase-17
AK	Actinic keratosis
BCC	Basal cell carcinoma
BrdU	Bromodeoxyuridine
CD44v3	CD44 splice variant 3
COL7	Type VII collagen protein
<i>COL7A1</i>	Type VII collagen gene
DEB	Dystrophic epidermolysis bullosa
DMBA	7,12-dimethylbenz[α]anthracene
DMEM	Dulbecco's modified Eagle's medium
EB	Epidermolysis bullosa
EBS	Epidermolysis bullosa simplex
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
gs-RDEB	Generalized severe recessive dystrophic epidermolysis bullosa
HaCaT	Non-tumorigenic immortalized human keratinocyte cell line
HEK293	Human embryonic kidney 293 cell line
HB-EGF	Heparin binding epidermal growth factor –like growth factor
IHC	Immunohistochemistry
JEB	Junctional epidermolysis bullosa
NC-1	Non-collagenous domain-1 of COL7
NC-2	Non-collagenous domain-2 of COL7
NHEK	Normal human epidermal keratinocyte
NMSC	Nonmelanoma skin cancer
MMP	Matrix metalloproteinase
MMP-7	Matrix metalloproteinase-7
MMP-9	Matrix metalloproteinase-9
MMP-13	Matrix metalloproteinase-13
MT1-MMP	Membrane type-1 matrix metalloproteinase
MTT assay	Nonradioactive cell proliferation assay
p53	Tumor protein 53
PDT	Photodynamic therapy
RAdMMP13	Recombinant adenovirus coding for MMP-13
rCOL7	Recombinant type VII collagen
rMMP-13	Recombinant matrix metalloproteinase-13
RDEB	Recessive dystrophic epidermolysis bullosa
RDEB-O	Recessive dystrophic epidermolysis bullosa, severe other
RT-PCR	Real-time polymerase chain reaction
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
serpin	Serine proteinase inhibitor
serpinA1	Serine proteinase inhibitor, clade A, member 1 protein

Abbreviations

<i>SERPINA1</i>	Serine proteinase inhibitor, clade A, member 1 gene
sHB-EGF	Soluble heparin binding epidermal growth factor –like growth factor
siRNA	Small interfering RNA
TIMP	Tissue inhibitor of metalloproteinases
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
<i>TP53</i>	Tumor protein 53 gene
TMA	Tissue microarray
UT-SCC	Human cutaneous squamous cell carcinoma cell line
UV	Ultraviolet

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV.

- I Kivisaari, A.K., Kallajoki, M., Mirtti, T., McGrath, J.A., Bauer, J.W., Weber, F., Königová, R., Sawamura, D., Sato-Matsumura, K.C., Shimizu, H., Csikós, M., Sinemus, K., Beckert, W., and Kähäri, V-M. (2008). Transformation-specific matrix metalloproteinases (MMP)-7 and MMP-13 are expressed by tumour cells in epidermolysis bullosa-associated squamous cell carcinomas, *Br J Dermatol* 158(4):778-85.
- II Kivisaari, A.K., Kallajoki, M., Ala-aho, R., McGrath, J.A., Bauer, J.W., Königová, R., Medvecz, M., Beckert, W., Grénman, R. and Kähäri V-M. (2010). Matrix metalloproteinase-7 activates heparin-binding epidermal growth factor-like growth factor in cutaneous squamous cell carcinoma, *Br J Dermatol* 163(4):726-35.
- III Kivisaari, A.K., Fritsch, A., Ala-aho, R., Farshchian, M., Marjomäki, V.S., Heino, J., Peltonen, J., Grénman, R., Bruckner-Tuderman, L., Kähäri, V-M. Type VII collagen is a novel substrate for cutaneous squamous cell carcinoma associated matrix metalloproteinase (MMP)-13. (manuscript)
- IV Farshchian, M.¹, Kivisaari, A.K.¹, Ala-aho, R., Riihilä, P., Kallajoki, M., Grénman, R., Peltonen, J., Pihlajaniemi, T., Heljasvaara, R. and Kähäri, V-M. (2011). Serpin peptidase inhibitor clade A member 1 (serpinA1) is a novel biomarker for progression of cutaneous squamous cell carcinoma, *Am J Pathol* 179(3):1110-19.

¹These authors contributed equally to this work.

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1 INTRODUCTION

The rising incidence and morbidity of nonmelanoma skin cancers (NMSC) has created great interest in resolving the pathogenesis of these diseases. NMSC consists mainly of basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (SCC) but also cutaneous lymphomas, adnexal tumors, Merkel-cell carcinomas and other rare primary nonmelanoma neoplasms belong to this group of malignancies. This study has focused on SCC of the skin on the grounds that SCC is responsible for the majority of NMSC related deaths.

Ultraviolet (UV) radiation is the most important risk factor for the pathogenesis of sporadic cutaneous SCC and its precursors, actinic keratoses (AK) and Bowen's disease. Other important risk factors for the development of cutaneous SCC are chronic ulcers and immunosuppression. Sporadic cutaneous SCC is generally a disease of the elderly, but also younger individuals are at risk of developing precursor lesions and eventually skin cancer due to the increased recreational sun-exposure. The majority of cutaneous SCC is fairly easy to detect and manage with a simple surgical excision. Though a portion of cutaneous SCCs are clinically aggressive and may metastasize and turn up lethal. The causes for the progressive behaviour of this sub-group of cutaneous SCCs are not well understood.

Generalized severe recessive dystrophic epidermolysis bullosa (gs-RDEB) is a severe inherited skin disorder where a minor trauma induces formation of blisters and skin erosions. Reason for skin fragility is absence of functional anchoring fibrils in the sublamina densa due to mutations of type VII collagen gene (*COL7A1*). RDEB patients are prone to develop clinically progressive cutaneous SCCs into the sites of chronic skin ulceration. These cutaneous SCCs tend to develop into bony prominences rather than into sun exposed areas.

Here, we have studied gs-RDEB –associated cutaneous SCCs representing clinically progressive cutaneous SCCs to elucidate the aggressive molecular background of these carcinomas. The role of matrix metalloproteinase-7 (MMP-7, matrilysin-1) in the pathogenesis of cutaneous SCC was studied both *in vivo* and *in vitro*. Type VII collagen (*COL7*) was identified as a novel substrate for transformation specific endopeptidase matrix metalloproteinase-13 (MMP-13, collagenase-3). Furthermore, additional novel biomarker for the progression of cutaneous SCC, serpinA1, was identified.

2 REVIEW OF THE LITERATURE

2.1 PROGRESSION OF THE CUTANEOUS SQUAMOUS CELL CARCINOMA (SCC)

2.1.1 Squamous cell carcinoma of the skin

Malignancies of the skin are divided into melanoma and nonmelanoma skin cancers (NMSC). NMSC is a heterogeneous group of skin cancers including basal cell carcinomas (BCC), cutaneous squamous cell carcinomas (SCC), skin lymphomas, adnexal tumors, Merkel-cell carcinomas, and other infrequent primary cutaneous neoplasms. Most of NMSC are either BCCs (80%) or SCCs (20%) (Kwa et al., 1992). BCCs grow locally and they usually never metastasize and are often completely curable (Telfer et al., 2008). In contrast, cutaneous SCCs have potential to metastasize and recur and therefore SCCs are responsible for the majority of NMSC related deaths (Weinberg et al., 2007). The major risk factor for the development of sporadic cutaneous SCC is ultraviolet (UV) –light. Typically, these sporadic cutaneous SCCs develop into the sun exposed skin areas of the elderly. Other notable risk factors for the development of cutaneous SCC are chronic ulcers and immunosuppression. Cutaneous SCC can normally be treated with a simple excision, but some of the cases are clinically progressive and may recur or metastasize even after initial surgical removal. At present, there are no generally accepted biomarkers for the assessment of cutaneous SCCs (Dooley et al., 2003). Particularly aggressive cutaneous SCCs develop into the individuals that suffer from generalized severe recessive dystrophic epidermolysis bullosa (gs-RDEB) which is a hereditary mechanobullous blistering disorder (Fine et al., 2009). The gs-RDEB –associated cutaneous SCCs differ from sporadic cutaneous SCCs as gs-RDEB –associated SCCs are not UV-induced but these SCCs develop into the sites of chronic skin ulceration. In this thesis the molecular mechanisms of rapid progression of cutaneous SCC has been studied.

2.1.2 Increasing incidence of cutaneous SCC

The incidence of NMSC, including cutaneous SCC, is increasing worldwide (Alam and Ratner, 2001; Madan et al., 2010). In the United States, NMSC is the most common cancer and the estimated incidence of cutaneous SCC is approximately 300 000 per year (2009) (American Cancer Society, 2011). The incidence of cutaneous SCC has been increasing also in Finland during the past decades and the number of new cases is still increasing (Table 1) (Finnish Cancer Registry, 2011). A 3-8% yearly increase in the incidence of cutaneous SCC has been reported in Finland which is comparable to the reports worldwide (Madan et al., 2010; Finnish Cancer Registry, 2011). Recreational sun-exposure is one of the main reasons for the increasing incidence of cutaneous SCC despite the growing public awareness of the harmful effects of UV-light (Lin et al., 2011). In addition, the incidence of cutaneous SCC

doubles with each 10-degree decrement in geographic latitude and is the highest at the equator (Johnson et al., 1992).

Table 1. Number of cutaneous SCC cases in Finland yearly (mean, years 1983-2009)

	1983-1987	1993-1997	2003-2007	2009
Male	186	321	497	713
Female	225	391	495	669

Reference: Finnish Cancer Registry, www.syoparekisteri.fi (accessed May 2011)

In Finland, cutaneous SCC was the 4th most common cancer in men and the 5th most common cancer in women in the year 2009 (Table 2) (Finnish Cancer Registry, 2011). If the NMSC is taken as a combination of BCC and cutaneous SCC, NMSC was the 2nd most common cancer in males and the most common cancer in females in the year 2009, as 3535 BCC cases were reported in males and 4003 BCC cases in females, respectively (Finnish Cancer Registry, 2011). In concordance with international registration policy, BCC are not included in the official cancer statistics in Finland.

Table 2. Most common cancers in Finland 2009

A. Male (top 7 cancers)

Primary site	Cases	% of all cases	Incidence/100 000 ¹
Prostate	4595	30.9	89.5
Lung	1676	11.3	32.0
Colon	811	5.5	16.0
<i>Cutaneous SCC</i>	713	4.8	12.6
Urinary	708	4.8	13.6
Non-Hodgkin lymphoma	650	4.4	14.6
Melanoma of skin	631	4.2	14.2

B. Female (top 7 cancers)

Primary site	Cases	% of all cases	Incidence/100 000 ¹
Breast	4469	32.0	92.1
Colon	827	5.9	11.9
Uterus	808	5.8	14.0
Lung, trachea	691	5.0	11.0
<i>Cutaneous SCC</i>	669	4.8	7.3
Central nervous system	591	4.2	13.9
Non-Hodgkin lymphoma	559	4.0	9.7
Melanoma of skin	531	3.8	11.5

¹ Age adjusted incidence (“world general population”)

Reference: Finnish Cancer Registry, www.syoparekisteri.fi (accessed May 2011)

2.1.3 The risk factors of cutaneous SCC

Excessive exposure to UV-light is the most common cause of SCC of the skin (Preston and Stern, 1992). UVB radiation (wavelength 315 nm – 280 nm) damages directly DNA and RNA and leads to the generation of mutagenic photoproducts such as cyclopyrimidine dimers and pyrimidine-pyrimidine adducts (Rünger, 2007). UVA (wavelength 400 nm – 315 nm) causes indirect DNA damage via a photo-oxidative-stress-mediated mechanism and is thus less mutagenic than UVB (Ridley et al., 2009). Theoretically, UV-light as a major causative risk factor could be largely avoided, but in practice, sun-avoidance is a huge challenge (Lin et al., 2011). Nevertheless, a physician is obligated to educate the parents on the sun-protection of infants and the education should continue later in life to minimize the risk of sun burn (Mahé et al., 2011). In addition to UV-exposure, chronic ulcers, immunosuppression and certain chronic skin diseases and genetic disorders are important SCC risk factors (Alam and Ratner, 2001). Table 3 summarizes the risk factors for the development of cutaneous SCC.

Table 3. Risk factors for the development of cutaneous squamous cell carcinoma

Exposure to UV radiation (UVA, UVB)
Therapy with methoxalen and UVA
Fair skin type
Ionizing radiation
Genodermatosis (albinism, xeroderma pigmentosum, epidermolysis bullosa)
Chronic inflammation of skin (lupus erythematosus, epidermolysis bullosa)
Chronically injured skin with ulcers (burn scars, leg ulcers, epidermolysis bullosa)
Human papilloma virus infection
Exposure to chemical carcinogens
Immunosuppression
Immunosuppressive medications
Organ transplantation
Osteomyelitis
Sinus tracts
Precursor lesions (actinic and arsenical keratoses)
<i>In situ</i> SCC (Bowen's disease and Erythroplasia Queyrat)
Tobacco smoking
Leukemia and lymphoma
HIV/AIDS

(Modified from Alam and Ratner, 2001)

2.1.4 Pathways involved in the development of invasive cutaneous SCC

Inactivation of tumor protein 53 gene (*TP53*) has probably the most important role in the induction of cutaneous SCC (Benjamin and Ananthaswamy, 2007; Brash, 2006). Tumor protein 53 is also known as tumor suppressor protein 53 (p53). *TP53* mutations occur mainly due to UV radiation. *TP53* mutations are observed in roughly 90% of cutaneous SCCs. Here, the first mutation of the gene encoding p53 leads to the apoptosis of the keratinocytes and the damaged keratinocyte will not undergo malignant transformation. If the keratinocyte already has dysfunctional p53 when additional *TP53* mutation occurs as a result of UV irradiation, keratinocyte will not be

able to undergo apoptosis and instead clonal expansion will occur which is clinically manifested as the development of precursor lesion, actinic keratosis (AK) (Alam and Ratner, 2001). The important p53 pathway is influenced also by cyclin-dependent kinase inhibitor 2A locus which encodes p16INK4a and p14ARF proteins, known to function as tumor suppressors. Inactivation of cyclin-dependent kinase inhibitor 2A locus has been detected in cutaneous SCCs (Pacifico et al., 2008).

Xeroderma pigmentosum patients are at great risk of developing SCC of the skin (Bradford et al., 2011). Here, the major pathway responsible for SCC induction is Xeroderma pigmentosum complementation group C protein. Xeroderma pigmentosum complementation group C protein is crucial in DNA repair and failure to repair UV damaged DNA leads finally to cutaneous SCC development.

Normally, aging is accompanied by a gradual loss of telomeres. The physiological role of human telomerase is to compensate the telomere loss during cell division. In cutaneous SCC, telomerase activity is elevated and this can lead to immortalization of tumor cells (Parris et al., 1999).

Melanocortin-1 receptor variants are associated with fair skin, red hair, and increased risk of developing melanoma. In addition, melanocortin-1 receptor variants are an independent risk factor of developing cutaneous SCC (Bastiaens et al., 2001). The major pathways involved in cutaneous SCC development are shown in Figure 1.

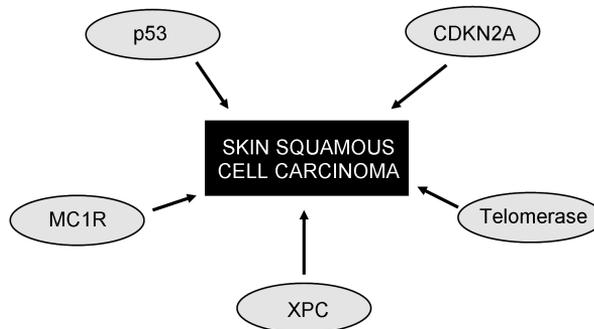


Figure 1. Major pathways involved in the pathogenesis of cutaneous SCC (p53=tumor protein 53, CDKN2A=cyclin-dependent kinase inhibitor 2A, MC1R=melanocortin-1 receptor, XPC=xeroderma pigmentosum complementation group C). (Modified from Madan et al., 2010)

2.1.5 The progression of SCC precursor lesions

Actinic keratosis (AK), also known as solar or senile keratosis, is acknowledged as a precursor lesion of cutaneous SCC, but AK is also suggested to be classified as an early *in situ* SCC (Röwert-Huber et al., 2007). Indeed, AK is increasingly being recognized as an early clinical manifestation of a biological continuum that may ultimately lead to the development of invasive SCC of the skin (Ackerman and Mones, 2006; Moy, 2000). Clinically, UV-induced AK is mainly an asymptomatic erythematous lesion covered with scale. Incidence of AK is increasing (Kim et al.,

2010), as is the incidence of cutaneous SCC, but accurate estimation of the incidence of AK in Finland is not available, as AKs are not reported to Finnish Cancer Registry.

It has been estimated that up to 20% of AK may progress to invasive cutaneous SCC in ten years (Callen et al., 1997). This also means that approximately 80% of AK does not progress to invasive cutaneous SCC during ten year follow-up. On the other hand, AK may develop already to a 40-year-old individual and if extensive sun-exposure continues and AK is left untreated, the corresponding individual has a significant risk of developing cutaneous SCC later in life. At present, there are no molecular markers for a physician to assess which AK will progress to SCC and which will not progress. Currently, this means that all AKs diagnosed should be treated, if possible, as one cannot rule out the possibility of progression to invasive cutaneous SCC (de Berker et al., 2007).

There are several treatment modalities for AK which are summarized in Table 4 (Stockfleth et al., 2008). The treatment of AK can be divided to lesion therapy or field therapy where lesion therapy is intended for treating the visible AK lesions whereas field therapy refers to treatment modalities where not only the visible lesions are treated but also the surrounding sun-damaged skin area is treated according to the principle of field cancerization (Table 4) (Stockfleth et al., 2008). Although there are a number of treatment options available for AK, development of new drugs is ongoing process and ingenol mebutate (PEP005) is probably the next novel drug to encounter clinical use (Siller et al., 2009).

Table 4. Summary of treatment options for actinic keratosis

Treatment	Mode of action	Lesion vs. field therapy	Response rate	Recurrence rate
Cryotherapy	cold-induced disruption	lesion	75-98%	1.2-50%
Laser therapy	Infrared ablation	lesion	~90%	10-15%
Curettage, excision	Surgical removal	lesion	Undocumented	Undocumented
Topical 5-fluorouracil	Interrupts DNA synthesis	field	50%	55%
Chemical peeling	Caustic destruction of epidermis	field	~75%	25-35%
Topical retinoids	Antioxidant effect	field	0-85%	Undocumented
Topical diclofenac	NSAID-COX inhibition	field	50-79%	Undocumented
Photodynamic therapy	Photochemical and photothermal destruction	field	10-99%	Undocumented
Topical imiquimod	Immune response modifier	field	55-84%	10%
Sun protection	Prevention of new AK	field	NA	NA

(Modified from Stockfleth et al., 2008)

Increasing incidence of AK leads to ascending economic burden to the communities, as AK treatments are generally not inexpensive. In clinical assessment, it could be valuable if one could determine at the precursor stage, whether the management of AK is necessary or if the lesions could be followed up safely without active treatment.

2.1.6 Recurrence and metastasis of cutaneous SCC

SCC of the skin is a locally invasive, malignant tumor and it has potential to recur and metastasize, with an overall 5-year recurrence rate of 8 percent for primary tumors and a 5-year rate of metastasis of approximately 5 percent (Rowe et al., 1992). The relatively low general risk of recurrence and metastatic spread is substantially higher if the primary lesion is large, as lesions with a diameter >2 cm recur at a rate of 15 percent, and they metastasize at a rate of 30 percent (Rowe et al., 1992). The location of a primary tumor is also relevant in the assessment of the risk of recurrence and metastasis, as the cutaneous SCC of the lip and ear recur and metastasize at a rate of 10 to 25 percent. If the cutaneous SCC recurs, then the risk of metastasis is elevated up to 25-45 percent depending on the location of the tumor (Rowe et al., 1992). Moreover, cutaneous SCCs arising in injured or chronically diseased skin are associated with a risk of metastasis that reaches 40 percent (Königová and Rychterová, 2000; Novick et al., 1977).

The cutaneous SCC is often taken as a single, uniform disease, but actually there are many unique histopathological features by which SCC can be divided into distinct subtypes (Yanofsky et al., 2011). Distinguishing histopathological variants through microscopic evaluation has clinical importance, as cutaneous SCC subtypes have different risk of recurrence and metastasis (Lohmann and Solomon, 2001). Poorly differentiated appearance, depth of more than 4 mm and perineural invasion are all features that increase significantly the risk of cutaneous SCC recurrence and metastasis (Alam and Ratner, 2001).

Table 5. Risk factors for recurrence and metastasis of cutaneous SCC

Variable	Approximate relative risk*	
	Recurrence	Metastasis
Rapid tumor growth	-	-
Tumor size >2 cm	2	2
Tumor location (lip/ear)	2	3
Immunosuppression	-	2
Previous radiation treatment	-	-
Previously treated SCC	3	4
RDEB –associated SCC	-	-
Tumor depth > 4 mm	2	5
Poor histologic differentiation	2	3
Acantholytic features	-	-
Spindle-cell features	-	-
Perineural invasion	5	5

*A relative risk of 1 is defined as the likelihood of recurrence or metastasis of a small primary cutaneous SCC. Dashes indicate an association with increased risk, but of which there are insufficient data to estimate the relative risk. (Modified from Alam and Ratner, 2001)

In order not to underestimate the risk of recurrence and metastasis in the cases pointed out above, the elevated risk should be taken into account when a physician makes a plan for follow-up after the initial surgical treatment. The risk factors and relative risks associated with recurrence and metastasis of cutaneous SCC are summarized in Table 5.

2.1.7 Cutaneous SCC arising from chronic ulcers

UV-induced SCCs are practically always preceded by precursor lesions. This is not usually the case with chronic ulcer related cutaneous SCCs, as these SCCs generally lack the precursor lesion. Though, an *in situ* SCC has been reported on a patient with RDEB (Souza et al., 2005). SCC may develop into the skin sites which are chronically injured, like burn scars (Marjolin's ulcer) or chronic leg ulcers (Baldursson et al., 1995; Königová and Rychterová, 2000; Ogawa et al., 2006).

In the year 1828, a French surgeon Jean-Nicolas Marjolin characterized ulcer with malignant degeneration which developed into a burn scar. The typical feature of a burn scar SCC of the skin is the latency period of 20-50 years after the skin burn (Copcu et al., 2003). Burn scar SCC act clinically aggressively which is also the case with gs-RDEB –associated cutaneous SCC which are introduced in the next section. SCC of the skin can also occur in chronic scarring inflammatory disease like discoid lupus erythematosus (Simpson et al., 2010).

2.2 EPIDERMOLYSIS BULLOSA (EB) –ASSOCIATED PROGRESSIVE CUTANEOUS SCC

2.2.1 Inheritable epidermolysis bullosa (EB)

Epidermolysis bullosa (EB) is a heterogenous group of mechanobullous skin disorders associated with widespread skin blistering, erosions and chronic wounds originally classified by Pearson (Pearson, 1962). The modern classification of inheritable EB was formed by the Third International Consensus Meeting on Diagnosis and Classification of EB, where EB was divided into four major types and several subtypes relied on the combination of clinical findings, targeted proteins and the ultrastructural level where the blisters arise (Fine et al., 2008). The major types are EB simplex (intraepidermal type), junctional EB (dermo-epidermal junction type), dystrophic EB (dermolytic type) and Kindler syndrome (mixed type) (Fine et al., 2008). The level of blistering in corresponding EB types is shown in Figure 2 and the proteins which are mutated in different major types of EB are listed in Table 6.

In dystrophic EB (DEB), the level of blister formation is deeper than in the other EB forms. In DEB, the blisters form in the sublamina densa and in the upper dermis while in EBS the skin separation occurs in the epidermis and in JEB the separation level is in the dermo-epidermal junction. In Kindler syndrome a specific skin cleavage site cannot be pointed out as the cleavage can occur at different levels. Due to the deep

level of blistering in DEB, the blisters may heal with mutilating scar formation and chronic ulcers often develop (Bruckner-Tuderman et al., 1989).

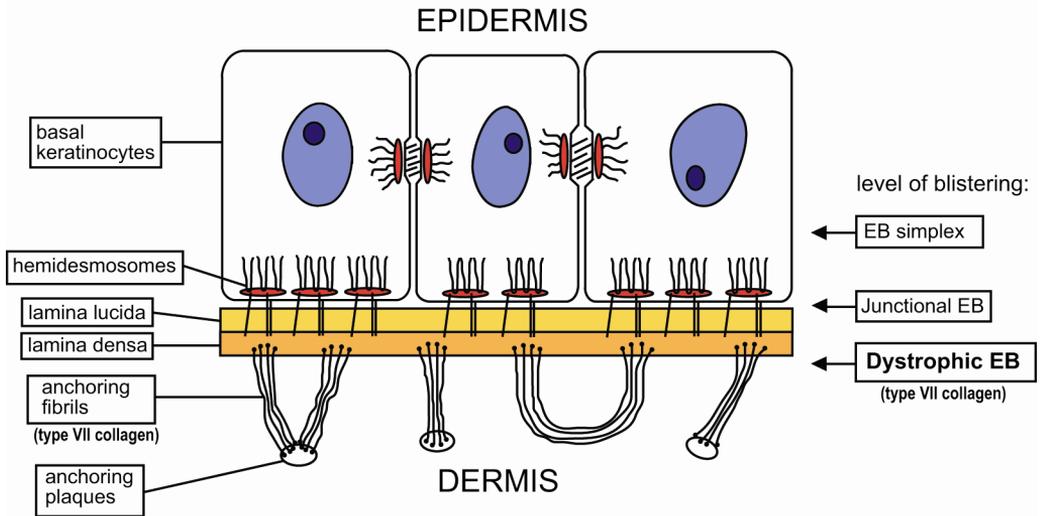


Figure 2. The schematic representation of the dermal-epidermal junction and the major epidermolysis bullosa (EB) subtypes according to the level of blistering. In addition to EB simplex, junctional and dystrophic EB, Kindler syndrome is also classified as a major EB subtype, but in Kindler syndrome the level of blistering is mixed.

Table 6. The level of basement membrane cleavage and targeted proteins of major EB types

Level of skin cleavage	Major EB type	Targeted protein(s)
Intraepidermal	EBS	Keratins 5 and 14; plectin; $\alpha 6 \beta 4$ integrin; plakophilin-1; desmoplakin
Intra-lamina lucida	JEB	Laminin-332; type XVII collagen; $\alpha 6 \beta 4$ integrin; bullous pemphigoid antigen 1-e (BPAG1-e, BP230)
Sub-lamina densa	DEB	Type VII collagen (COL7)
Mixed	Kindler syndrome	Kindlin-1

EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa, DEB, dystrophic epidermolysis bullosa (Modified from Fine et al., 2008 and Groves et al. 2010)

The mutations in the gene encoding the type VII collagen protein (COL7), *COL7A1* gene, are the molecular basis of DEB (Uitto et al., 1994). The types of mutations in *COL7A1* range from premature termination codon causing mutations, small insertions or deletions or splice junction mutations to subtle missense mutations. In fact, over 500 distinct mutations have been disclosed (Von Bartenwerffer et al., 2011). DEB is divided into dominant or recessive forms according to the mode of inheritance (Fine et

al., 2008). Recessive DEB (RDEB) is generally clinically more severe than dominant DEB, but the genotype-phenotype correlations are not yet fully understood (Von Bartenwerffer et al., 2011). The most severe subtype of DEB is generalized severe RDEB (gs-RDEB), previously designated as RDEB, Hallopeau-Siemens.

The recessive forms of EB are relatively common in Scandinavia. Especially recessive JEB has been noted in the northern part of Sweden and Norway, but also a rise in RDEB has been noted in Scandinavian countries due to the immigration from countries where cousin marriage is common (Vahlquist and Tasanen, 2010). The estimated prevalence of EB in different countries is shown on Table 7. It is apparent that the prevalence of DEB in Finland is above the average prevalence worldwide. In Finland, the prevalence of DEB was evaluated in 1980's, when it was 8.8 per million (Kero, 1984).

Table 7. Prevalences of EB per million inhabitants

	DEB	JEB	EBS
Scotland	24.4	0.4	32.0
Norway	9.3	-	24.3
Finland	8.8	0.2	15.1
Croatia	6.6	1.5	1.5
Saudi Arabia	3.7	0.0	1.7
Japan	3.5	0.2	4.0
North Ireland	3.3	0.7	28.0
U.S.A.	2.4	0.4	4.6
South Africa	1.2	0.7	0.8

DEB, dystrophic epidermolysis bullosa; JEB, junctional epidermolysis bullosa, EBS, epidermolysis bullosa simplex. (Modified from Horn and Tidman 2002)

In addition to the inherited forms of epidermolysis bullosa described above, there is also an acquired form of dystrophic epidermolysis bullosa, epidermolysis bullosa acquisita, which is an autoimmune disease (Woodley et al., 1984). In epidermolysis bullosa acquisita, there are autoantibodies directed against COL7 which results in subepidermal blistering. Cutaneous SCC is a very rare complication in epidermolysis bullosa acquisita (Yamada et al., 2005).

2.2.2 Type VII collagen (COL7) in human skin

The expression of COL7 gene (*COL7A1*) is restricted to a number of different epithelia. In skin, COL7 is synthesized by both human epidermal keratinocytes and dermal fibroblasts (Ryynänen et al., 1992). COL7 is one of the 29 genetically distinct extracellular matrix (ECM) collagens which have been described in vertebrates (Myllyharju and Kivirikko, 2004; Söderhäll et al., 2007).

Characteristic feature for all collagens is a triple helical conformation but they often contain so called non-collagenous domains which are not triple helical (Uitto, 2008a). All collagen molecules consist of three subunits which form either homotrimers or heterotrimers. COL7 is a homotrimeric molecule as it is formed by three monomers

encoded by a single gene, *COL7A1* (Christiano et al., 1994). COL7 monomer consists of a non-collagenous domain-1 (NC-1) in the aminoterminal end and following triple helical domain which is interrupted by a non-collagenous hinge region and then followed by the non-collagenous domain-2 (NC-2) in the carboxyterminal end (Lapiere et al., 1994). Altogether the size of COL7 monomer is approximately 290 kDa (Chung and Uitto, 2010). COL7 monomer, $\alpha 1(\text{VII})$ polypeptide, and its subdomain organization is presented in Figure 3.

In human skin, COL7 is the exclusive component of anchoring fibrils. The main function of anchoring fibrils is to take part in the attachment of the epidermis to the dermis (Sakai et al., 1986). To ensure the attachment of the epidermal layer to the dermis, anchoring fibrils do not only affix the basement membrane to the underlying upper dermis, but anchoring fibrils also interconnect type I collagen containing dermal fibrils (Villone et al., 2008). Type I collagen is the most abundant collagen in the body and it is also a major ECM component in human skin (Weber et al., 1984).

During the formation of anchoring fibrils, COL7 monomers first form homotrimer molecules which are secreted to the extracellular space where they build up tail-to-tail anti-parallel homodimers with the amino-terminal NC-1 domains at both ends of the dimer molecule (Colombo et al., 2003). Subsequently, a large number of these dimers aggregate laterally to make the functional unit, anchoring fibril. In RDEB, as both *COL7A1* alleles are mutated, full length COL7 monomers are not produced and thus normally functioning anchoring fibrils are not made and anchoring fibrils are either totally absent or abnormal and reduced in number.

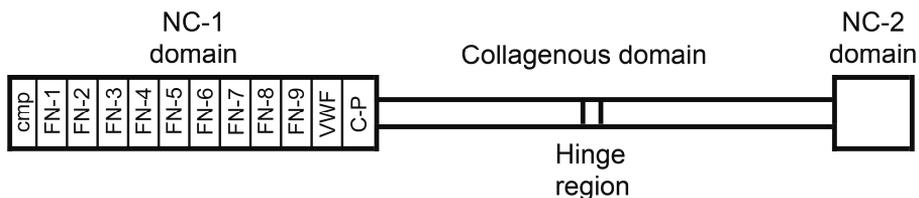


Figure 3. Type VII collagen (COL7) monomer, $\alpha 1(\text{VII})$ polypeptide, and its subdomain organization. The non-collagenous domain-1 (NC-1) consists of several subdomains with homology to the cartilage matrix protein (cmp), nine consecutive segments with homology to the fibronectin type III domains (FN1-9), a segment with homology to the A domain of von Willebrand factor (VWF), and a cysteine-proline rich region (C-P). The triple helical collagenous domain is interrupted by non-collagenous hinge region. The non-collagenous domain-2 (NC-2) does not have any subdomains. (Modified from Lapiere et al., 1994)

2.2.3 Recessive dystrophic epidermolysis bullosa (RDEB) and skin cancer

RDEB is subdivided to generalized severe RDEB (gs-RDEB, previously designated as Hallopeau-Siemens RDEB) and RDEB –severe other (RDEB-O, previously designated as RDEB, non-Hallopeau-Siemens). In addition, there are rare variants of RDEB: inversa, pretibial, pruriginosa, centripetalis and bullous dermolysis of the new born (Fine et al., 2008). As these rare variants do not have an increased tendency for skin malignancies, they are not further discussed here.

gs-RDEB is a severe inherited disorder which clinically manifests with skin blistering after a minor mechanical trauma (Bruckner-Tuderman, 1999; Horn and Tidman, 2002). Skin blistering may lead to mutilating scarring and often poorly healing chronic skin ulcers develop. The keratinocytes in these chronic ulcers display a growth-activated phenotype (Smoller et al., 1990). In gs-RDEB, the anchoring fibrils are practically absent while in RDEB-O anchoring fibrils may be present but are poorly formed and reduced in number (Bruckner-Tuderman, 1999; Uitto et al., 1994).

The gs-RDEB patients are prone to develop progressive cutaneous SCC into the sites of chronic ulceration (Reed et al., 1974). The cumulative risk of developing cutaneous SCC and subsequent death in patients with gs-RDEB by the age of 55 is greater than 90% and 78%, respectively (Fine et al., 2009). This magnitude of developing SCC of the skin in a genetic disease is only exceeded by patients suffering from xeroderma pigmentosum, where xeroderma pigmentosum complementation group C mutations result in dramatic hypersensitivity to sunlight and a greater than 2000-fold increase in the risk of developing skin cancers (South and O'Toole, 2010). In contrast to xeroderma pigmentosum, RDEB –associated cutaneous SCCs develop into the sites of chronic friction and to the bony prominencies but not to the sun-exposed areas (McGrath et al., 1992).

A clinically fundamental difference between sporadic, UV-induced cutaneous SCC and RDEB –associated cutaneous SCC is the age when the cancer is diagnosed. Sporadic cutaneous SCC is almost exclusively a disease of elderly while RDEB – associated cutaneous SCC develops often at 3rd or 4th decade of life (Mallipeddi, 2002). The youngest RDEB –associated SCC case reported is a 6-year-old patient, pointing out the importance of monitoring the entire skin of RDEB-patients on a regular basis already in childhood (Shivaswamy et al., 2009).

In addition to the substantially increased risk of SCC of the skin in RDEB, the risk of melanoma is moderately increased during the childhood, as the risk of developing melanoma is 2.5% by the age of 12 in RDEB while a life time risk in general population is 1.35-2.7% (Chorny et al., 1993; Fine et al., 2009). SCCs occur with increased incidence not only in RDEB patients but also in JEB patients, while dominant DEB do not have an increase in cutaneous SCC incidence (Fine et al., 2009). The risk of skin cancer by the age of 30 in DEB subtypes is summarized on Table 8.

Table 8. Risk of skin cancer by age 30 and risk of EB related death in dystrophic epidermolysis bullosa (DEB)

	DDEB, generalized	RDEB, severe generalized	RDEB, generalized other
Cutaneous SCC	none	3+	2+
Malignant melanoma	none	1+	none
Basal cell carcinoma	none	none	none
Death related to EB	none	4+	2+

DDEB, dominant dystrophic epidermolysis bullosa
RDEB, recessive dystrophic epidermolysis bullosa

Risk scale: none, 1+, 2+, 3+, 4+ (Modified from Fine et al., 2008)

2.2.4 The special characteristics of RDEB –associated cutaneous SCC

Most RDEB –associated cutaneous SCCs are histologically well differentiated (McGrath et al., 1992). Although poor differentiation is a risk factor for recurrence and metastasis in skin SCC in general (Alam and Ratner, 2001), even histologically well differentiated SCC in RDEB can end up in secondary spread and thus it is not possible to predict tumor behaviour simply on the basis of histological grade alone in RDEB –associated SCC (Mallipeddi, 2002).

Repetitive tissue damage and tissue stress has been proposed as a background for tumor promotion in RDEB in earlier studies (Goldberg et al., 1988). The tissue stress hypothesis was further supported by the observation of growth-activated keratinocytes in biopsies from RDEB scarred skin (Smoller et al., 1990). The reduced activity of natural killer cells has been noted in severe EB forms which may have a role in tumor growth, as natural killer cells play a key role in the immune response against certain cancers and viruses (Tyring et al., 1989). Furthermore, elevated basic fibroblast growth factor levels were noted in the urine of RDEB patients which may contribute to the elevated fibroblast derived collagenase and the development of cutaneous SCC, but it remains unproven whether this finding is a result of repetitive tissue injury or a distinct RDEB related pathology (Arbiser et al., 1998).

Overexpression of breast cancer associated musin 1, cell surface associated, has been documented in RDEB –associated SCC, but the expression level was comparable with the sporadic cutaneous SCC studied and therefore musin 1 overexpression seemingly does not explain the increased aggressiveness of the RDEB –associated cutaneous SCCs (Cooper et al., 2004). Comparing the gene expression data of RDEB –associated cutaneous SCC to sporadic cutaneous SCC revealed that in RDEB –associated cutaneous SCC the expression of insulin-like growth factor-binding protein-3 is reduced, which in physiologic situation has proapoptotic effects (Mallipeddi et al., 2004). This study concluded that reduced expression of insulin-like growth factor-binding protein-3 in RDEB –associated cutaneous SCC may partly explain the aggressive behaviour of these tumors.

Matrix metalloproteinases (MMP) are expressed during ECM remodelling and during keratinocyte, cancer cell, fibroblast and inflammatory cell migration (Ala-aho and Kähäri, 2005; Toriseva and Kähäri, 2009; Vihinen and Kähäri, 2002). Increased amounts of MMP-1, MMP-2, MMP-3, and MMP-9 have been found in RDEB skin with accompanied lower level of tissue inhibitor of metalloproteinase-1 (TIMP-1) (Bodemer et al., 2003). These findings could be related to the phenotypic variability of RDEB, but also to cutaneous SCC formation. In addition, a genetic polymorphism increasing the activity of the MMP-1 promoter has been shown to modify the severity of RDEB disorder as such, but it remains unclear whether the MMP-1 promoter activating polymorphism is related to SCC development in the skin (Titeux et al., 2008).

Though COL7 may be totally absent in gs-RDEB, it has been proposed that COL7 is required for human epidermal tumorigenesis (Ortiz-Urda et al., 2005). This conclusion was derived from observations on the tumorigenic conversion of keratinocytes cultured from RDEB patients and xenotransplanted to immunodeficient mice. In this experimental model, keratinocytes expressing NC-1 domain of COL7 developed cancer while keratinocytes which did not express NC-1 domain of COL7

did not develop cutaneous SCC. However, it has been later shown in actual clinical situation that RDEB patients do develop cutaneous SCC even when COL7 is totally absent (Pourreyaon et al., 2007). The tumor samples studied with the NC-1 domain specific antibody was negative in these clinical cases which proves that NC-1 domain of COL7 is not a prerequisite for cutaneous SCC development in these RDEB cases.

The literature is somewhat controversial, whether COL7 is considered as a tumor suppressor or tumor promoter. Recent experimental data provide evidence that the loss of COL7 using small interfering RNA technique promotes cutaneous SCC growth and invasion, suggesting a role for COL7 as a tumor suppressor (Martins et al., 2009). In summary, many possible explanations for the aggressive behaviour of RDEB – associated SCC has been suggested but comprehensive understanding is still lacking.

2.2.5 The diagnosis and management of RDEB –associated cutaneous SCC

The clinical diagnosis of a cutaneous SCC in RDEB patient is not an easy task, as there are practically always many healing erosions and chronic ulcers simultaneously present on the patient skin. Regular skin checks (every 3-6 months) are recommended from the age of 10 years and every 3 months from 16 years onwards and skin biopsies should be taken whenever malignancy is suspected (Venugopal and Murrell, 2010). Photography may be a useful tool for monitoring the poorly healing ulcers of RDEB patients (Murrell, 2003).

There are no good ways of preventing RDEB –associated cutaneous SCC as long as chronic ulceration takes place and this will happen until effective restorative gene therapy is available. Though, isotretinoin up to a dose of 0.5 mg/kg/d can be used in order to delay RDEB –associated cutaneous SCCs as this regimen was found safe for RDEB patient in a phase I trial (Fine et al., 2004). Further studies are though needed to evaluate the possible efficacy of isotretinoin treatment in chemoprevention of skin cancer in RDEB patients.

Bowen's disease (*in situ* SCC) has been reported on RDEB patients and photodynamic therapy (PDT) with 5-aminolevulinic acid has been used for therapy for these lesions (Souza et al., 2005). While PDT is effective for the management of UV-induced Bowen's diseases, excision is rather recommended for the therapy of Bowen's disease in RDEB patient as the biopsy may not have contained the invasive SCC adjacent to the *in situ* SCC (Venugopal and Murrell, 2010).

Once cutaneous SCC has been diagnosed, the patient should be quickly managed by a team including a plastic surgeon familiar with EB and a dermatologist. The standard treatment for a primary RDEB –associated SCC is full thickness excision with wide margins (Fine et al., 2009; Yamada et al., 2004). Mohs micrographic surgery has been used and reported for the treatment of RDEB –associated SCC (Saxena et al., 2006), but conventional plastic surgery may be sufficient as a longitudinal follow-up showed that Mohs surgery did not reduce local recurrence or number of regional metastases (Fine et al., 2009). The diagnostic procedure includes the staging of the tumor via imaging guided by clinical suspicion with methods including ultrasound, computerized tomography, magnetic resonance imaging and positron emission tomography (Venugopal and Murrell, 2010). Biopsies of sentinel nodes have also been reported for staging and prognostic purposes, but the value remains inconclusive (Fine, 2004;

Rokunohe et al., 2008). Because of the progressive nature of RDEB –associated SCC, patients requiring surgical treatment may also need adjunctive chemotherapy and/or radiotherapy (Bastin et al., 1997; Lentz et al., 1990).

If the RDEB –associated cutaneous SCC is unresectable, one may consider using epidermal growth factor receptor (EGFR) inhibitors. Cetuximab is a monoclonal antibody against EGFR and it is approved for the treatment of head and neck cancers, colorectal cancers and lung cancers (Reuter et al., 2007) and it has also been used for cutaneous SCC (Suen et al., 2007). EGFR is overexpressed in SCC tumor cells, which leads to the stimulation of SCC tumor cell growth (Quadros et al., 2004). Successful cetuximab therapy of unresectable RDEB –associated SCC of a 24-year old patient has been reported (Arnold et al., 2009). Acneiform rash is reported as a common cetuximab side effect, but no additional problems have been noted in RDEB patients with cetuximab therapy (Arnold et al., 2009; Venugopal and Murrell, 2010).

In addition to cetuximab, also other EGFR inhibitors erlotinib and gefitinib as well as multi-tyrosine kinase inhibitor dasatinib could be considered for treatment of RDEB –associated cutaneous SCC as clinical trials on recurrent or metastatic cutaneous SCCs are either completed or going on with these drugs (Clinical Trials registry, accessed 2011).

2.2.6 The cure of RDEB – future therapies

Until recently, RDEB management has been limited to palliative methods such as bandaging, nutritional support, analgesia, the treatment of infections, esophageal dilatation, the surgical treatment of syndactylies and eventually the treatment of cutaneous malignancies (Eisenberg and Llewelyn, 1998; Ingen-Housz-Oro et al., 2004; Lin and Golianu, 2006; Mellerio et al., 2007; Okada et al., 2006; Vahlquist and Tasanen, 2010). During the last decade, promising interventions are being developed for the restoration of COL7 and anchoring fibrils using allogenic or autologous gene therapy, protein therapy and cellular transplantation (De Luca et al., 2009; Ferrari et al., 2006; Uitto, 2008b). Advances in gene therapy are obviously eagerly waited as successful gene correction would benefit the patients in their everyday life and eventually gene correction might also prevent the development of skin cancer.

COL7 production has been successfully restored by lentiviral transduction of *COL7A1* gene into RDEB keratinocytes and these gene-corrected cells were able to regenerate human skin on immuno-deficient mice (Chen et al., 2002). Genomic DNA vector containing entire *COL7A1* locus in a P1-derived artificial chromosome has been transferred to RDEB keratinocytes *in vitro* by microinjection, and biosynthesis of $\alpha 1(\text{VII})$ procollagen was detected for one year in a cell culture (Mecklenbeck et al., 2002). Additional viral *COL7A1* induction has been done by injecting a self-inactivating lentiviral vector intradermally into immunodeficient mice and into a human RDEB skin equivalent grafted onto immunodeficient mice (Woodley et al., 2004b). A non-viral approach to stable correction of *COL7A1* gene in RDEB epidermal cells has been accomplished by using phi C31 bacteriophage integrase-based gene transfer (Ortiz-Urda et al., 2002).

Protein based therapy has also been studied, where COL7 protein has been manufactured *in vitro* and thus injected intradermally into a mouse skin and RDEB

human skin equivalent on mice (Woodley et al., 2004a). This approach has though a little value on clinical aspect as no intrinsic COL7 production is induced.

As a cell based strategy, genetically engineered RDEB fibroblasts have been injected intradermally into intact RDEB skin and this method normalized locally the COL7 production in skin (Ortiz-Urda et al., 2003). A similar approach has been tried in RDEB patients, who received intradermal injections of allogenic fibroblasts which resulted in increase in anchoring fibrils (Wong et al., 2008). The fibroblast based cell therapy has been successfully repeated in another dermatology clinic (Yan and Murrell, 2010). Moreover, allogenic mesenchymal cells have been administered intradermally to chronic ulcerated skin sites of RDEB sufferers which led to re-epithelization and COL7 was detected along the basement membrane (Conget et al., 2010).

In addition to gene correction and protein based therapy, skin grafting on five RDEB patients has been done using bilayered living human skin construct characterized by a normal *COL7A1* expression which resulted in a temporary relief of clinical symptoms (Fivenson et al., 2003).

The locally administered gene therapies may indeed have a role in ameliorating the symptoms, but greater effect on the patient would be achieved by systemic gene correction. Gene therapy using embryonic bone marrow cell transplantation on fetal circulation diminished the skin symptoms of a dominant negative *Col7a1(-/-)* mice (Chino et al., 2008). In another murine model, systemically administered congenic bone marrow cells were efficient in homing to the skin and they improved the epidermal-dermal integrity (Tolar et al., 2009). The *in vitro* studies encouraged for trying allogenic bone marrow stem-cell transplantation for RDEB patients. Here, allogenic bone marrow stem cell transplantation increased COL7 deposition on the recipient's skin, but not without complications as one patient out of seven died as a consequence of graft rejection and infection (Wagner et al., 2010). Recently, patient-specific stem cells (induced pluripotent stem cells) have been generated from three RDEB patients and these induced pluripotent stem cells have been successfully gene corrected to express *COL7A1* which has been a first autologous approach for hematopoietic and nonhematopoietic grafting (Tolar et al., 2011).

Nevertheless, curative gene therapy is not yet an available method for treating RDEB, but promising steps has been taken towards better management of this devastating disease. While the cure is not available, one has to focus on palliating measures and early diagnosis and treatment of complications such as cutaneous SCC.

Another way of going about the issue of any severe inherited disorder is advancing the prenatal diagnostics which has been done also in RDEB (Pfundner et al., 2003). Prenatal diagnostics are obviously embodied with profound ethical aspects to pay attention to (Greely, 2011).

2.3 ENDOPEPTIDASES IN SCC OF THE SKIN

2.3.1 Role of matrix metalloproteinases (MMPs) in tumor growth

MMPs contribute to the homeostasis of many tissues and participate in several physiological processes, such as the proteolysis of ECM scaffold in skin (Löffek et al., 2011). Cancer, including skin cancer, arises from the mutations in genes that regulate the essential pathways of cell function leading to uncontrolled growth of cells (Hanahan and Weinberg, 2000). During the development of cancer, as uncontrolled growth occurs, ECM proteolysis is simultaneously altered (Kessenbrock et al., 2010). Here, tumor cells participate in several interactions with the surrounding microenvironment involving ECM scaffold, growth factors and cytokines associated with ECM, as well as surrounding cells in the tumor stroma, like fibroblasts, endothelial cells and inflammatory cells (Kessenbrock et al., 2010; Murphy, 2008). The coordinated breakdown and remodelling of ECM are crucial events for skin cancer invasion and metastasis (Ala-aho and Kähäri, 2005; Kerkelä and Saarialho-Kere, 2003).

Matrix metalloproteinases (MMP) are a family of endopeptidases, which contain a zinc-ion in the catalytic site of the enzyme (Kähäri and Saarialho-Kere, 1997). MMP gene family consists of 24 members and is divided into six subgroups according to their domain structure and ability to degrade a particular subset of ECM proteins: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs (Sternlicht and Werb, 2001). As MMPs are collectively capable of cleaving essentially all ECM proteins, it is conceivable that MMPs are associated with tumorigenesis, including skin cancer invasion and metastasis (Vihinen and Kähäri, 2002). MMP-inhibitors have been developed and clinical cancer treatment trials have shown that certain MMP-inhibitors limit the growth of the tumor which implies that MMP-inhibitors may come to clinical use in the future (Gialeli et al., 2011; Vihinen et al., 2005).

MMP-upregulation is seen in many different types of malignant tumors, including cutaneous SCC (Ala-aho and Kähäri, 2005; Kerkelä and Saarialho-Kere, 2003), malignant melanoma (Hofmann et al., 2005; Zigrino et al., 2009), breast cancer (Köhrmann et al., 2009), gastric cancer (Yasui et al., 2005), colorectal cancer (Zucker and Vacirca, 2004), thyroid cancer (Cho Mar et al., 2006) and prostate cancer (Littlepage et al., 2010). Classically, MMPs are thought to have a major role in ECM degradation (Liotta et al., 1980), but now it is evident that MMPs do more than just degrade physical barriers (Kessenbrock et al., 2010). MMPs contribute to the tumor development by taking part to epithelial-mesenchymal transition (Kalluri and Weinberg, 2009), activating growth signals like TGF- β (Yu and Stamenkovic, 2000), regulating apoptosis (Mitsiades et al., 2001), contributing to vascular events (Littlepage et al., 2010), interacting with adipocytes (Wu and Smas, 2008) and by participating in metastatic niche formation (Heissig et al., 2002; Kaplan et al., 2005). On the other hand, MMPs may also have a protective role at some stages of tumor development (Decock, 2011).

Certain MMPs are suggested to have important role in invasion and metastasis of tumors. In this study, we have focused on MMP-7, MMP-13 and MMP-9 as these MMPs take part in all essential steps of tumor development as a group (Table 9)

(Gialeli et al., 2011). Interestingly, both MMP-7 (Wang et al., 2005) and MMP-13 (Hernández Ríos et al., 2009) are capable of activating the pro-form of MMP-9 which makes studying the co-expression of these MMPs relevant.

MMP-7, also known as matrilysin-1, is the smallest member of MMP family lacking the hinge region and the hemopexin domain (Woessner and Taplin, 1988). MMP-7 is synthesized as an inactive 28 kDa zymogen form (proMMP-7) which is proteolytically activated into 19 kDa active form (Imai et al., 1995; Ueno et al., 1999; Yamamoto et al., 2003). In healthy skin, MMP-7 is not produced by keratinocytes but it is produced in the dermal sweatglands (Saarialho-Kere et al., 1995). In cancer, MMP-7 has been implicated in the early stages of intestinal tumorigenesis (Fingleton et al., 1999) and in metastasis of endometrial and gastrointestinal cancers (Shiomi and Okada, 2003). In addition MMP-7 expression is upregulated in several epithelial cancers, such as breast cancer (Basset et al., 1990; Köhrmann et al., 2009), lung cancer (Muller et al., 1991) and skin cancer (Karelina et al., 1994).

Table 9. The possible role of MMP-7, MMP-13 and MMP-9 in cancer progression

MMP	Activity	Effect
<i>Cancer cell invasion</i> MMP-9, MMP-13	Proteolytic	Degrade barriers
<i>Cancer cell proliferation</i> MMP-7, -9	Shedding of membrane-anchored ligands	Proliferation
<i>Cancer cell apoptosis</i> MMP-7	Cleavage of Fas ligand	Anti-apoptotic
<i>Tumor angiogenesis</i> MMP-13	Degradation of type IV collagen	Regulation of angiogenesis
<i>Cell adhesion, migration, epithelial-mesenchymal transition</i> MMP-7, -13	Degradation of type IV collagen, shedding of E-cadherin	Promote migration, induce epithelial-mesenchymal transition
<i>Immune surveillance</i> MMP-7	Cleavage of chemokines, release of α 1-proteinase inhibitor	Affect leukocyte infiltration, decrease cancer cell sensitivity to natural killer cells

(modified from Gialeli et al., 2010)

In skin cancer, MMP-7 overexpression has been noted in extramammary Paget's disease (Kuivanen et al., 2004), melanoma (Kawasaki et al., 2007), in Merkel-cell

carcinoma (Fernández-Figueras et al., 2007) and also in BCC and cutaneous SCC (Impola et al., 2005; Hartmann-Petersen et al., 2009). MMP-7 may have multiple roles on human tumorigenesis like in invasion, intravasation, regulation of inflammation, tumor cell proliferation, apoptosis and angiogenesis (Table 9) (Ii et al., 2006; Rémy and Trespeuch, 2005). The effect of MMP-7 may not always benefit the tumor growth, as there is a discrepancy whether the overexpression of MMP-7 in lung cancer cells correlates with good or poor prognosis (Liu et al., 2007; Stenvold et al., 2011).

MMP-13, also known as collagenase-3, was originally found in the human breast cancer tissue (Freije et al., 1994). MMP-13 is produced as a 60 kDa zymogen form (proMMP-13) that is activated via a 50 kDa intermediate form to active 48 kDa form (Knäuper et al., 1996a). *MMP13* gene has a very restricted expression pattern and MMP-13 protein has broad substrate specificity compared with other members of MMP family (Ala-aho and Kähäri, 2005; Tardif et al., 2004). In physiological situations, MMP-13 is produced only during bone development and bone remodelling (Stähle-Bäckdahl et al., 1997).

MMP-13 is expressed in various pathological conditions like gingival and fetal wound repair (Ravanti et al., 1999; Ravanti et al., 2001), osteoarthritis (Reboul et al., 1996; Troeberg and Nagase, 2011), rheumatoid synovium (Kim et al., 2011b; Lindy et al., 1997), chronic skin ulcers (Vaalamo et al., 1997), intestinal ulcers (Vaalamo et al., 1998), ulcerative colitis (Rath et al., 2010), chronic periodontitis (Silva et al., 2008; Uitto et al., 1998), atherosclerosis (Ketelhuth and Bäck, 2011; Sukhova et al., 1999) and aortic aneurysms (Mao et al., 1999; Rizas et al., 2009).

In malignancies, MMP-13 expression has been found in BCC of the skin (Airola et al., 1997; Chu et al., 2007), head and neck SCC (Dünne et al., 2003; Johansson et al., 1997; Junttila et al., 2007, Stokes et al., 2010), malignant melanoma (Corte et al., 2005), vulvar SCC (Johansson et al., 1999), breast carcinoma (Freije et al., 1994; Nannuru et al., 2010), urothelial carcinoma (Boström et al., 2000; Wallard et al., 2006), chondrosarcoma (Söderström et al., 2001), esophageal carcinoma (Etoh et al., 2000), gastric cancer (Elnemr et al., 2003), lung cancer (Thomas et al., 2000), prostate cancer (Escaff et al., 2010), neuroectodermal tumors (Bodey et al., 2000), and in thyroid cancer cell lines (Baldini et al., 2004). Notably, MMP-13 is not expressed by keratinocytes or stromal cells in healthy human skin (Impola et al., 2005).

MMP-9, also known as gelatinase-B, is a 92-kDa gelatinase with elastolytic activity (Shipley et al., 1996). Progelatinase, proMMP-9, forms a complex with TIMP-1 (Lambert et al., 2009) while TIMP-1 unencumbered MMP-9 is capable of inducing angiogenesis (Ardi et al., 2009). MMP-9 is produced by inflammatory cells like monocytes and macrophages (Shim et al., 2011), mast cells (Souza Freitas et al., 2011) and T-lymphocytes, many transformed cells (Lamar et al., 2008) as well as by keratinocytes during wound healing (Salo et al., 1994).

In malignant tumors, MMP-9 overexpression has been noted in bladder cancer (Seiler et al., 2011), ovarian cancer (Hoskins et al., 2011), breast cancer (Kim et al., 2011a), pancreatic cancer (Saito et al., 2011), head and neck SCC (Souza Freitas et al., 2011, Stokes et al., 2010), gastric cancer (Kang et al., 2011), colorectal cancer (Dragutinović et al., 2011) and prostate cancer (Aalinkeel et al., 2011). In thyroid cancer, anti-MMP-9 therapy using 3,3'-diindolylmethane has been studied (Rajoria et

al., 2011). Recently, MMP-9 has been implicated in tumor tolerance in which MMP-9 seems to have a significant role in tumor survival (Wang et al., 2011). High-risk human

Table 10. Selected cutaneous SCC related matrix metalloproteinases and their inhibitors

Enzyme	Substrates	Activated by	Inhibited by
MMP-7 (matrilysin-1)	E-cadherin, serpinA1, COL1, COL2, COL4, COL18, elastin, fibronectin, fibrinogen β -chain, fibulin-2, laminin β -3, entactin, HB-EGF, osteonectin, annexin, aggrecan, apolipoproteins A-1 and C-II, vitronectin, MBP, decorin, versican, nidogen, osteopontin, plasminogen, tenascin, β 4 integrin, α -prodefensin, Fas ligand, pro-TNF- α , connective tissue growth factor, insulin-like growth factor-binding protein 2, proMMP-1, MMP-9	MMP-3, plasmin, catecholamine	tissue inhibitors of matrix metalloproteinases 1-4, α 2-macroglobulin
MMP-13 (collagenase-3)	COL1, -2, -3, -4, -6, -9, -10, -14, -18, serpins, α -1 antichymotrypsin, aggrecan, biglycan, decorin, fibrillin, fibronectin, fibrinogen β -chain, gelatin, laminin γ -2, large tenascin C, osteonectin, PAI, fibrinogen, versican, opticin, prolactin, α 2-macroglobulin, casein, coagulation factor XII, C1q, MCP-3, SDF-1, interleukin-8, proMMP-9, proMMP-2, proMMP-13	MMP-2, MMP-3, MMP-10, MMP-14, MMP-15, MT-1-MMP, MT2-MMP, plasmin	tissue inhibitors of matrix metalloproteinases 1-3, α 2-macroglobulin
MMP-9 (gelatinase-B)	aggrecan, plasminogen activator inhibitor precursor, COL2, -3, -4, -5, -7, -11, -14, -17, α -crystallin β -chain, decorin, α -synuclein, amyloid β A4, biglycan, elastin, fibrillin, gelatin, laminin α -1, laminin β -1, link protein, myelin basic, osteonectin, vitronectin, fibrin, fibronectin, fibrinogen, plasminogen, platelet basic protein, insulin, substance P, IL-1 β , IL-8, CXC motif chemokine 5 and 9, casein, prolactin, C1q, ADAM-TS-4 precursor, MMP-9	MMP-7, MMP-13, MMP-3, MMP-2, MMP-26	tissue inhibitors of matrix metalloproteinases 1-4, α 2-macroglobulin, pregnancy-zone protein

(modified from Ala-aho and Kähäri, 2005 and Sternlicht and Werb, 2001 and added with data from Merops database <http://merops.sanger.ac.uk/>, accessed September 2011)

papilloma viruses (HPV-16, -18) are known to cause cervical cancer and certain head and neck cancers and HPV-16 has been shown to activate *MMP9* promoter which may play a role in the invasion and metastasis of these carcinomas (Mühlen et al., 2010).

Table 10 summarizes the substrates of the cancer associated MMPs studied in this thesis. All MMP substrates listed in Table 10 may not be true *in vivo* substrates, as part of the original studies are carried out using *in vitro* methods. In addition, the activators and inhibitors of the given endopeptidases (MMP-7, MMP-13 and MMP-9) are presented in Table 10 (Rawlings et al., 2010; Merops database, accessed 2011).

2.3.2 Role of serine proteinase inhibitors (serpins) in cancer

Serine proteinases are endopeptidases which contain a serine residue in the catalytic centre. Serine proteinase inhibitors (serpins) inhibit mainly serine proteinases but some serpins inhibit also caspases (Ray et al., 1992), and papain-like cystein proteinases (Schick et al., 1998). Serpins are a superfamily of proteinase inhibitors with two clades of extracellular serpins ‘clade A’ and intracellular serpins ‘clade B’ (Silverman et al., 2001). Serpins are broadly distributed proteinase inhibitors that use a conformational change to inhibit target enzymes (Law et al., 2006). In humans, several serpin related diseases have been identified, including alpha-1-antitrypsin (AAT, serpinA1) deficiency which is manifested with emphysema and liver abnormalities (Kueppers et al., 1964; Sharp et al., 1969; Sveger, 1976). SerpinA1 is a highly effective inhibitor of neutrophil elastase and it also inhibits the activity of plasmin, thrombin, chymotrypsin and plasminogen activator (Silverman et al., 2001).

The elevated expression of serpinA1 is associated with the invasive and metastatic potential or poor prognosis in HLA-positive cervical carcinoma, lung cancer, colorectal cancer and gastric carcinoma (Higashiyama et al., 1992; Karashima et al., 1990; Kloth et al., 2008; Tahara et al., 1984). Recently, serpinA1 screening test has been proposed for identifying gastric cancer patients (Hsu et al., 2010). Upregulation of serpinA1 in thyroid cancer has been verified by a critical meta-analysis (Griffith et al., 2006). In addition serpinA1 upregulation, elevated expression of serpinB4, also known as squamous cell carcinoma antigen, has been noted in the SCC of cervix, the SCC of lung, the head and neck SCC and recently in the human breast cancer (Catanzaro et al., 2011). In cutaneous SCC and in AK, the overexpression of serpinB13 (hurpin) has been shown by using human tissue samples (Moussali et al., 2005).

In skin, serpinA1 deficiency may lead to necrotizing panniculitis (Fregonese and Stolk, 2008). Here, spontaneous mixed panniculitis will manifest preferably to gluteal areas, trunk or to limbs. Secondary skin vasculitis in panniculitis lesion may result in necrosis which resolves transepidermally. A case report has shown a manifestation of keratoacanthoma which developed after the immunosuppressive therapy of such skin vasculitis (Guitart et al., 1991). Here, the development of keratoacanthoma may well be mainly induced by immunosuppression rather than the serpinA1 (AAT) deficiency itself.

2.4 MOLECULAR PROFILING OF CUTANEOUS SCC

2.4.1 Gene chip microarray

The development of gene expression techniques has enabled rapid analysis of the whole human genome. These methods are mainly based on the detection of complementary DNA with gene specific probes. In cancer research, gene expression profiling studies have been utilized widely and the data from different studies have also been collected and analyzed together and this way new insights for cancer biology have been gained (Virtanen and Woodgett, 2008). It has been suggested that there are few hundred of differently expressed genes in skin cancer (Hudson et al., 2010). The experimental planning and selection of the control specimens is crucial when gene chip based profiling of cancer tissues or cancer cell lines is done.

Firstly, to avoid the degradation of RNA prior to the analysis, the original specimens have to be of good quality. The RNA studied with gene chip technology has to be intact and the sample must not contain genomic DNA. After the actual probing of the genes, the signal values must be normalized before analyzing the data. Though, probably the most demanding part of high-throughput gene expression profiling is to figure out, which changes and differences in gene expression profiles are of possible clinical importance.

As gene chip microarray method is based on the comparison of studied samples and control samples, choosing the suitable control samples is a crucial event. As thousands of genes are studied simultaneously, one has to use data analysis platform to manage the vast amount of data received. Before analysing the data, one has to determine the level of meaningful signal fold-changes to limit the number of genes for further analysis. In addition to commercial programs, there are also open source data analysis platforms, like Chipster, available (<http://chipster.csc.fi/index.shtml>). Data analysis platform enables the quality control of the studied samples as well as normalization, preprocessing and statistical analyses (Kallio, 2011). More importantly, data analysis platform helps to find the genes of interest via several interactive visualizations and workflows (Kallio, 2011). For example, the genes can be ranged according to the signal fold-changes and visualized as heatmaps which allows to pick up the mostly upregulated or the mostly downregulated genes.

2.4.2 Tissue microarray (TMA)

Advances in high-throughput genetic technologies, such as oligonucleotide gene chip microarrays, has lead to generation of enormous amount of data. Though, the genome-wide expression data must be validated in some other experimental ways *in vitro*, like quantitative real time-PCR. To make judgements on identified potential candidate genes, one should also show the relevance of the gene expression profiling findings *in vivo*. One way of validating *in vitro* data at the anatomical level is conventional immunohistochemistry (IHC). However, it is time and resource consuming to stain dozens or even hundreds of large paraffin sections. To make the IHC step faster and more reliable, Kononen et al. (1998) developed a technique called

tissue microarray (TMA) which can be used to detect molecular targets *in vivo* in a more high-throughput fashion (Kononen et al., 1998).

Immunohistochemical studies using TMA blocks have many advantages compared with conventional IHC. The TMA block may contain up to 1000 specimen cores, if the punch size is small enough (0.3-0.6 mm) (Hoos and Cordon-Cardo, 2001). In our study, we used larger punches (1.5 mm) to make the specimens more representative. Even so, TMA has relatively small sample size per tumor which may diminish the reliability of the results. Nevertheless, the simultaneous staining of multiple samples enables optimizing of pre-treatment conditions, antibody dilutions and detection systems. Moreover, the analysis of the specimens will be faster and the amount of valuable antibodies used will lessen substantially with use of TMA technique.

3 AIMS OF THE PRESENT STUDY

Cutaneous SCCs are generally indolent cancers of the elderly. Nevertheless, SCCs account for the majority of nonmelanoma skin cancer –related metastatic disease and death. Moreover, the incidence of SCC precursor lesions and invasive SCCs is arising constantly worldwide and also in Finland. At present, there are no generally accepted biomarkers for detecting those cutaneous SCCs that are prone to behave aggressively and have elevated potential to metastasize. In contrast to the UV-induced sporadic cutaneous SCCs, recessive dystrophic epidermolysis bullosa –associated cutaneous SCCs manifest early in life, and are distinguished by a particularly progressive clinical course. Only a little is known about the mechanisms behind the progressive nature of RDEB –associated cutaneous SCC. Here, RDEB –associated cutaneous SCCs were considered as a clinical model of progressive cutaneous SCC in search for novel biomarkers for progression of cutaneous SCC. In addition, this study was conducted to elucidate the mechanisms of the progression of cutaneous SCC.

The specific aims of the study were:

1. To examine the role of selected MMPs in RDEB –associated and sporadic SCC of the skin.
2. To elucidate the specific mechanisms, how MMPs contribute to the growth and invasion of cutaneous SCC.
3. To examine the role of COL7 in the progression and growth of cutaneous SCC.
4. To identify novel biomarkers for the progression of cutaneous SCC.

4 MATERIALS AND METHODS

4.1 ETHICAL ISSUES

The study was conducted according to the Declaration of Helsinki. Archival human tissue specimens were used in studies I, II and IV. The use of archival tissue samples was approved by the Ethics committee of the Hospital District of Southwest Finland. Informed consent was given prior to the surgery, when cutaneous SCC cells were obtained for the establishment of SCC cell lines. Similarly, informed consent was given prior to the surgery when normal human epidermal keratinocyte cells were obtained. The animal experiments performed in study IV were approved by the State Provincial Office of Southern Finland.

4.2 HUMAN TISSUE SAMPLES (I, II and IV)

4.2.1 Sample collection

The clinical material of this study consisted of tissue samples of RDEB –associated cutaneous SCC from 17 individuals, altogether 25 tumors (Csikós et al., 2003; Kawasaki et al., 2003; Königová and Rychterová, 2000; McGrath et al., 1992; Tomita et al., 2003; Weber et al., 2001). Due to the rarity of the RDEB, these tissue samples were collected by international collaboration (I). The mean age of RDEB individuals was 33 years (range 12-56 years) and the sex ratio was 7 male vs. 10 female. Sites of the tumors are presented in publication I (I, Table 1). RDEB –associated SCC samples were taken during the surgical management of the tumors between the years 1988 and 2004.

All other human tissue samples were collected from the archives of the Department of Pathology, Turku University Hospital, Finland. These tissue samples were taken for diagnostic and clinical purposes during the years 2004-2007. For comparison to the aggressive type of SCCs (RDEB –associated SCC), 71 sporadic (non-EB, UV-induced) cutaneous SCCs from 71 individuals (mean age 80 years, range 59-92 years, sex ratio 35 male vs. 36 female) were collected. In addition, 29 Bowen's diseases (*in situ* SCC) from 29 non-EB individuals (mean age 76 years, range 59-92 years, sex ratio male 15 vs. female 14) and 36 SCC precursor lesions, AKs (mean age 74 years, range 48-89 years, sex ratio 19 male vs 17 female) were collected.

4.2.2 TMA construction

Individual tissue areas were selected from formaldehyde fixed, paraffin embedded samples using hematoxylin-eosin stained tissue slides. The selection of the representative tissue areas was done in following donor blocks: RDEB-associated cutaneous SCC n=12, sporadic cutaneous SCC n=71, Bowen's disease n=29 and AKs n=36. In addition, tissue samples for staining control purposes were included (breast cancer n=2, prostate cancer n=2, liver n=2, adjacent benign skin n=27). 1.5 mm punched cores from donor blocks were transferred to recipient TMA blocks. TMA blocks, each consisting of 31-47 separate cores, were constructed with a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, U.S.A.).

4.3 IMMUNOHISTOCHEMISTRY (IHC) (I, II and IV)

4.3.1 Large paraffin sections and TMAs

The tissue material of RDEB –associated cutaneous SCCs for immunohistochemical studies on I and II consisted in part of large paraffin embedded tumor tissue sections (13 tumors in I and 8 tumors in II). The reason for not including these cases in TMAs was that the original tissue blocks of these samples were not available. Instead, corresponding 5 µm thick large paraffin sections ready for immunostaining were received and studied by immunohistochemistry (IHC).

All other RDEB –associated cutaneous SCC samples (n=12) and all sporadic cutaneous SCCs, Bowen's diseases, actinic keratoses and staining control samples were studied by IHC as TMA blocks and slides. The mouse chemical carcinogenesis tissue samples (detailed data below) were all studied as large paraffin embedded tissue sections by IHC.

4.3.2 Manual and automated immunostaining

IHC stainings were performed manually on studies I and II. Prior to the immunolabelling, 5 µm thick paraffin sections were deparaffinized and rehydrated. Antigen retrieval was carried out using the microwave oven. Immunolabelling was done on tissue sections using primary antibodies shown on Table 11 and avidin-biotin-peroxidase system (StreptABCComplex, Dako, Glostrup, Denmark or CTS kit, R&D Systems, Minneapolis, MN, U.S.A.), as previously described (Wood and Warnke, 1981). 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine was used as a chromogen and Mayer's haematoxylin as a nuclear counterstain.

The automated immunostaining device was used on study IV (Ventana Medical Systems SA, Illkirch, France). Here, the slides were pretreated with alkaline endopeptidase (enzyme protease 3, Ventana Medical Systems SA, Illkirch, France) and the primary antibody was detected using Ventana Ultraview Universal DAB kit and Ventana Amplification kit.

All IHC samples were independently examined by two observers (Atte Kivisaari and Markku Kallajoki on studies I and II, Atte Kivisaari and Mehdi Farshchian on study IV). IHC slides were analysed and photographed digitally under a light microscope (Olympus BX60). The semiquantitative analysis of the IHC stainings was carried out as described in the original publications (I, II and IV). Primary antibodies used in IHC stainings of tissue samples in the original publications are presented in Table 11.

Table 11. Primary antibodies used in immunohistochemistry

Primary antibody	Antigen	Source	Dilution	Used in
MMP-7, Ab-3, IM71	MMP-7	Oncogene	1:50	I, II
MMP-13, Ab-3, IM64L	MMP-13	Oncogene	1:80	I
MMP-9, abm-213	MMP-9	Reseach Diagnostics	1:200	I
E-cadherin	E-cadherin	Novocastra	1:25	I
Syndecan-1	syndecan-1	Oxford Biotechnology	1:200	I
HB-EGF, AF-259-NA	HB-EGF	R&D Systems	1:25	II
NCL-CD44v3	CD44v3	Novocastra	1:50	II
Phospho-p44/42 MAPK	ERK1/2	Cell Signaling	1:100	II
Alpha-1-antitrypsin	serpinA1	DAKO	1:800	IV

4.4 HUMAN CELL CULTURES (II-IV)

4.4.1 Human cutaneous SCC cell lines (II-IV)

Human cutaneous SCC cell lines used in this study were established from surgically removed tumors in the Department of Otorhinolaryngology, Turku University Hospital, as previously described (Lansdorf C.D et al., 1999). These original tumors consisted of 5 primary cutaneous SCCs (UT-SCC12A, UT-SCC91, UT-SCC118, UT-SCC105 and UT-SCC111) and 3 metastatic cutaneous SCCs (UT-SCC7, UT-SCC115 and UT-SCC59A). UT-SCC91 and UT-SCC111 were used in original publication II and all SCC cell lines were used in original publications III and IV. SCC cells were cultured in DMEM supplemented with 6 mmol/l glutamine, non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum.

4.4.2 Normal human epidermal keratinocytes (NHEK) (III, IV)

NHEK cells from four patients (NHEK 45B, NHEK 59, NHEK 64 and NHEK 70) were obtained from the normal skin samples of patients undergoing surgery for mammoplasty at the Department of Surgery, Turku University Hospital, by a modification of a previously described method (Boyce and Ham, 1983). In addition, NHEK cells were purchased from Promocell (NHEK PC, Promocell, Heidelberg, Germany). NHEK cells were cultured in Keratinocyte Basal Medium 2 (KBM®-2,

Cambrex, NJ, U.S.A.), supplemented with SingleQuots® (Cambrex Bioscience, MD, U.S.A.).

4.4.3 HaCaT and Ha-RAS-transformed HaCaT cell lines (IV)

HaCaT cell line, a spontaneously immortalized nontumorigenic human adult epidermal keratinocyte cell line, and Ha-RAS-transformed tumorigenic cell lines A5, II-4, and RT3 were kindly provided by Dr. Norbert Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Here, A5 cell line is a RAS-transformed benign tumorigenic HaCaT-derived cell line, II-4 cell line forms invasive malignant tumors and RT3 cell line represents metastatic tumors *in vivo* (Mueller et al., 2001). All these cell lines were cultured in DMEM containing 10% FCS and antibiotics.

4.5 PROTEIN ANALYSES (II-IV)

4.5.1 Western blot analysis (II-IV)

Equal aliquots of the conditioned media of cultured cells or cell extract were fractionated on 7.5% - 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), transferred to a nitrocellulose membrane (Hybond ECL filter, Amersham, U.K.) and blotted with primary antibodies listed on Table 12 (II-IV). Primary antibodies were detected with corresponding peroxidase-conjugated secondary antibodies (anti-mouse, anti-rabbit or anti-goat) and visualized by enhanced chemiluminescence (ECL Western Blotting kit, GE Healthcare Life Sciences, Amersham, U.K.).

Table 12. Primary antibodies used in Western blotting (II-IV)

Primary antibody	Antigen	Source	Dilution	Used in
MMP-7, Ab-3, IM71	MMP-7	Oncogene	1:200	II
MMP-13, Ab-4	MMP-13	Calbiochem, Merck	1:1000	III
MT1-MMP, AB815	MT1-MMP	Chemicon	1:200	II
TIMP, AB8116	TIMP	Chemicon	1:1000	II
HB-EGF, AF-259-NA	HB-EGF	R&D Systems	1:1000	II
NCL-CD44v3	CD44v3	Novocastra	1:250	II
Phospho-p44/42 MAPK	pERK1/2	Cell Signaling	1:1000	II
Phospho-EGFR	pEGFR	Cell Signaling	1:1000	II
Alpha-1-antitrypsin	serpinA1	DAKO	1:1000	IV
LH7.2	COL7, NC-1	I. Leigh	1:1000	III
NC2-10	COL7, NC-2	L. Bruckner-Tuderman	1:1000	III
Anti-β-actin, AC-15	β-actin	Sigma-Aldrich	1:1000	II-IV

4.5.2 Analysis of cell surface proteins (biotinylation) (II)

To study the alterations in HB-EGF located specifically on the cell surface, the cultured cells were biotinylated (EZ-Link Sulfo-NHS-LC-Biotin, Pierce Biotechnology,

IL, U.S.A.) and splitted by the lysis buffer containing the proteinase inhibitor (Complete Proteinase Inhibitor Mini, Roche Applied Science, Mannheim, Germany). Subsequently, the biotinylated proteins were immobilized with streptavidin beads (Streptavidin Sepharose High™ Performance, GE Healthcare Life Sciences, Amersham, U.K.) and washed with the lysis buffer. Cell surface proteins were detached from the streptavidin beads by boiling them at 95°C in SDS-PAGE buffer containing 2.5% β-mercaptoethanol. Finally, after centrifugation, the supernatant was electrophoresed as described above. Thus, the specific bands detected by Western blotting, could be designated as proteins that localize in the cell surface.

4.6 CELL PROLIFERATION ANALYSIS (II)

4.6.1 Cell viability assay

To study the effect of MMP-7 in the growth of SCC cells, 1×10^5 UT-SCC91 or UT-SCC111 cells were seeded on 96-well plates and the cell viability was determined while the activity of MMP-7 was inhibited (II). The MMP-7 was inhibited either by using the broad spectrum MMP inhibitor (GM6001, ilomastat, Chemicon/Millipore, MA, U.S.A.) or with specific MMP-7 siRNA, as described in II. The number of viable cells was determined by CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, WI, U.S.A.) according to the manufacturer's instructions.

4.6.2 Cell proliferation assay

To determine the effect of intrinsic MMP-7 on SCC cell proliferation, 1×10^5 UT-SCC91 or UT-SCC111 cells were seeded on 96-well plates. The SCC cells were incubated for 24 hours and then treated with the general MMP inhibitor (GM6001) or transfected with MMP-7 specific siRNA for specific knockdown of MMP-7, as described on II. To quantify the effect of MMP-7 inhibition on the proliferation of the SCC cells, the quantity of DNA synthesis was measured by adding bromodeoxyuridine (BrdU) for five hours. BrdU incorporation into SCC cell DNA was analyzed using a colorimetric immunoassay.

4.7 COL7 DIGESTION ASSAY (III)

4.7.1 Recombinant type VII collagen (rCOL7) production

Human embryonic kidney 293 cell line (HEK293) was transfected to express recombinant *COL7A1* gene using pcDNA3.1/Zeo®(-) expression vector according to the manufacturers instructions (Invitrogen Corporation, U.S.A.). Stable *COL7A1* expressing HEK293 cell lines were generated and selected with Phleomycin (50

mg/ml). Transfected *COL7A1* expressing HEK293 cell lines were maintained in GIBCO® DMEM:HAM's F12 1:1 media (containing 10% fetal calf serum, antibiotic/antimycotic, glutamate and pyruvate). HEK293 cells were passaged at confluency using poly-L-lysine or poly-D-lysine coated plates, harvesting the rCOL7 containing medium every 2-3 days. The collected medium was stored at -80°C and later the medium was precipitated with ammoniumsulphate and dialysed against PBS. rCOL7 was detected with Western blotting using specific antibodies against NC-1 domain (Heagerty et al., 1986) and NC-2 domain. rCOL7 was purified using Flag agarose chromatography columns and eluted with Flag® Peptide (Sigma-Aldrich). The concentration of purified rCOL7 (150 µg/µl) was determined with NanoDrop® ND-1000 Spectrophotometer according to manufacturer's instructions (Thermo Fisher Scientific INC, Wilmington, DE, U.S.A.).

4.7.2 Recombinant MMP-13 (rMMP-13) production

Bac-To-Bac baculovirus expression system (GIBCO BRL, Gaithersburg, MD, U.S.A.) was used for generating the recombinant baculovirus for human proMMP-13 as previously described (Toriseva et al., 2007). Shortly, for the production of rMMP-13, the generated recombinant baculovirus was added in *Spodoptera frugiperda* cell line (Sf9) insect cell cultures for 3 days at 27°C temperature. Subsequently, the *Spodoptera frugiperda* cell line cells were harvested and pelleted and the collected supernatant was analyzed by Western blotting using MMP-13 specific antibody. The catalytic activity of produced rMMP-13 was verified by radioactive collagen cleavage assay as previously described (Ala-Aho et al., 2002) and the rMMP-13 concentration was determined using Biotrak™ ELISA system according to the manufacturer's instructions (Amersham Pharmacia Biotech, UK). The detected concentration of rMMP-13 was 0.1 µg/µl which is sufficient for digestion assays.

4.7.3 Adenoviral gene delivery of MMP-13

As an alternative MMP-13 source for digestion assays, adenovirally infected human fibrosarcoma cells (HT-1080) were used. Here, MMP-13 gene was delivered to HT-1080 cells by infecting HT-1080 cells with recombinant adenovirus (RAdMMP13) coding for *MMP13*, as previously described (Ala-Aho et al., 2002). MMP-13 containing conditioned media were collected after 24 hours and media aliquots were used for rCOL7 digestion assay.

4.7.4 rCOL7 digestion with rMMP13

Digestion assays were performed to study whether rMMP-13 (enzyme) can cleave rCOL7 (substrate). The enzymatic reaction was carried out on a shaker for 6 hours or for 20 hours at room temperature (25°C). Different amounts of rMMP-13 were added to the assay chamber (Eppendorf tube), 0.05 pg, 0.1 pg or 0.2 pg, respectively (III, Figure 2). The amount of the substrate, rCOL7, was equal in every assay performed (3 mg in 20 µl of purified media). As rMMP-13 was produced as a latent inactive form, it had to be activated and here this was carried out using 10 mM p-aminophenylmercuric

acetate and incubation was started. The reaction was stopped by freezing the samples at the 6 hour time point or continued until 20 hours. The digestion assay output was fractionated with SDS-PAGE and the digested fragments were detected with specific antibodies.

As a positive control for the insect cell derived rMMP-13, we used MMP-13 containing media originated from human cells. This MMP-13 was produced by human fibrosarcoma cell line (HT-1080) as described above. To inhibit the enzymatic activity of rMMP-13 or HT-1080 derived MMP-13 on rCOL7, we used the specific MMP-13 inhibitor, pyrimidine dicarboxamide compound (C₂₂H₂₀F₂N₄O₂) which potently inhibits MMP-13 at 4μM concentration (444283 MMP-13 inhibitor, Calbiochem/Merck, Germany).

4.8 GENE CHIP MICROARRAY (III, IV)

To search for genes which may have an important role in tumor growth and progression, gene expression profiling was used (III, IV). Here, the gene microarray data of human SCC cell lines (n=8) was compared with the data from normal primary human epidermal keratinocytes (NHEK, n=5), where the mean signal level of NHEK cells was used as the control for signal levels of each individual SCC cell line. The gene expression profiling was performed with The GeneChip® Human Genome U133 Plus 2.0 Array (HG-U133 Plus 2.0 Array, Affymetrix INC, Santa Clara, CA, U.S.A.) at Finnish Microarray and Sequencing Centre, Turku Centre for Biotechnology. HG-U133 Plus 2.0 Array analyzes the relative expression level of more than 47,000 transcripts and variants of the whole human genome. The raw Affymetrix data was normalized using Robust Multichip Average –assay (Chipster software, CSC, Finland).

4.9 RNA STUDIES (quantitative real-time PCR) (IV)

Prior to the RNA extraction from cultured cells, the conditioned medium was replaced by serum-free culture media for 24 hours. Total RNA was isolated from cultured cells (IV, Figure 1 and 2) or from fresh tissue samples (IV, Figure 6) using RNeasy kit (Qiagen, Chatworth, CA, U.S.A.) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed using reverse transcriptase M-MLV RNase H minus reverse transcriptase and random hexamers (Promega Corporation, Madison, WI, U.S.A.). The primers and probes for studying the expression of *SERPINA1* (human) and *Serpina1* (mouse) were designed based on human and murine sequences as described on study IV. The specific primers and probes were purchased from Oligomer (Helsinki, Finland). Quantitative real-time PCR (RT-PCR) was performed using ABI 7700 sequence detector (Applied Biosystems, Warrington, U.K.) as described previously (Junttila et al., 2007). β-actin or glyceraldehyde-3-phosphate-dehydrogenase was used as a reference gene (Zhong et al., 2011).

4.10 MOUSE SKIN CHEMICAL CARCINOGENESIS (IV)

Normal mouse skin (n=5) and hyperplastic mouse skin (n=6) and mouse skin SCC samples were produced and collected from FVB/N HanHsd mice (maintained at the Laboratory Animal Centre, the University of Oulu). The mouse skin chemical carcinogenesis was performed as previously described (Abel et al., 2009).

Briefly, to induce hyperplasia, the shaved dorsal skin was treated four times, at two day intervals, with 5 µg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich, St. Louis, MO, U.S.A.) in 100 µl of acetone. The control mice were treated in a similar way four times with acetone, without TPA. To induce mouse skin SCC, a single dose of 100 µg of 7,12-dimethylbenz[α]anthracene (DMBA, Sigma-Aldrich, St. Louis, MO, U.S.A.), a known potent carcinogen, in 100 µl of acetone was administered topically on the shaved dorsal mouse skin, followed by weekly TPA treatments (5 µg in 100 µl of acetone) for 20 week period. The SCC development and progression, and the health status of the mice were monitored once per week for the duration of the experiment.

The mice were sacrificed at the end of the experiment at week 32 or earlier when tumor load was excessive, when invasive carcinomas appeared, or when the diameter of an individual tumor exceeded 10 mm. Tumors and other skin samples were removed and fixed in phosphate-buffered 4% paraformaldehyde for 24 hours and embedded in paraffin. The samples were evaluated in a blinded manner on the basis of hematoxylin and eosin-stained tissue sections and the skin alterations were classified as representing benign mouse skin, hyperplasia or SCC.

4.11 STATISTICAL METHODS (I-IV)

In study I, statistical analysis was performed using SPSS for Windows program (15.01, SPSS Inc., IL, U.S.A.). The non-parametric Pearson χ^2 test was used to compare staining intensities or staining immunopositivity.

In study II, parametric Student's t-test was used to compare the results of *in vitro* studies and Pearson χ^2 test in *in vivo* studies (Microsoft® Excel; Microsoft Corporation, WA, U.S.A.).

In study III, the statistical analysis of Affymetrix gene array data were done using T-test (Microsoft® Excel; Microsoft Corporation, WA, U.S.A.).

In study IV, Student's t-test was used to evaluate the differences between mRNA levels on studied cell lines and Pearson χ^2 test was used for the comparison of the semiquantitative IHC results.

5 RESULTS

5.1 INCREASED EXPRESSION OF MMP-7 IN PROGRESSIVE CUTANEOUS SCC *IN VIVO* (I)

MMP-7 is the smallest member of the MMP family and it can cleave several ECM components. The elevated expression of MMP-7 has been shown in tumors of epithelial origin in the gastrointestinal and respiratory tracts as well as in skin cancer (Impola et al., 2005). MMP-7 is constitutively expressed in sweat glands which serves as an internal staining control in immunohistochemistry (Saarialho-Kere et al., 1995).

The expression of MMP-7 in the tissue samples was studied using immunohistochemistry (IHC). The cytoplasmic immunostaining was analysed in RDEB –associated cutaneous SCC (n=25), sporadic UV-induced cutaneous SCCs (n=61), in *in situ* cutaneous SCC (Bowen’s disease, n=28) and adjacent nonmalignant skin (n=27). IHC was performed in large paraffin sections in 13 cases of RDEB – associated cutaneous SCCs, all other samples were studied using TMA technique. MMP-7 staining was evaluated semiquantitatively, where moderate staining (++) referred to the normal staining intensity of MMP-7 in sweat glands, strong staining (+++) was stronger than (++) , and weak staining (+) was weaker than (++) , and negative staining (-) was negative. Semiquantitative analysis revealed that MMP-7 expression is upregulated more in the aggressive type of cutaneous SCCs than in sporadic UV-induced cutaneous SCCs (P=0.001) Moreover, MMP-7 expression correlated with the tumor grade in the RDEB –associated skin SCCs: the less differentiated tumor, more MMP-7 staining was noted (I).

In Bowen’s disease, MMP-7 expression was elevated compared with adjacent normal skin but the immunostaining in Bowen’s disease was less abundant compared with invasive skin SCC. Nonmalignant keratinocytes did not generally express MMP-7 (I).

5.2 INCREASED EXPRESSION OF MMP-13 IN PROGRESSIVE CUTANEOUS SCC *IN VIVO* (I)

MMP-13 is a transformation specific MMP for keratinocytes, as it is not expressed by normal keratinocytes but its expression is strongly upregulated in tumor cells of cutaneous SCC (Airola et al., 1997; Johansson et al., 1997). Here, MMP-13 staining was noted in 24 of 25 (96%) RDEB –associated cutaneous SCCs, whereas the staining was strong or very strong in 15 of 25 (60%) cases (I, Figure 5a). Comparable results were detected from sporadic cutaneous SCCs, as all 61 (100%) samples showed staining for the MMP-13 antibody and the staining was either strong or very strong in 48 of 61 (79%) cases (I, Figure 5b). No statistically significant difference in MMP-13

immunopositivity was found between RDEB –associated and sporadic cutaneous SCCs. Consistent to the previous findings, no MMP-13 staining was noticed in keratinocytes in normal adjacent epidermis.

5.3 LOSS OF E-CADHERIN AND SYNDECAN-1 IN THE PRESENCE OF MMP-7 (I)

E-cadherin and syndecan-1 are cell surface glycoproteins and proteoglycans which together form a powerful invasion suppressor complex (Brouxhon et al., 2007; Furukawa et al., 1997; Stepp et al., 2010). The downregulation of E-cadherin and syndecan-1 has been associated to the tumor development and invasion, but on the other hand MMP-7 is known to cleave these cell surface molecules (Li et al., 2002; Noë et al., 2001).

Here, immunostaining of both E-cadherin and syndecan-1 was strongly reduced in cutaneous SCC tumor areas, where MMP-7 was expressed (I, Figure 1 and 2). Strong, membranous staining of both E-cadherin and syndecan-1 was observed in the normal, adjacent epidermis which serves as a positive internal staining control for these antibodies. The loss of E-cadherin and syndecan-1 in MMP-7 positive tumor areas suggests that MMP-7 has shed these cell surface proteins in the cutaneous SCCs studied. This implies that the loss of E-cadherin and syndecan-1 may not only be a consequence of diminished expression but at least in part a result of the increased shedding of these cell surface proteins by endopeptidases, like MMP-7.

5.4 MMP-9 IS EXPRESSED BY INFLAMMATORY CELLS IN CUTANEOUS SCC (I)

MMP-9 expression was studied in the tissue samples using immunohistochemistry. Here, MMP-9 expression was not noted in the adjacent normal epidermal keratinocytes or in the tumor cells. Interestingly, strong MMP-9 staining was noted in the peritumoral inflammatory cells (I, Figure 5c). The number of MMP-9 immunopositive inflammatory cells was calculated and there was a three-fold increase in Bowen's disease and a nine-fold increase in both SCC groups when compared with adjacent normal epidermis. This suggests that MMP-9 may have a role in altering the peritumoral environment of cutaneous SCC.

5.5 MMP-7 ACTIVATES HEPARIN BINDING EPIDERMAL GROWTH FACTOR –LIKE GROWTH FACTOR (HB-EGF) IN SCC CELLS (II)

Since MMP-7 expression was more upregulated in RDEB –associated cutaneous SCCs, compared with sporadic cutaneous SCCs, it was decided to study further the possible molecular mechanisms how MMP-7 influences the growth of cutaneous SCCs. A cell surface proteoglycan CD44v3 is known to facilitate the interaction between MMP-7 and HB-EGF in a female reproductive organ model (Yu et al., 2002). The interaction between CD44v3, MMP-7 and HB-EGF has been proposed to result in the release of soluble HB-EGF, which can promote cell proliferation and survival. Here, the goal was to study whether CD44v3 mediates the activation of HB-EGF by MMP-7 in cutaneous SCC.

The immunohistochemical study of RDEB –associated cutaneous SCC (n=20), sporadic cutaneous SCC (n=60), Bowen’s disease (n=28) and adjacent nonmalignant skin (n=30) showed that MMP-7 co-localizes with CD44v3 in the SCC tumors *in vivo* (II, Figures 1 and 2). HB-EGF immunostaining was noted in the cutaneous SCCs, but the staining was lost in MMP-7 – CD44v3 double-positive tumor areas suggesting that HB-EGF has been cleaved and activated in these tumor areas (II, Figures 1 and 2). In addition, in the corresponding MMP-7 positive tumor areas ERK activation was noted (II, Figure 3).

To verify these *in vivo* IHC findings, *in vitro* experiments using SCC cell lines (n=2) were performed. Here, the general inhibition of MMPs resulted in the accumulation of inactivated HB-EGF to the cell surface of SCC cells (II, Figure 4a). Cell surface proteins were studied using biotinylation as described on the methods section above. The unspecific inhibition of MMP-7 was further validated by using MMP-7 specific small interfering RNA (siRNA), where the specific knockdown of MMP-7 resulted in the accumulation of uncleaved HB-EGF to the SCC cell surface and simultaneously diminished release of soluble, activated sHB-EGF to the conditioned medium (II, Figure 4b). In concordance to the hypothesis, the specific knockdown of MMP-7 with siRNA resulted in markedly reduced phosphorylation of ERK1/2 which may well be a result of inhibited HB-EGF cleavage (II, Figure 4b).

To demonstrate the functional significance of MMP-7 inhibition on the growth of SCC cells, cell viability and cell proliferation assays were executed (MTT assay and BrdU incorporation assay) which attested that specific MMP-7 inhibition leads to the diminished viability and proliferation of SCC cells. From these results, it was deduced that the overexpression of MMP-7, as is the case cutaneous SCCs, favors the growth and viability of aggressive type of cutaneous SCCs.

5.6 HUMAN CUTANEOUS SCC CELLS PRODUCE COL7 AND MMP-13 (III)

As RDEB –associated SCCs are clinically aggressive and the major pathogenic alteration in RDEB are mutations in *COL7A1* gene, it is feasible to speculate that COL7, the lack of it or its fragments, may have an essential role in the progressiveness of RDEB –associated cutaneous SCCs. In this context, it was decided to investigate, if UV-induced, sporadic cutaneous SCCs express COL7. Here, the hypothesis was that sporadic SCC tumor cells would express COL7 as the fragments of COL7 might be beneficial for the tumor growth and invasion.

To begin with, the expression profile of *COL7A1* on NHEKs (n=5) and UT-SCC cell lines (n=8) was analyzed using GeneChip® Human Genome U133 Plus 2.0 Array. According to the gene array, *COL7A1* is expressed in SCC cell lines studied and there was a trend to higher expression level of *COL7A1* in SCCs compared with NHEKs though there was no statistical difference between the two groups (III, Table 1). Gene array results clearly show that the *COL7A1* expression is not downregulated in SCC cell lines. In addition, it was noticed that *MMP13* expression level was significantly higher in SCC cell lines compared with NHEKs, as described earlier (III, Table 1, $P<0.05$).

Further, the gene array data were verified with NHEKs (n=4) and SCC cell lines (n=7) with Western blotting. On protein level, it was shown that COL7 is produced by SCC cell lines (III, Figure 1). MMP-13 was produced by SCC cell lines but not by NHEK cells, as the gene array data suggested (III, Figure 1). Interestingly, COL7 fragments were detected in the conditioned media of UT-SCC cell lines but not in NHEK media. This shows that COL7 molecules are cleaved in the extracellular milieu of SCC lines but not in NHEKs (III, Figure 1). The COL7 fragments in the extracellular milieu, which refers to the tumor stroma, may have pro-invasive properties.

5.7 MMP-13 CLEAVES COL7 *IN VITRO* (III)

The *in vitro* studies with NHEKs and UT-SCC cell lines showed that SCC cell derived COL7 is cleaved to its fragments in the conditioned media which lead to the assumption that the breakdown of COL7 monomers may be due to enzymatic cleavage by MMP-13. COL7 is not a known substrate of MMP-13, but the excess of MMP-13 in these SCC cell lines implies that COL7 might be a substrate for MMP-13.

By using activated, recombinant MMP-13 (rMMP-13) and recombinant COL7 (rCOL7), digestion assays were carried out which demonstrated that rCOL7 is cleaved by rMMP-13 dose dependently (III, Figure 2). Moreover, the specific inhibition of rMMP-13 with the small molecular MMP-13 inhibitor prevented the cleavage of rCOL7 (III, Figure 3). As a positive control for rMMP-13, HT-1080 cell derived human MMP-13 was used. Adenovirally induced human MMP-13 was able to cleave identical fragments from COL7 as the rMMP-13 (III, Figure 3). The enzymatic activity

of MMP-13 produced by adenovirally infected human cells was effectively inhibited by the MMP-13 inhibitor (III, Figure 3).

The cleavage of rCOL7 by rMMP-13 occurred inside the NC1-domain and at least in two distinct sites of the triple helical domain (just next to the NC-1 domain and close to the aminoterminal end of NC2-domain) (III, Figure 4).

5.8 SERPINA1 PRODUCTION IS ENHANCED IN HUMAN SCC OF THE SKIN (IV)

In the search for novel biomarkers for the progression of cutaneous SCC, gene chip microarray expression profiling for the whole human genome was used (GeneChip® Human Genome U133 Plus 2.0 Array). The gene expression profile of primary human epidermal keratinocyte cell lines was the standard (mean of 5 NHEKs). In this study, the entire serpin family consisting of 36 members was analyzed and the mean levels of the gene expression of the SCC cell lines (n=8) were compared with the NHEK gene expression levels (IV, Figure 1A). mRNA levels of *SERPINA1* and *SERPINA3* were elevated in SCC cell lines and these genes were chosen for further studies.

The gene array results were validated with quantitative real-time PCR (RT-PCR) comparing the mRNA levels of 5 NHEKs and 8 skin SCC cell lines. RT-PCR results revealed significant (50-fold, P=0.03) upregulation of *SERPINA1* mRNA in SCC cell lines (IV, Figure 1B). In contrast, *SERPINA3* mRNA expression was not elevated and thus it was omitted from the further experiments.

SERPINA1 mRNA expression studies were followed by showing the serpinA1 production on protein level. Here, serpinA1 protein production was shown by Western blotting in all SCC cell lines studied (n=8) while only 1 out of 5 NHEKs produced serpinA1 (IV, Figure 2A).

To study the possible correlation of *SERPINA1* expression for the tumorigenic potential of transformed keratinocytes *in vitro*, four different types of cell lines which have a distinct growth pattern were used. *SERPINA1* mRNA levels in these cell lines were measured with RT-PCR. Here, HaCaT cells represented nontumorigenic but immortalized human epidermal keratinocytes, A5 cell line was a benign tumorigenic HaCaT –derived cell line, II-4 represented invasive SCC and finally RT3 cell line was a model of metastatic SCC. Interestingly, *SERPINA1* mRNA expression was low in nontumorigenic HaCaT cell line while *SERPINA1* mRNA expression was higher in Ha-RAS-transformed cell lines and the expression level correlated with tumorigenic potential as *SERPINA1* mRNA expression was the highest in RT-3 cell line (IV, Figure 2B).

The regulation of serpinA1 expression in cutaneous SCC cells was also studied. The UT-SCC cells were treated with the inhibitor of p38 α and p38 β mitogen-activated protein kinases (SB203580) or with the inhibitor of mitogen-activated protein kinase kinase MEK1/2 (PD98059). SB203580 treatment resulted in potent downregulation in basal serpinA1 expression when compared with the control cells (IV, Figure 3C). Interestingly, the inhibition of MEK1/2 did not alter significantly the serpinA1 production (IV, Figure 3C). As p38 β is not expressed in cutaneous SCC cells, these

results implicate that p38 α mitogen-activated protein kinase regulate serpinA1 expression in cutaneous SCC cells.

To investigate the role of serpinA1 in the progression of cutaneous SCC from precursor lesion AKs to *in situ* SCCs (Bowen's disease) and further to invasive cutaneous SCCs *in vivo*, we analyzed serpinA1 production with IHC. IHC was performed using serpinA1 specific antibody on tissue microarray (TMA) sections consisting of AKs (n=36), *in situ* SCCs (n=29), UV-induced, sporadic cutaneous SCCs (n=71) and RDEB –associated cutaneous SCCs (n=12). Correlation with tumor progression was noted also *in vivo*, since cytoplasmic serpinA1 staining was progressively more intense, when the clinical diagnosis proceeded from premalignant lesions towards invasive and aggressive tumors (IV, Figure 4, Table 1). Moreover, the strongest serpinA1 staining was detected in the aggressive-type SCCs which originated from the RDEB patients.

5.9 SERPINA1 OVEREXPRESSION CORRELATES WITH PROGRESSION OF CHEMICALLY INDUCED MOUSE SKIN SCC (IV)

To widen the insight into the role of serpinA1 in the progression of SCC of the skin, the well characterized, multi-stage mouse model of chemically induced skin carcinogenesis was employed (Abel et al., 2009). First, *SerpinA1* mRNA from fresh tissue samples was analyzed with quantitative RT-PCR. *SerpinA1* mRNA expression of normal mouse skin (n=8), hyperplastic benign mouse skin (n=6) and DMBA-TPA –treated mouse skin SCCs (n=14) were analyzed. The mean mRNA level of the *SerpinA1* was significantly higher in chemically induced mouse skin SCCs than in the normal mouse skin or hyperplastic benign mouse skin (IV, Figure 6).

To verify the *SerpinA1* gene expression data, IHC on paraffin block slides of untreated normal mouse skin (n=5), acetone-treated (vehicle) mouse skin (n=2), TPA-treated hyperplastic benign mouse skin (n=6) and DMBA-TPA –treated mouse skin SCCs (n=17) was performed. SerpinA1 immunostaining was analyzed semi-quantitatively and independently by two observers (IV, Table 2). No serpinA1 staining was noted in the keratinocytes of untreated or vehicle treated mouse skin, while mainly weak serpinA1 staining was noted in hyperplastic, TPA-treated mouse skin (III, Figure 5A-C). Interestingly, serpinA1 immunostaining was noted in all chemically induced mouse skin SCCs and the staining was significantly stronger in mouse skin SCCs compared with all benign mouse skin samples as a group (P=0.002, IV, Figure 5D).

6 DISCUSSION

6.1 COLLECTION OF CLINICAL MATERIAL AND EVALUATION OF IMMUNOSTAINING METHODS

This study relies on the invaluable RDEB –associated SCC tumor samples collected from our international collaborators (Csikós et al., 2003; Kawasaki et al., 2003; Königová and Rychterová, 2000; McGrath et al., 1992; Tomita et al., 2003; Weber et al., 2001). The amount of RDEB –associated cutaneous SCCs collected was considered sufficient (n=25), as the numbers are comparable to the other RDEB-SCC related works (Purdie et al., 2010; Slater et al., 1992). The RDEB –associated cutaneous SCC tissue samples were all embedded in paraffin and the samples were received either as the whole tissue blocks (n=12) or as large pre-cut paraffin sections (n=13). The first RDEB –associated cutaneous SCC samples in this study date back to the year 1988, and the latest are from the year 2004. As RDEB –associated cutaneous SCCs are clinically often aggressive and lethal, RDEB –associated tissue samples were considered as a clinical model of progressive type of cutaneous SCC in this study.

In the future, the strength of similar type of studies can be enhanced, as an international effort has been set up to collect EB –associated tumor samples to one database which will be under control of dystrophic epidermolysis bullosa research association. Such database would allow to study not only RDEB –associated cutaneous SCCs, but also JEB and Kindler syndrome –associated cutaneous SCCs as also in these genodermatoses cutaneous SCCs may develop at young age and these SCCs may act aggressively (Yuen and Jonkman, 2011; Emanuel et al., 2006; Has et al. 2011).

Selection and collection of control tissues is always of great importance when an immunohistochemical study is planned (Sherman et al., 2010). Here, the obvious tumor material to compare with would be sporadic, UV-induced cutaneous SCCs. To gain a better overview of the development of cutaneous SCC, it is justified to study the clinical phases from normal skin to the precancerous AKs and Bowen’s disease (*in situ* SCC) and eventually invasive cutaneous SCC. The control samples including AK, Bowen’s disease and sporadic cutaneous SCC were collected from the archives of the Department of Pathology of the University of Turku.

After the collection of paraffin embedded tissue blocks, it was decided to generate tissue microarray (TMA) blocks (Idikio, 2011). The donor-tissue locations, where the samples were punched, were carefully selected and thus the cores were inserted into the TMA recipient blocks. The evaluation and selection of the punch locations was done using haematoxylin-eosin stained slides of the original tissue blocks. In this thesis, it was decided to use 1.5 mm punch cores rather than smaller 0.5-1.0 mm cores to gain larger specimens that would allow better insight to the original sample. Use of the TMA technique allowed performing immunohistochemical analysis of the whole tissue material by staining just 8 TMA slides at a time. In addition to TMA technique,

conventional IHC staining using 8 or 13 RDEB –associated cutaneous SCC large paraffin embedded slides was performed in study I and II.

The advantage of using TMA blocks in immunohistochemical studies in this study was saving time and resources, as the staining procedure was much faster and the amount of valuable primary antibodies needed was much less (Kononen et al., 1998). Time was saved considerably also when the immunostainings were analysed as multiple samples were placed on a single slide. Another advantage was that the staining procedure was performed under the same conditions at the same time which made the staining procedure more reliable and there was less room for disturbing contingent events. A downside of using TMA blocks was that one could not evaluate the whole tissue sample but one had to accept that only relatively small part of the original tissue was to be analyzed. To enhance the representativeness of TMA blocks, duplicates of the original tumor samples were inserted to the recipient TMA blocks.

In study I and II, conventional manual immunostaining methods were used. The benefit of using the conventional immunostaining method in the first studies was to gain better understanding of the immunohistochemical methods as the immunostaining procedures were executed manually starting from deparaffinization and rehydration and proceeding to pretreatments, antibody incubations and chromogen reactions, counter staining and ending up mounting the slides. In study IV, it was decided to use automated immunostaining device which allowed enhancing the quality of the staining procedure as the machine was capable of doing the immunostaining simultaneously to all of our tissue samples.

The evaluation of the immunostained samples under the light microscope is crucial step in the analysis of the data (Prasad and Prabhu, 2011). The evaluation of manually and automatically stained samples was carried out similarly, ie. all samples were independently analyzed by two observers. If a discrepancy was noted between the results of two independent observers, the given samples were re-analyzed and a consensus was achieved. Whenever warranted, the immunohistochemical stainings were analyzed semiquantitatively and statistical comparison between the studied sample groups was done. Here, the major issue was to choose an adequate way of scoring the staining intensities (Bhargava et al., 2006). In study I, MMP-7 staining was scored moderate, if the cytoplasmic immunostaining in the tumor cells was comparable with the staining in the dermal sweat glands. Here, the dermal sweat glands served as an internal staining control as sweat glands are known to express MMP-7. Further, the staining in the tumor cells was considered strong, if the staining was stronger than noted in the sweat glands and weak, if the tumor cell staining was weaker than in sweat glands. If the tumor cells did not have MMP-7 immunostaining, the semiquantitative score was negative. The study I was based solely on the immunohistochemical analysis of the given samples.

6.2 VALUE OF THE TUMOR CELL LINES

As the study I was based on immunohistochemical staining of the tissue samples, the study II was more complex from methodological aspect. Also in study II, the basic

findings were reached by immunohistochemical studies of the clinical *in vivo* tumor samples and control tissues, but the study was continued by verifying the findings with SCC cell line (UT-SCC) *in vitro* studies. As it was shown in study I that MMP-7 is overexpressed particularly in the aggressive type cutaneous SCCs, RDEB –associated SCCs, the cell line experiments in study II were planned to elucidate the mechanisms how MMP-7 promotes the growth and survival of SCC cells using two UT-SCC cell lines. These cell line studies relied on the UT-SCC cell lines established from surgically removed SCCs of the skin in the Department of Otorhinolaryngology (Lansdorf C.D et al., 1999).

In study III, the value of using UT-SCC cell lines was that the mechanistic role of MMP-13 in cutaneous SCC could be studied. By using SCC cell lines, it was demonstrated that COL7 and MMP-13 are expressed and produced by the cutaneous SCC tumor cells. Without the gene expression profiling done on these cell lines and the Western blot studies showing the protein production, our *in vitro* digestion assays would have been of little clinical relevance.

Studies on UT-SCC cell lines, NHEKs, and Ha-RAS-transformed HaCaT cell lines in study IV were essential steps in producing evidence that serpinA1 expression correlates with progression of cutaneous SCC.

Eventhough cell line studies are a convenient way to study the behaviour of tumor cells, one must keep in mind that tumor cells in cell lines may act differently *in vitro* than the actual tumor cells *in vivo*. This is the case when earlier tissue based results showing serpinB13 overproduction in skin SCC (Moussali et al., 2005) are compared with the cell line based gene expression analysis in this study (IV) which does not demonstrate *SERPINB13* overexpression in UT-SCC cell lines (IV, Figure 1A). The explanation for the fact that tumor cell lines may show different expression profile than the actual tumor *in vivo*, is that one cannot choose the cell population that grows in the cell line and this cell population may not represent the most important cells of the tumor.

6.3 PROPOSED MODEL OF ACTIVATION OF HB-EGF BY MMP-7 IN CUTANEOUS SCC

In study II, the interest was focused on the mechanisms by which elevated MMP-7 expression in tumor cells would benefit the growth and survival of cutaneous SCC. Here, HB-EGF was chosen as proposed target of MMP-7, as shedded HB-EGF has been noted to contribute in the progression of other cancers, like ovarian cancer (Tanaka et al. 2005). In addition, HB-EGF has been proposed to have a significant role in certain drug-resistant breast cancers (Yotsumoto et al., 2010). HB-EGF is synthesized as a membrane-anchored protein which can be cleaved by certain metalloproteinases such as a disintegrin and metalloprotease-17 (ADAM-17) and MMP-7 that leads to the release of soluble HB-EGF (sHB-EGF) (Sunnarborg et al., 2002). The membrane-anchored HB-EGF is biologically active, but it can act only on neighboring cells via juxtacrine signalling while cleaved sHB-EGF can diffuse to distant locations and so its mitogenic activity can spread over an extensive area

(Higashiyama et al., 2008). sHB-EGF binds strongly to heparin as well as to cell surface heparan sulphate proteoglycans which enhances its binding to EGFR and may enhance its bioactivity (Nishi and Klagsbrun, 2004). Interestingly, the monoclonal antibodies specific for human HB-EGF have been shown to bind and inactivate membrane-anchored HB-EGF, but fail to inhibit the activity of sHB-EGF (Hamaoka et al., 2010). This phenomenon is relevant, when anti-HB-EGF treatments are developed as uncleaved HB-EGF is appearing as an easier target than sHB-EGF.

In earlier studies, HB-EGF expression has been shown in the basal cells of the normal human epidermis and in the epithelial cells lining epidermal appendages (Downing et al., 1997). Interestingly, specific HB-EGF staining has been detected in skin cancers derived from the basal epithelial cell layer, including basal and squamous cell carcinomas of the skin (Downing et al., 1997).

As sHB-EGF seemingly is mitogenically more active than the membrane-anchored HB-EGF and the activity of sHB-EGF is more difficult to inhibit, it is justified to study the role of HB-EGF cleavage in the progression of cutaneous SCCs.

CD44v3 is a cell surface proteoglycan implicated in cell adhesion and trafficking (Lesley et al. 1993). CD44v3 has also been associated with growth and migration of head and neck SCCs (Wang et al., 2007). In addition, earlier studies on human cancer cell lines (neuroblastoma and Burkitt lymphoma cell lines) and with Cd44^{-/-} mice have shown that CD44v3 can serve as an anchoring site for MMP-7 to cleave HB-EGF (Yu et al., 2002). Moreover, recent studies on mouse skin keratinocytes showed that keratinocyte proliferation is CD44-dependent phenomenon and requires the presence of MMPs and HB-EGF (Barnes et al., 2010).

In this study (II), it was demonstrated by IHC that HB-EGF is expressed in cutaneous SCCs, but there was no HB-EGF immunoreactivity in MMP-7 – CD44v3 double-positive areas. This absence of HB-EGF immunoreactivity was interpreted as shedding of membrane-anchored HB-EGF. This interpretation is somewhat speculative as IHC staining represents only the actual moment when the biopsy was taken and it does not reflect the past situation. Nevertheless, the IHC findings in this study supported the hypothesis that CD44v3 enables cleavage of membrane-anchored HB-EGF by MMP-7. As IHC findings in this study were not conclusive, the IHC results were endorsed with cell line experiments that showed HB-EGF accumulation to the cell surface when MMP-7 was inhibited. The proposed mechanism, where MMP-7 is anchored by CD44v3 to the tumor cell surface to facilitate HB-EGF shedding, is presented on Figure 4.

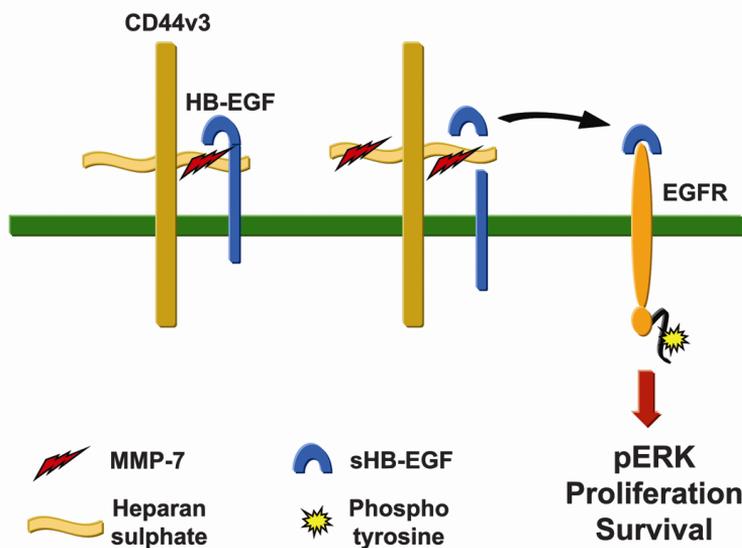


Figure 4. The schematic representation of the proposed mechanism, how matrix metalloproteinase-7 (MMP-7) is expected to contribute to SCC cell proliferation and survival by shedding HB-EGF. Here, CD44 splice variant 3 (CD44v3) serves as an anchoring site for MMP-7 on the cell surface of the cutaneous SCC cell. Immobilized MMP-7 is thought to shed the heparin binding epidermal growth factor-like growth factor (HB-EGF) and release a soluble, active form of HB-EGF (sHB-EGF) which is then capable of attaching to its receptors like the epidermal growth factor receptor (EGFR) in an autocrine or alloctine manner. EGFR activation will subsequently lead to the phosphorylation of extracellular signal-regulated kinase (pERK) which will favor the proliferation and survival of cutaneous SCC cells.

6.4 ROLE OF MATRIX METALLOPROTEINASES IN GROWTH AND INVASION OF CUTANEOUS SCC

MMPs are suggested to play an important role in tumor cell growth and invasion by degrading various ECM components and by activating growth factors (Gialeli et al., 2011; Ii et al., 2006; Kerkelä and Saarialho-Kere, 2003). In this study the overexpression of MMP-7 and MMP-13 was noted in the tumor cells of cutaneous SCCs and the overexpression of MMP-7 was most abundant in RDEB –associated cutaneous SCCs which are referred to the progressive type cutaneous SCCs (I). In addition to the tumor cell associated MMP-7 and MMP-13, increased staining for MMP-9 in the inflammatory cells adjacent to the cutaneous SCCs was noted (I). These findings support the proposed theories that MMPs may have an active role in the tumor development and growth.

As significantly more MMP-7 immunopositivity was noted in the RDEB –associated cutaneous SCCs, it was decided to study the mechanistic role of MMP-7 in these carcinomas, as discussed above. To further evaluate the role of MMP-7 in SCC tumor cell viability, the function of MMP-7 was inhibited in UT-SCC cell lines.

General MMP-inhibition and specific knock-down by MMP-7 specific siRNA were utilized to prove that MMP-7 is responsible for releasing soluble HB-EGF from the cutaneous SCC tumor cell surface (II). The observation that general MMP inhibition by GM6001 resulted in more potent inhibition of HB-EGF shedding than specific MMP-7 inhibition can be explained by the fact that GM6001 inhibits among other MMPs also ADAM-17 which is capable of shedding HB-EGF (Maretzky et al., 2011).

For further evaluation of the role of MMP-7 in the growth and survival of cutaneous SCC cells, cell survival and viability assays were performed. BrDU incorporation assay enabled demonstrating that the specific knock-down of MMP-7 leads to diminished SCC cell proliferation. Moreover, the use of CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT assay) allowed to confirm that specific knock-down of MMP-7 affects the cutaneous SCC cell viability in the manner that cell proliferation rate is lower when MMP-7 is specifically inhibited (II).

The study II elucidated one of the mechanisms by which MMP-7 may promote cutaneous SCC tumor cell growth. Findings in this study (II) are in concordance with studies on colon cancer cell line, where MMP-7 catalyzed the release of HB-EGF that induced proliferation of cancer cells (Cheng et al., 2007). There are definitely other mechanisms by which MMP-7 may promote tumor cell growth and survival, like mediating cell apoptosis by processing Fas ligand (Wang et al., 2006), but the role of this mechanism in cutaneous SCC is not known.

MMP-13 is not expressed by normal keratinocytes in physiological situation but its expression is turned on when malignant transformation occurs (Impola et al., 2005). In addition to cutaneous SCC, overexpression of MMP-13 has been noted in head and neck SCCs which are closely related to cutaneous SCC (Culhaci et al., 2004; Johansson et al., 1997; Junttila et al., 2007; Luukkaa et al., 2006; Stokes et al., 2010). MMP-13 clearly has a significant role in the SCC invasion by cleaving ECM components. According to the results in this study (I), the overexpression of MMP-13 does not serve as a biomarker of the aggressive behaviour of the RDEB –associated cutaneous SCCs as there was no significant difference in MMP-13 expression between RDEB –associated SCCs and sporadic, UV-induced SCCs.

In other cancers than cutaneous SCC, tumor-derived MMP-13 expression may serve as a marker of aggressive tumor phenotype. This is the case with breast cancer, where MMP-13 expression correlates with aggressive tumor phenotypes, and correlates inversely with the overall survival (Zhang et al., 2008). In addition, MMP-13 expression associates with poor prognosis in non-small cell lung cancer (Hsu et al., 2006). On the other hand, MMP-13 expression does not serve as a prognostic factor in all types of cancer, as primary neuroendocrine carcinomas of the skin do not express MMP-13 (Massi et al., 2003).

6.5 CLEAVAGE OF COL7 BY MMP-13 MAY FAVOR THE INVASION OF CUTANEOUS SCC

Invasion of cancer cells is a multi-step process in which proteolysis of ECM and interaction between cancer cells and stromal cells are essential for preparing the way for cancer cell movement (Ala-aho and Kähäri, 2005). Anchoring fibrils that are made

of COL7 monomers, are crucial for the normal attachment of epidermis to the dermis and thus anchoring fibrils ensure the homeostasis of the skin (Chung and Uitto, 2010). The results of this study (III) demonstrate for the first time that cutaneous SCC associated MMP-13 is capable of cleaving COL7 *in vitro*. The digestion assays in study III demonstrate clearly that MMP-13 can cleave COL7, but this study does not show conclusive evidence that this phenomenon takes place in the actual tumor environment. Though, it was shown *in vitro* that both MMP-13 and COL7 are expressed by UT-SCC cells. Moreover, the fragments of COL7 were found in the culture media of SCC cells but not in culture media of NHEKs which supports the hypothesis that MMP-13 produced by tumor cell could cleave COL7.

It is possible that the cleavage of COL7 by MMP-13 is beneficial for the cancer invasion. Firstly, the fully formed anchoring fibrils form a mechanistic obstacle to the cutaneous SCC in the upper dermis and the cleavage of COL7 would enable the invasion of the tumor cells. On the other hand, the COL7 fragments cleaved by MMP-13, especially the NC-1 domain, may have tumorigenic potential (Ortiz-Urda et al., 2005). NC-1 domain may enhance the movement of the tumor cells by interacting with the dermal fibroblasts via $\alpha 2\beta 1$ integrin (Chen et al., 1999). In study III, the molecular size of one of the COL7 fragments cleaved by MMP-13 was equivalent to NC-1 domain, suggesting that MMP-13 can cleave this particular domain from the COL7 monomer.

Further studies using frozen sections of cutaneous SCC samples are needed to show the putative localization of COL7 and its fragments in the tumor margin. The hypothesized co-localization of MMP-13 and COL7 could also be studied using frozen sections, as antibodies that work in frozen sections are readily available (Wu et al., 2002; Onodera et al., 1992).

6.6 NOVEL BIOMARKERS FOR CUTANEOUS SCC PROGRESSION

In this study (IV), an effort to find additional novel biomarkers for cutaneous SCC progression was made. Here, focus was on serine proteinase inhibitor (serpin) family, as serpins have been implicated on invasive and metastatic potential of several malignancies (Hsu et al., 2010; Hsu et al., 2007; Kloth et al., 2008), but only scarce data were available on serpins in skin cancer (Moussali et al., 2005). In physiological situation, serpins are involved in numerous processes including fibrinolysis, blood coagulation and inflammation which implies that *SERPIN* gene family may have a significant role in the tumorigenesis (Gettins, 2002; Law et al., 2006; Silverman et al., 2001).

The search for novel biomarkers was started with gene expression profiling, where the gene expression profiles of whole serpin family on SCC cell lines was compared to normal human epidermal keratinocytes. The following step with quantitative real-time PCR helped to focus on one serpin, serpinA1, also known as alpha-1-antitrypsin (AAT). Studies on transformed HaCaT cell lines, *in vivo* immunohistochemical studies on premalignant and SCC tissues and finally the use of chemically induced mouse skin SCC model led to a conclusion that serpinA1 expression correlates with cutaneous

SCC progression and the aggressiveness of the tumors. It is possible that serpinA1 may be used as a biomarker for detecting cutaneous SCC with metastasizing potential or potential to invade rapidly.

Further studies combining clinical follow-up data with immunohistochemical analysis of archival tumor samples are needed in finding biomarkers which could be used for reliable detection of cutaneous SCC that has a potential to behave aggressively. It is likely that one cannot predict the prognosis of cutaneous SCC on a base of one single biomarker but instead, a panel of several biomarkers is needed (Rodríguez-Enríquez et al., 2011). Prognostic cutaneous SCC biomarker panel would have substantial clinical value. In practice, biomarker panel could be used after cutaneous SCC surgery when it could help to estimate the need for adjunctive treatments and plan the follow-up.

6.7 PUTATIVE ROLE OF SERPINA1 IN THE PROGRESSION OF CUTANEOUS SCC

As it has been shown in study IV that there is a correlation between serpinA1 production and cutaneous SCC progression, it is conceivable to suggest that serpinA1 is a biomarker for cutaneous SCC progression. What has not been shown nor studied here is the actual functional role of serpinA1 in the cutaneous SCC tumor growth and invasion. Therefore, the functional role of serpinA1 in the progression of cutaneous SCC can be discussed here only on the basis of the literature.

The possible clinical role of serpinA1 in tumor growth can be evaluated by observing patients who have genetic disorder with serpinA1 (AAT) deficiency. More aggressive phenotype of colorectal cancer has been proposed to correlate with serpinA1 (AAT) deficiency, but this suggestion has been proved false in a large case-control study which showed that there was no difference in frequency of *SERPINA1* deficiency alleles between the non-hereditary colorectal cancer patients and unrelated matched controls (Lindor et al., 2010). Association of serpinA1 (AAT) deficiency and non-small cell lung cancer (NSCLC) has been shown, but interestingly, being a carrier of *SERPINA1* deficiency alleles did not have a significant effect on NSCLC survival (Li et al., 2011). In serpinA1 (AAT) deficiency disorder, serpinA1 generally accumulates to the hepatocytes which may lead to liver cirrhosis in adulthood and eventually hepatocellular cancer may develop (Abdel-Hamid, 2009). Here, the development of hepatocellular cancer is caused by liver cell damage due to accumulation of misfolded serpinA1 aggregates.

The role of serpinA1 in cutaneous SCC has not been studied previously. In other malignancies, it seems that serpinA1 overexpression is associated with poor prognosis but serpinA1 (AAT) deficiency does not have a significant effect to the prognosis. Interestingly, serpinA1 inhibits plasmin (Silverman et al., 2001) which is a well-known activator of several MMPs, including tumor-associated MMP-7 and MMP-13 (Kerkelä and Saarialho-Kere, 2003). Moreover, MMP-7 (Zhou et al., 2010) can cleave and thus inactivate serpinA1 and also MMP-13 is capable of cleaving serpins (Knäuper et al., 1996b) which makes a regulatory loop possible. It is conceivable, that the

overexpression of serpinA1 in cutaneous SCC is balancing the overexpression of MMPs in cutaneous SCC as serpinA1 can diminish activation of MMPs via inhibiting plasmin. On the other hand, MMPs may attempt to control the inhibitory effect of serpinA1 by cleaving serpinA1. The proposed regulatory loop between MMPs and serpinA1 is shown in Figure 5.

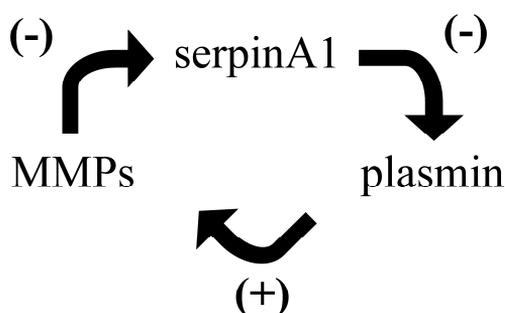


Figure 5. Proposed regulatory connection between matrix metalloproteinases (MMP) and serine proteinase inhibitors (serpins). Here, serpinA1 is capable of inhibiting plasmin which can activate MMPs that have inhibitory effect on serpinA1. In the proposed regulatory loop, serpinA1 inhibits plasmin which leads to diminished activation of MMPs while MMPs attempt to lessen the inhibitory the effect of serpinA1 by cleaving it.

As both proteinases, MMP-7 and MMP-13, and proteinase inhibitor serpinA1 are upregulated in cutaneous SCC, it raises a question whether these players are acting together or against each other. The regulation of MMPs is much more complex process than the proposed regulatory loop shown on Figure 5 (Kessenbrock et al., 2010). In cancer invasion, it is likely that maximal proteinase level will not yield maximal desired effect, i.e. both proteinases and their inhibitors are needed for controlled proteolysis of the surrounding ECM and successful invasion. This is the case in an earlier study on cutaneous SCC, where overexpression of serine proteinase inhibitor, clade E, member 1, also known as plasminogen activator inhibitor-1, was accompanied by overexpression on matrix metalloproteinase-10 (Freytag et al., 2009). In that work, it was proposed that serine proteinase inhibitor, clade E, member 1 regulates the invasive phenotype of human cutaneous SCC in a delicate balance with transforming growth factor- β and epidermal growth factor dependent matrix metalloproteinase-10 elevation (Freytag et al., 2009). In this context, it is logical that both proteinases and proteinase inhibitors are overexpressed in the same tumor.

As serpinA1 may diminish the activation of MMP-7, it is justified to argue that serpinA1 could lessen the activation of HB-EGF by MMP-7 in cutaneous SCC. Here, one has to acknowledge that various MMPs and a disintegrin and metalloproteinases have same substrates. In cutaneous SCC, it is likely that overexpression of serpinA1 probably is not able to prevent shedding of HB-EGF by diminishing the activation of MMP-7, as HB-EGF can be shedded and activated by ADAM-17 (Hinkle et al., 2004)

which activity is not dependent on plasmin as ADAM-17 is activated mainly by phorbol esters (Horiuchi et al. 2007) and inhibited by tetraspanin CD9 (Gutiérrez-López et al., 2011).

Previous study has documented an antiapoptotic effect of serpinA1 via inhibition of caspase-3 (Petrache et al., 2006). SerpinA1 would favor the growth of cutaneous SCC, if serpinA1 would prevent the apoptosis of cutaneous SCC cells as it does to the lung endothelial cells. Moreover, serpinA1 may promote cancer cell survival by inhibiting natural killer cell activity (Laine et al., 1990; Zelvyte et al., 2004). It is also conceivable that overexpression of serpinA1 stimulates malignant cell proliferation by its C-terminal 26-residue peptide which has mitogenic properties (Congote and Temmel, 2004). Further functional studies are needed for revealing the functional role of serpinA1 in the progression of cutaneous SCC.

6.8 TREATMENT OF RDEB –ASSOCIATED CUTANEOUS SCC

The starting point for this thesis was the lack of knowledge, why RDEB patients develop aggressive cutaneous SCCs. On the RDEB sufferers perspective, this study was made in the hope of finding the explanation for the aggressive behaviour of the malignancy that they are expected to develop. On the other hand, the RDEB – associated cutaneous SCCs were studied here as an example of progressive cutaneous SCC which might serve the general population in case that we would find the grounds for the progression of cutaneous SCC that could be extrapolated to the cutaneous SCC growth in general.

Results of this study suggest that MMP-7 may partially explain the aggressive behaviour of RDEB –associated cutaneous SCC via activating HB-EGF. As HB-EGF can activate epidermal growth factor receptor (EGFR) in the cutaneous SCC tumor cells, this piece of information supports the use of EGFR antagonists (cetuximab, erlotinib, gefitinib) in the treatment of unresectable RDEB –associated cutaneous SCCs and even other unresectable cutaneous SCCs (Arnold et al., 2009; Clinical Trials, accessed 2011).

At present, the golden standard of treating RDEB –associated cutaneous SCC remains careful excision accompanied by additional treatment modalities such as chemotherapy and radiotherapy according to the individual needs (Saxena et al., 2006; Yamada et al., 2004). In the future, the need for the treatment of RDEB –associated cutaneous SCC may change fundamentally if the restorative *COL7A1* therapies could be employed successfully at young age.

7 SUMMARY AND CONCLUSION

The present study investigated the role of MMPs in cutaneous SCC and especially in the progressive type cutaneous SCCs, RDEB –associated SCCs. MMP-7, MMP-13 and MMP-9 expression was studied in the precancerous and malignant skin lesions and overexpression of MMP-7 and MMP-13 was noted in the cutaneous SCC tumor cells and MMP-9 was overexpressed in peritumoral inflammatory cells. Interestingly, MMP-7 expression was higher in the RDEB –associated cutaneous SCCs than in sporadic, UV-induced SCCs. It is possible that MMP-7 is partially responsible for the aggressive behaviour of RDEB –associated cutaneous SCCs.

To gain deeper insight, the role of MMP-7 in the cutaneous SCC cell growth and proliferation was investigated. It was shown that CD44v3 may act as an anchoring site for MMP-7 in a manner that HB-EGF is cleaved from the tumor cell surface and thus activated. The activated soluble HB-EGF was shown to promote the SCC cell growth via activating EGFR. Direct inhibition of HB-EGF or inhibition of MMP-7 might be specific targets in the combination therapy of aggressive cutaneous SCCs, but further studies are needed to assess the possible clinical benefit of HB-EGF or MMP-7 inhibition.

Anchoring fibrils that are securing the integrity of dermal-epidermal attachment consist of type VII collagen (COL7). MMP-13 is an effective collagenase which is not expressed by normal keratinocytes, but its expression is turned on when malignant transformation occur. In this study, a novel substrate, COL7, for skin cancer associated MMP-13 was found. It was shown that both COL7 and MMP-13 are expressed and produced by sporadic SCC tumor cells and COL7 fragments were found in the SCC cell line media. Moreover, it was shown that MMP-13 can cleave COL7 monomer at least from three distinct locations. COL7 fragments cleaved by MMP-13, particularly the NC-1 domain, may have protumorigenic potential.

In addition, the expression of the whole serine proteinase inhibitor gene family in cutaneous SCCs was studied. Here, it was found that serpinA1, also known as alpha-1-antitrypsin (AAT), is overexpressed and produced by SCC tumor cells. In addition, serpinA1 (AAT) immunostaining intensities correlated on the progression of cutaneous SCCs towards malignant and progressive forms of cutaneous SCC. SerpinA1 (AAT) production correlated also to the malignant and invasive properties of Ha-RAS-transformed HaCaT keratinocyte cell lines as well as malignant progression of chemically induced mouse skin SCCs.

In summary, the observations of the present study show that MMP-7 may act as a biomarker for the aggressive behaviour of RDEB –associated cutaneous SCC and that MMP-13 may have a role in the remodelling of ECM during the cutaneous SCC growth. ECM remodelling includes the cleavage of COL7 by MMP-13, which has been shown here for the first time. In addition to the studies on matrix metalloproteinases and COL7, it was shown that the upregulation of serine proteinase inhibitor serpinA1 expression correlates with the malignant transformation of epidermal keratinocytes and their progression to invasive cutaneous SCC. Our results suggest that serpinA1 may serve as a novel biomarker for the diagnosis of rapidly progressing or unresectable cutaneous SCC as a part of a panel of other cutaneous SCC biomarkers.

In conclusion, better tools are needed to evaluate which cutaneous SCCs are prone to metastasize or invade rapidly. Here, validated panel of biomarkers would help to assess the prognosis and risk of metastasis and recurrence of cutaneous SCC. Further studies combining large scale immunohistochemical analysis of skin cancers with the clinical follow-up data are needed to achieve that goal.

8 ACKNOWLEDGEMENTS

This work was carried out at the Turku Centre for Biotechnology, the MediCity Research Laboratory, the Department of Dermatology and Venereology, the University of Turku, during the years 2004-2011. The work would not have been possible without great supervision: I wish to express my sincerest gratitude to my supervisor, professor Veli-Matti Kähäri, for his obliging guidance through the years in the laboratory. In addition to being a mentor in the field of science, professor Kähäri has been a great colleague and teacher during my clinical residency in dermatology.

I was lucky to have such experienced thesis committee members, professor Olli Carpén and docent Sirkku Peltonen, who gave their vast knowledge to my use. Docents Jouko Lohi and Olli Saksela are greatly acknowledged for reviewing this thesis and providing valuable criticism.

I express my appreciation and thanks to all co-authors Risto Ala-aho, Johann W. Bauer, Leena Bruckner-Tuderman, Wolfgang Beckert, Mehdi Farshchian, Anja Fritsch, Reidar Grénman, Jyrki Heino, Ritva Heljasvaara, Markku Kallajoki, Radana Königová, Varpu S. Marjomäki, Márta Medvecz, John A. McGrath, Tuomas Mirtti, Juha Peltonen, Taina Pihlajaniemi, Pilvi Riihilä, Kazuko C. Sato-Matsumura, Daisuke Sawamura, Hiroshi Shimizu, Kathrin Sinemus and Florian Weber.

I sincerely thank the members of the Cell invasion –group: Risto Ala-aho, Mehdi Farshchian, Niina Hieta, Juho Joutsa, Melissa Junttila, Janne Kallio, Song-Ping Li, Suvi-Katri Leivonen, Marjaana Mako, Johanna Markola, Liisa Nissilä, Petri Nykvist, Sari Pitkänen, Pilvi Riihilä, Elina Siljamäki, Mervi Toriseva and Esko Veräjänkorva.

I owe a dept to the National Graduate School of Clinical Investigation (CLIGS) as the school provided the secure background for my thesis and being a CLIGS student made it possible to combine the clinical residency and the thesis project seamlessly.

My parents Elias and Pirjo have always supported me towards my own interests which I am thankful for. My father served as an unofficial reviewer of my thesis which helped me to avoid some obstacles and many misspellings. I express my gratitude also to my brothers Ilkka and Eino and to my sister Aija.

Finally, I want to thank the person who probably has supported me the most, my beloved wife Paula. And Toivo, you have been the greatest inspiration and your laughter always gave me hope (as your name implies) and thrust to continue the work.

The work was financially supported by the National Graduate School of Clinical Investigation, the Academy of Finland, the Dystrophic Epidermolysis Bullosa Research Association (DeBRA, U.K.), the Sigrid Juselius Foundation, the Cancer Research Foundation, Turku University Hospital, the Southwestern Finland Cancer Societies, the Finnish Dermatological Society, the Finnish Medical Foundation, Orion-Farmos Research Foundation, K.Albin Johansson Foundation and the Finnish Society of Dermatopathology.

Raisio, December 2011



Atte Kivisaari

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