Turun yliopisto
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ROLES OF NOVEL BIOMARKERS IN PROGRESSION OF CUTANEOUS SQUAMOUS CELL CARCINOMA

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TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1179 | Medica - Odontologica | Turku 2015

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The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6170-2 (PRINT)
ISBN 978-951-29-6171-9 (PDF)
ISSN 0355-9483
Painosalama Oy - Turku, Finland 2015

To my family

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Roles of novel biomarkers in progression of cutaneous squamous cell carcinoma

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ABSTRACT

Cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer. The incidence of cSCC is increasing worldwide due to lifestyle changes such as recreational exposure to sunlight and the aging of the population. Because of an emerging need for molecular markers for the progression of cSCC, we set our goal to characterize three distinct novel markers overexpressed in cSCC cells.

Our results identified overexpression of serpin peptidase inhibitor clade A member 1 (SerpinA1), EphB2 and absent in melanoma 2 (AIM2) in cSCC cell lines compared with normal human epidermal keratinocytes (NHEKs). Immunohistochemical analysis of SerpinA1, EphB2 and AIM2 revealed abundant tumor cell-specific expression of cytoplasmic SerpinA1 and AIM2 and cytoplasmic and membranous EphB2 in cSCC tumors *in vivo*. The staining intensity of SerpinA1, EphB2 and AIM2 was significantly stronger in cSCC as compared with carcinoma *in situ* (cSCCIS) and actinic keratosis (AK). Tumor cell-associated SerpinA1 and EphB2 was noted in chemically induced mouse skin SCC, and the staining intensity was stronger in mouse cSCCs than in untreated skin. AIM2 staining intensity was significantly more abundant in cSCC of organ transplant recipients (OTR) than in sporadic cSCC *in vivo*. EphB2 knockdown resulted in inhibition of migration in cSCC cells. In addition, knockdown of EphB2 and AIM2 was found to inhibit the proliferation and invasion of cSCC cells and to delay the growth and vascularization of cSCC xenografts *in vivo*. Altogether, these findings identify SerpinA1 as a novel biomarker for cSCC. In addition, characterization of the roles of EphB2 and AIM2 in the progression of cSCC was implicated them as possible therapeutic targets for the treatment of cSCC particularly in unresectable and metastatic tumors.

Keywords: cutaneous squamous cell carcinoma, SerpinA1, EphB2, AIM2, matrix metalloproteinase

Mehdi Farshchian

Ihon levyepiteelisyövän kehittymiseen liittyvät uudet merkkitekijät

Iho- ja sukupuolitautioppi, Kliininen laitos, Lääketieteellinen tiedekunta, Turun yliopisto; MediCity tutkimuslaboratorio, Turun yliopisto; Turun yliopiston kliininen tohtoriohjelma (TKT) ja Valtakunnallinen kliininen tutkijakoulu (VKTK), Suomi

TIIVISTELMÄ

Keratinosyyttiperäinen ihon okasolusyöpä on yleisin metastasoituva ihosyöpä. Sen ilmaantuvuus kasvaa maailmanlaajuisesti elintapojen muutoksen kuten auringonvalolle altistumisen sekä väestön ikääntymisen takia. Tarvitaankin uusia merkkitekijöitä tämän syövän etenemisen ennustamiseksi. Työssämme löysimme kolme uutta merkkitekijää ja selvitimme niiden roolia ihon okasolusyövässä.

Havaitsimme, että seriiniproteaasin estäjä A1 (SerpinA1), EphB2 ja absent in melanoma 2 (AIM2) ovat voimakkaasti koholla ihon okasolusyövästä eristetyissä solulinjoissa verrattuna ihmisen normaaleihin epidermaalisiin keratinosyytteihin. Tämän ohella havaitsimme immunohistokemiallisessa analyysissä SerpinA1:n, EphB2:n ja AIM2:n ilmentyvän spesifisti kasvaimen soluissa sekä ilmentyvän merkitsevästi enemmän okasolusyövissä verrattuna *in situ* karsinoomaan ja aktiiniseen keratoosiin *in vivo*. Lisäksi SerpinA1:n ja EphB2:n havaittiin olevan yliekspressoituja hiiren ihoon kemiallisesti aiheutetussa okasolusyövässä. AIM2:n värjäytymisintensiteetti oli merkitsevästi voimakkaampi elinsiirtopotilaista peräisin olevissa ihon okasolusyövissä kuin sporadisissa okasolusyövissä. EphB2:n hiljentäminen esti merkitsevästi okasolusyövästä eristettyjen solujen migraatiota. Lisäksi EphB2:n ja AIM2:n hiljentäminen syöpäsoluissa vähensi merkitsevästi solujen jakaantumista ja invaasiota sekä tuumorien vaskularisaatiota ja kasvua xenograftimallissa *in vivo*. Yhdessä nämä havainnot antavat aiheen otaksua, että SerpinA1 voisi toimia merkkitekijänä ihon okasolusyövässä. Lisäksi havaitsimme, että EphB2:lla ja AIM2:lla on tärkeä rooli ihon okasolusyövän kehittymisessä, joten ne voisivat mahdollisesti toimia uusina hoidon kohteina erityisesti metastaattisten ja vaikeasti kirurgisesti poistettavien ihon okasolusyöpien hoidossa.

Avainsanat: ihon oksasolusyöpä, SerpinA1, EphB2, AIM2, matriksin metalloproteinaasi

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ABBREVIATIONS

AAT	Alpha-1-antitrypsin
ADAM	A disintegrin and metalloproteinase
AIM2	Absent in melanoma 2
AK	Actinic keratosis
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
BCC	Basal cell carcinoma
Bcl-2	B-cell lymphoma 2
COX2	Cyclo-oxygenase-2
cSCC	Cutaneous squamous cell carcinoma
cSCCIS	Cutaneous squamous cell carcinoma *in situ*
DMBA	7, 12-dimethylbenz [α] anthracene
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Eph	Erythropoietin-producing hepatocellular
ERK1/2	Extracellular signal-regulated kinase 1/2
FU	Fluorouracil
HPV	Human papillomavirus
Hras	Harvey rat sarcoma virus oncogene
IFI16	Interferon -inducible protein 16
IFIX	Interferon -inducible protein X
IFN	Interferon
IHC	Immunohistochemistry
IP	Immunoprecipitation
IPA	Ingenuity Pathway Analysis
MAPK	Mitogen activated protein kinase

MMP	Matrix metalloproteinase
MNDA	Myeloid nuclear differentiation antigen
MT1-MMP	Membrane type-1 matrix metalloproteinase
NHEK	Normal human epidermal keratinocyte
NMSC	Non-melanoma skin cancer
NLR	Nod-like receptor
NSLC	Non-small cell lung cancer
OTR	Organ transplant recipient
PKC δ	Protein kinase C delta
PYD	Pyrin domain
qRT-PCR	Quantitative real-time PCR
RDEB	Recessive dystrophic epidermolysis bullosa
RIG-1	Retinoic acid-inducible gene 1
RLR	RIG-1-like receptor
RTK	Receptor tyrosine kinase
SAM	Sterile alpha motif
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficient
SerpinA1	Serpin peptidase inhibitor clade A member 1 protein
SERPINA1	Serpin peptidase inhibitor clade A member 1 gene
siRNA	Small interfering RNA
Srcasm	Src-activating and signaling molecule
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TMA	Tissue microarray
UV	Ultraviolet

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals (I-III).

I. Farshchian M, Kivisaari A, Ala-aho R, Riihilä P, Kallajoki M, Grénman R, Peltonen J, Pihlajaniemi T, Heljasvaara R, Kähäri VM. Serpin peptidase inhibitor clade A member 1 (SerpinA1) is a novel biomarker for progression of cutaneous squamous cell carcinoma.
Am J Pathol 2011;179(3):1110-9.

II. Farshchian M, Nissinen L, Siljamäki E, Riihilä P, Toriseva M, Kivisaari A, Ala-aho R, Kallajoki M, Veräjänkorva E, Honkanen HK, Heljasvaara R, Pihlajaniemi T, Grénman R, Peltonen J, Peltonen S, Kähäri VM. EphB2 promotes progression of cutaneous squamous cell carcinoma.
J Invest Dermatol 2015; doi: 10.1038/jid.2015.104.

III. Farshchian M, Nissinen L, Siljamäki E, Riihilä P, Kivisaari A, Kallajoki M, Grénman R, Peltonen J, Peltonen S, Quint KD, Bavinck JN, Kähäri VM. AIM2 promotes progression of cutaneous squamous cell carcinoma. Manuscript.

1. INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC), collectively referred to as non-melanoma skin cancer (NMSC), are by far the most common types of the human cancers in the Caucasian population. The incidence of cSCC is increasing worldwide making it the most common form of metastatic skin cancer.

Ultraviolet (UV) radiation and cumulative lifetime sun exposure, immunosuppression, human papillomavirus (HPV) infection and chronic ulcers are among the most important risk factors for the development of cSCC.

Surgical excision is the treatment of choice for primary cSCC. Destructive modalities and topical treatments are other therapeutic alternatives in the treatment of primary cSCC. However, treatment options for advanced, recurrent, unresectable and metastatic cSCCs are limited. Screening and diagnosis of cSCC at the early stages of tumor progression is potentially lifesaving. Patients with metastatic tumor who fail surgery and chemotherapy have a poor prognosis.

New molecular markers as prognostic parameters would be useful for early diagnosis, and to predict the progression of actinic keratosis (AK) and cSCC *in situ* (cSCCIS) to invasive cSCC. In this study three distinct markers serpin peptidase inhibitor clade A member 1 (SerpinA1), EphB2 and absent in melanoma 2 (AIM2) have been studied in cSCC. In addition, the role of EphB2 receptor tyrosine kinase and AIM2 in the progression and invasion of cSCC was examined in cSCC cells in culture and in cSCCs *in vivo*.

2. REVIEW OF THE LITERATURE

2.1 Structure and physiology of skin

Skin is the largest organ of our body and consists of three layers: the epidermis, dermis and subcutaneous fat. The epidermal and dermal layers are separated by a basement membrane (Figure 1). Apocrine units, hair follicles, sebaceous glands, sweat glands and arrector pili muscles are the main adnexal structures of the skin. In the epidermal layer most of the cells are keratinocytes (95%) at different differentiation states. Melanocytes, Merkel cells and Langerhans cells form the rest of cell population. The epidermal layers from the innermost layers form as follows: stratum basale (basal layer), stratum spinosum (squamous layer), stratum granulosum (granular layer) and stratum corneum (cornified layer) (Menon, 2002). The main component of the dermis is the extracellular matrix (ECM) consisting of collagen, glycosaminoglycans and elastin. Collagen type I is the most abundant collagen in the dermis. Collagen type I and III are both present in papillary and reticular dermis and their reduction due to the exposure to the UV light is one of the possible mechanisms for photoaging. Type VII collagen forms the major structural component of anchoring fibrils (Sakai et al, 1986). In addition, the dermal layer contains neurons, blood and lymphatic vessels and most of the skin adnexal structures (Menon, 2002). The subcutaneous fat consists of lipocytes, nerves and blood vessels. Skin serves as structural and functional barrier between the inside of the body and outside environment. It serves both as a physical barrier and chemical/biochemical barrier, which protects the body against exogenous

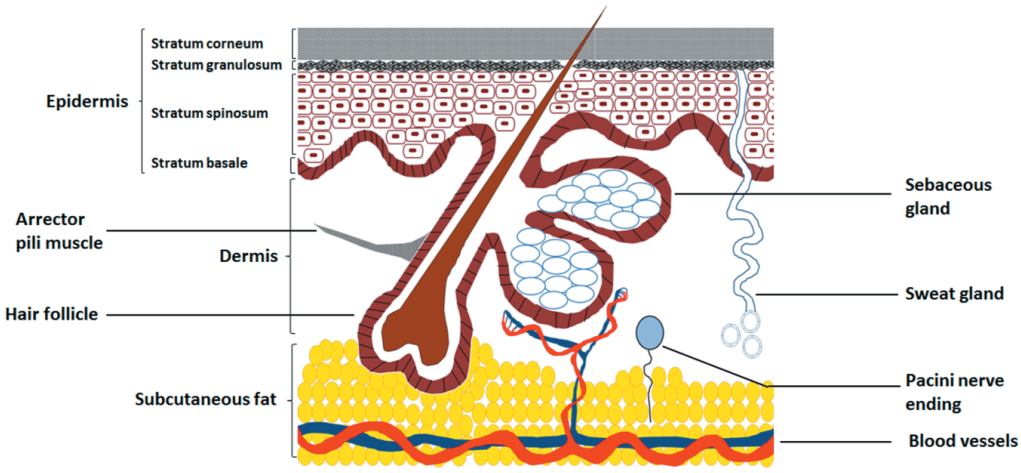


Figure 1. Structure of the human skin.

organisms. In addition, skin plays other important roles such as regulation of the body temperature, vitamin D metabolism and protecting against dehydration (Proksch et al, 2008).

2.2 Cutaneous squamous cell carcinoma (cSCC)

2.2.1 Risk factors and pathogenesis of cSCC

Non-melanoma skin cancers, including BCC and SCC, are the most common types of malignancies in the Caucasian population. cSCC is the second most common type of cutaneous malignancy and accounts for 20 percent of skin cancers (Alam & Ratner, 2001). cSCC is the most common metastatic skin cancer in the Caucasian population (Lim & South, 2014; Rogers et al, 2010). Exposure to solar UV radiation is the most common cause of cSCC (Alam & Ratner, 2001). Part of the epidermis protection against UV-induced DNA damage is provided by the melanocytes (Böhm et al, 2005). The risk of cSCC is markedly higher among individuals with a fair skin type, who live in the regions with high amount of UV radiation and occupations that require working outside many hours per day (Alam & Ratner, 2001). In addition, aging of the population and increased recreational exposure to UV light have notably increased the incidence of cSCC (Salasche, 2000). UVB radiation is the major risk factor and UVA augments the risk (Alam & Ratner, 2001; Salasche, 2000). UVB induces mutation of tumor protein 53 (*TP53)* tumor suppressor gene, resulting in irreversible inactivation of the tumor suppressor feature of the protein, which is potentially an important genetic event in the development of cSCC (Ratushny et al, 2012). UV radiation induces apoptosis in keratinocytes with one mutation in *TP53*. However, additional *TP53* inactivation renders the keratinocytes resistant to apoptosis and consequently uncontrolled proliferation, which is the early event in the progression of AK to cSCCIS and invasive cSCC (Figure 2) (Alam & Ratner, 2001; Boukamp, 2005; Madan et al, 2010). In addition, chronic exposure to UV light damages epidermal cells, resulting in the activation of inflammatory pathways such as NFκB, releasing of cyclo-oxygenase-2 (COX-2) and immunosuppression due to the changes in T-cell subsets as a result of cytokine dysregulation (Aggarwal et al, 2009; Berman & Cockerell, 2013). Loss-of-function mutation of *NOTCH1* is an early event in the progression of cSCC (South et al, 2014). The Ras mutation, especially *Hras*, is one of the key oncogenes in the development of cSCC (Boukamp, 2005; Pierceall et al, 1991; Spencer et al, 1995). However, *ras* mutation alone is not sufficient for the malignant transformation of the keratinocytes (Ratushny et al, 2012). In addition, c-myc, bcl-2, STAT-3, p63-FGFR2, ROS-induced PI3K/AKT-mTOR, Wnt/β-catenin, Shh/Gli1-3 and TGF-β-related signaling and PDGF-C pathway are the other known signaling pathways involved in the progression of cSCC (Lim & South, 2014; Ratushny et al, 2012).

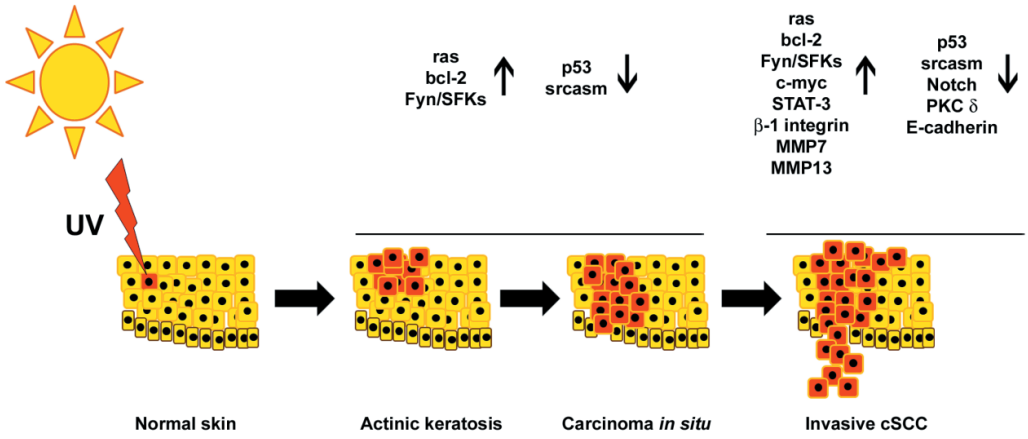


Figure 2. Common molecular features involved in development of AK, cSCCIS and cSCC. (Modified from Alam and Ratner, 2001; Boukamp, 2005; Ratushny, 2012)

Besides UV radiation, chronic ulcers, immunosuppression and HPV infection are the other risk factors for the development of cSCC (Kivisaari & Kähäri, 2013). In addition, similar to other malignancies, chronic inflammation and activation of inflammatory pathways are linked to tumorigenesis potential of cSCC and the progression of AK to cSCCIS and invasive cSCC (Ratushny et al, 2012) (Table 1). Furthermore, the inflammatory microenvironment is known as one of the possible mechanisms being used by cSCC tumor to protect the tumor against the immune system (Hofbauer et al, 2010).

Table 1. Risk factors for the development of cSCC. (Modified from Madan, 2010; Kivisaari and Kähäri, 2013)

Exposure to UV light
Fair skin type
Chronic ulcer
Chronic inflammation
Immunosuppression
Organ transplantation
Ionizing radiation
Chemical carcinogenesis
Human papilloma virus
AK and cSCCIS
Tobacco smoking
Arsenic
Occupational factors

2.2.2 Clinical features and progression of cSCC

The premalignant form of the cSCC, AK is known as the most common premalignant lesion of the skin. The lesion usually appears on the sun-exposed area of the body, such as face and back of the hands, manifests as skin-colored to reddish-brown scaly macules, papules or plaques. It is usually superficial, hard, indurated and has an elevated base with the size of few millimeters up to 2 centimeters. The surface of the lesion is covered by an adherent scale, but sometime it is shiny and smooth. At the early stages, the AK lesions are more palpable, but they may not be seen easily (Alam & Ratner, 2001; James et al, 2011; Madan et al, 2010; Stockfleth et al, 2011). The rate of AK progression to invasive cSCC is between 0.025 and 16 percent (approximately 10%), which usually takes place within two years. The risk is 100 fold higher in immunosuppressed patients (Fuchs & Marmur, 2007; Stockfleth et al, 2011).

If left untreated, the size of the lesion increases slowly and it develops to cSCCIS. The size of the cSCCIS increases gradually and becomes ulcerated and develops to cSCC, which invades the underlying layers. A diameter of >1cm, induration and inflammation, bleeding, ulceration, rapid increase in the size of the lesion and erythema are among the major clinical criteria for the progression of AK and cSCCIS to invasive cSCC. cSCC manifests itself as an enlarging papule or nodule or flat ulcer with a raised border, which becomes ulcerated, necrotic or keratoacanthoma-like (James et al, 2011; Stockfleth et al, 2011). Histopathological examination of the lesion is the gold standard for the diagnosis of cSCC (Madan et al, 2010).

2.2.3 Histopathology of cSCC

The premalignant lesion, AK presents with focal parakeratosis and thickening of epidermis (Figure 3). In the epidermis, there is loss of arrangement of normal orderly stratified accompanying with atypia of keratinocytes. The basal cells are mainly dysplastic (Figure 3). The grade of the intraepidermal keratinocyte atypia is classified as mild (AK I), moderate (AK II) or severe (AK III). Atypical keratinocytes are in basal and suprabasal layer in grade I. In grade II, atypical keratinocytes spread to the lower two-thirds of the epidermis, whereas in grade III, atypia occurs in full thickness through the epidermis. In the dermal layer, there is infiltration of inflammatory cells (James et al, 1978; Stockfleth et al, 2011). In cSCCIS the full epidermis is involved (Figure 3). The epidermal layer shows impairment in maturation and looks disorganized. Mitotic cells, multinuclear keratinocytes and dyskeratotic cells can be seen in epidermal layer. However, the border between epidermal and dermal layer (basement membrane) is intact (James et al, 2011).

cSCC is presented with a nest of atypical keratinocytes derived from epidermal keratinocytes invading into the dermis (Figure 3). The tumor cells have a large nucleus and eosinophilic cytoplasm. In addition, based on differentiation of the tumor, the area of

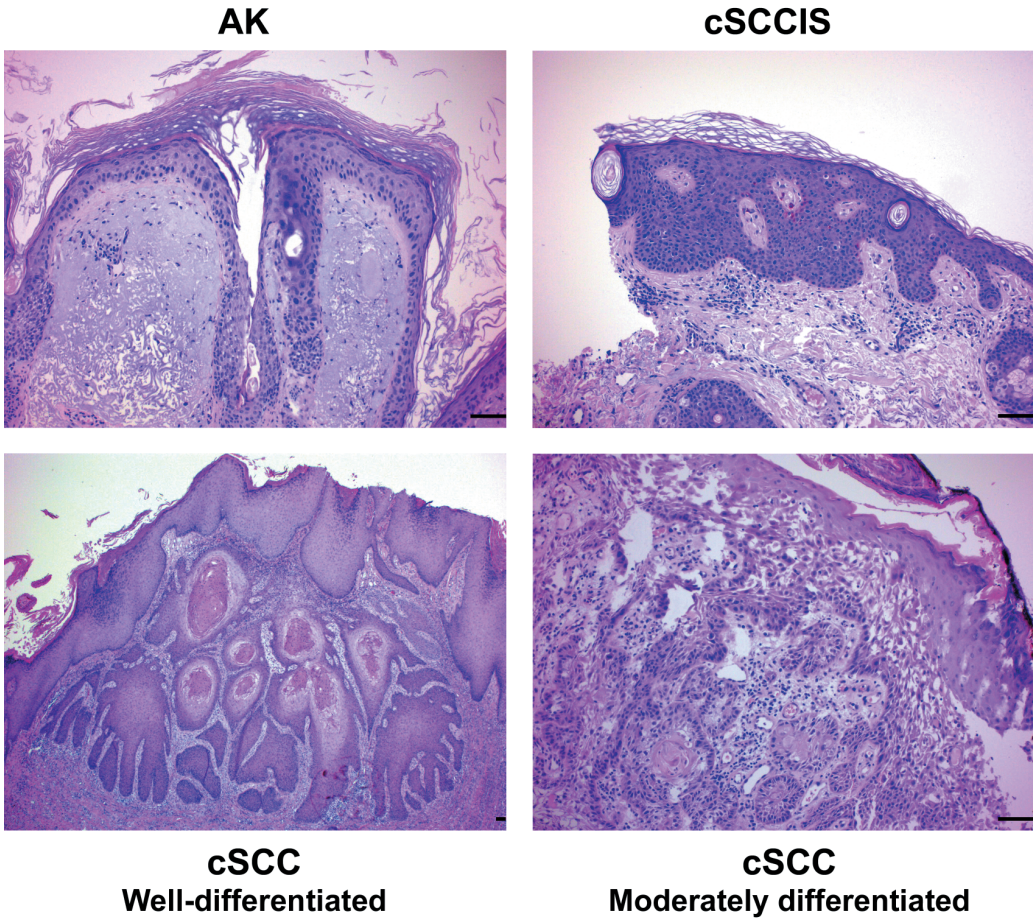


Figure 3. Histology of AK, cSCCIS and cSCC. Hyperkeratosis and thickening of the epidermis in AK, full epidermal layer involvement with mitotic cells in cSCCIS, keratin pearl formation in well-differentiated cSCC and invasive edges of moderately differentiated cSCC. Photographs by Mehdi Farshchian and Markku Kallajoki (Department of Pathology, University of Turku). (Scale bar = 100μM)

keratinization and keratin pearl formation can been seen. Using a subjective assessment cSCCs are classified into poorly, moderately and well-differentiated tumors (Figure 3). Well-differentiated cSCC manifests minimal pleomorphism and abundant keratinization presenting as keratin pearls. Poorly differentiated tumors are characterized by a high level of pleomorphism and nuclear atypia and very little or no nodular aggregates of keratinocytes (keratin pearls) (Dinehart et al, 1997; Lim & South, 2014; Stratigos et al, 2014).

2.2.4 Treatment of cSCC

Currently, there are limited tools available for prediction of which AK lesions will rapidly progress to invasive and metastatic cSCC. Therefore, all AKs should be treated.

The treatment options for the AK lesions are ablative procedures such as curettage, cryosurgery, excision and laser ablation and topical treatment with 5-fluorouracil (5-FU), diclofenac, imiquimod or ingenol mebutate (Lebwohl et al, 2012; Stratigos et al, 2014).

The principal treatment of the primary cSCC is treating the lesion in the early stages and preventing the recurrence of the tumor. The treatment of choice for the localized, low-risk tumors developed among AK and cSCCIS is destructive modalities such as cryotherapy, phototherapy and electrodessication and curettage as well as topical treatment with 5-FU, imiquimod, ingenol mebutate, diclofenac and chemical peels (Stratigos et al, 2014). These treatment modalities have as high as a 96 percent five-year control rate in the patients with low risk cSCC (Franco et al, 2013). The primary treatment for the cSCC tumors is surgical excision with a sufficient clear margin (4-5 mm for well-defined low risk lesions smaller than 2 cm and 6-10 mm for the lesions with more than 2 cm diameter or high risk lesions). Histological verification of the excised tumor can be done after performing the surgery or at the time of excision (Mohs micrographic surgery). Surgical excision is always prioritized over local destructive therapy unless the patient refuses the surgery or if there is any contraindication (Stratigos et al, 2014). Radiotherapy could be considered as an alternative therapeutic option for the patients with cSCC. However, it should be used with caution in patients with large tumors and immunosuppressed patients. In addition, a long list of contraindications and side effects such as age of the patient and location of the tumor apply to this treatment modality. Accordingly, radiotherapy should be considered only in patients with inoperable lesions and the ones who are poor candidates for the surgery (Parikh et al, 2014; Stratigos et al, 2014). Adjuvant radiotherapy should be taken into account in perineural involvement of cSCC and for the lesions, which cannot be excised with the free margin.

Although examination of the sentinel lymph node is beneficial for the early diagnosis of the metastatic cSCC (Parikh et al, 2014), there is no evidence supporting its value for the therapy and prognosis of the tumor (Stratigos et al, 2014). Surgical treatment, radiotherapy alone or together with chemotherapy and electrochemotherapy using bleomycin and cisplatin are the therapeutic modalities for the invasive metastatic SCC. Methotrexate, cisplatin, 5-FU, doxorubicin and bleomycin are the reported systemic chemotherapy drugs used for the treatment of metastatic cSCC (LeBoeuf & Schmults, 2011; Parikh et al, 2014). However, benefits of the chemotherapy should outweigh the toxicity and side effects of the treatment. Epidermal growth factor receptor (EGFR) is upregulated in metastatic and advanced SCC (Maubec et al, 2011; Toll et al, 2010) and its expression is associated with poor prognosis (Maubec et al, 2005). Targeting EGFR has been examined for the treatment of invasive and metastatic SCC. EGFR inhibitors have been developed both as small molecules TK inhibitors (erlotinib and gefitinib) and a monoclonal antibody (cetuximab), which is currently being used for the treatment of

metastatic head and neck SCC (Liu & Colegio, 2013; Stratigos et al, 2014). However, the effect of EGFR inhibitors in metastatic cSCC is under investigation in clinical trials.

2.2.5 Prognosis of cSCC

Although the prognosis of primary cSCC is favorable in the majority of cases (5-year cure rate of approximately 90%), the prognosis is poor for the metastatic tumors (Rogers et al, 2010). A recurrence rate of 4.6 percent, a lymph node involvement rate of 3.7 percent and disease-specific death of 2.1 percent have been reported in 10-year follow-up of primary cSCC (Schmults et al, 2013; Stratigos et al, 2014). Early diagnosis and treatment of AK, cSCCIS and primary cSCC lesions is the best strategy to prevent progression of the lesions toward invasive and metastatic cSCC. As the most metastatic form of skin cancer, the five-year metastatic rate of cSCC is approximately three to five percent (Madan et al, 2010; Ratushny et al, 2012; Stratigos et al, 2014). Regional lymph nodes (85%) are the major target organs for metastatic cSCC followed by metastasis to lung, liver, brain and bones (Stratigos et al, 2014). The size of the lesion (>2 cm), site of the lesion (lip, ear), deep infiltrating tumors, immunosuppression, history of radiation, histopathological features, poor differentiation, perineural invasion and lymph node involvement are among the most important indicators of the metastasis and recurrence of cSCC which requires routine close follow-up and examination of the patients (Alam & Ratner, 2001; Madan et al, 2010; Stratigos et al, 2014). Moreover, incomplete resection of the tumor is one of the main risk factors for the recurrence of cSCC. Ultrasound of the lymph nodes should be performed every three months in patients with local metastatic cSCC. In high risk patients, such as immunosuppressed patients and patients with multiple cSCC, regular follow-up is recommended every six months (Stratigos et al, 2014).

2.2.6 Recessive dystrophic epidermis bullosa (RDEB)-associated cSCC

Epidermolysis bullosa (EB) is a rare heterogeneous genetic disorder that manifests itself as blisters at the site of minor physical trauma, chronic wounds and erosions. The inherited EB is categorized as intraepidermal (EB simplex), junctional and dystrophic or dermolytic (Fine et al, 2008). The level of the epidermal separation can be detected by electron microscopy and immunofluorescent studies. In recessive dystrophic EB, the cleavage takes place in the deep layer (sublamina) (Bruckner-Tuderman et al, 1989). Dystrophic EB is due to the mutations in collagen type VII gene (COL7A1) (Uitto et al, 1994).

Patients with recessive dystrophic EB (RDEB) are at great risk for highly aggressive cSCC (Venugopal & Murrell, 2010). In contrast to the UV-induced sporadic cSCC, in RDEB patients, non-UV-induced aggressive cSCC is highly metastatic and is the main

cause of death in these patients (Rodeck & Uitto, 2007; Venugopal & Murrell, 2010). In RDEB patients the cumulative risk of developing cSCC until the age 55 years is 90.1 percent. cSCC in EB patients has been reported in the patients as early as age 12 years (Kawasaki et al, 2003). cSCC develops mainly at the region of chronic wounds and chronic skin scars (Venugopal & Murrell, 2010). Despite the fact that RDEB-associated SCCs spread and metastasize quickly, the primary lesions have the histopathological features of the well-differentiated lesions (McGrath et al, 1992). Due to the invasive and metastatic nature of the cSCC in RDEB patients, routine monitoring and diagnosis of the lesions in the early stages would be crucial (Venugopal & Murrell, 2010). Prompt excision of the primary cSCC with a sufficient and clear margin is the principle for treatment of RDEB patients (Saxena et al, 2006).

2.2.7 cSCC in immunosuppressed patients

Immunosuppression due to immunosuppressive medications after organ transplantation is associated with a markedly higher risk of cSCC (65-250 times) than the general population (Euvrard et al, 2003; Tessari et al, 2010). In contrast to the general population, SCC is more common than BCC (5:1) in immunosuppressed patients (Euvrard et al, 2003; Wisgerhof et al, 2009; Zamanian & Farshchian, 2007). In immunosuppressed patients, almost 40 percent of the AK lesions develop to cSCC compared with a 10 percent risk in immunocompetent patients (Stockfleth et al, 2011). In addition, cSCCs developed in immunosuppressed patients have more aggressive properties and are associated with a higher rate of recurrence, metastasis (5-10 folds) and mortality (Euvrard et al, 2003; Hameetman et al, 2013; Stratigos et al, 2014). As in immunocompetent patients, in immunosuppressed patients UV-induced *TP53* mutation takes place in the early stages of AK development to cSCC. However, the mutations occur more frequently in immunosuppressed patients (de Graaf et al, 2008). Loss of immune surveillance and HPV are two other risk factors known to play an important role in the development of cSCC in immunosuppressed patients (Stratigos et al, 2014). Among several HPV types, the role that Beta-PV plays in the progression of cSCC in immunosuppressed patients has been documented (Genders et al, 2015; Hofbauer et al, 2010). Beta-PV has been suggested as a marker for the prediction of cSCC development in organ transplant recipients (OTRs) (Genders et al, 2015). Apart from the effect of immunosuppressive drugs on the impairment of the immune system, reducing defense against the neoplasm and chronic inflammation, they promote progression of cSCC by increasing the level of TGFβ and VEGF (Hofbauer et al, 2010). Furthermore, activation of NFκB and TNF pathways has been identified in AK lesions as precursors of cSCC in immunosuppressed OTRs (Hameetman et al, 2013). With an average of eight years, the risk of cSCC increases over time following organ transplantation (Harwood et al, 2013). Among OTRs, heart, lung and kidney recipients

have the highest risk of developing cSCC (Krynitz et al, 2013). Pain sensation by the patient is one of the important warning signals for the development of cSCC in OTRs (Bouwes Bavinck et al, 2014). Patient education, close regular skin examination (every 6 months) and prompt treatment are the principles of the prevention and management of cSCC in immunosuppressed patients (Stratigos et al, 2014).

2.3 Proteinases in cSCC

2.3.1 Matrix metalloproteinases in skin cancer progression

Matrix metalloproteinases (MMPs) consist of a zinc-dependent family of endopeptidases responsible for degradation of ECM (Khokha et al, 2013). In humans, MMPs have 23 members that are categorized into subclasses such as: gelatinases, collagenases, stromelysins, and transmembrane (Klein & Bischoff, 2011; Nissinen & Kähäri, 2014). Collagenases (MMP-1, -8, and -13), which cleave collagen I, II and III, are involved in different physiologic and pathologic conditions (Ala-aho & Kähäri, 2005; Nissinen & Kähäri, 2014). MMPs are known to play a critical role in different physiological processes such as embryonal development, tissue remodeling, wound repair, organogenesis, host defense, homoeostasis and inflammation (Gialeli et al, 2011; Nissinen & Kähäri, 2014). In addition, MMPs are involved in the pathogenesis of several diseases with an increased turnover of ECM, e.g., osteoarthritis, rheumatoid arthritis, autoimmune blister diseases, periodontitis and photoaging (Ala-aho & Kähäri, 2005; Kähäri & Saarialho-Kere, 1997).

Increasing evidence implicates the role of MMPs in cancer progression (Kessenbrock et al, 2015). Cleavage of the ECM by MMPs produced by stromal cells is the key mechanism for the invasion of the tumor cells (Ala-aho & Kähäri, 2005). In benign tumors the basement membrane remains intact. However, invasive and metastatic cells secrete proteolytic enzymes, such as MMPs, to dissolve the basement membrane and ECM, which makes it possible for the tumor cells to invade into the adjacent stromal tissue. As the next step in tumor metastasis, tumor cells recruit these proteinases to distribute in ECM. Once the tumors cells enter the blood or lymphatic circulation and migrate to the distant tissues, they produce MMPs to cleave the basement membrane and ECM of the target tissue (Ala-aho & Kähäri, 2005; Gialeli et al, 2011; Kessenbrock et al, 2015). In addition, MMPs regulate chemokines and cytokines and inflammation and this way may promote cancer cell progression (Nissinen & Kähäri, 2014)

Several MMPs, such as MMP-1, -2, -3, -7, -9 and -13 are upregulated in primary and metastatic tumors. In addition, expression of different MMPs is associated with tumor progression, metastasis and poor prognosis (Deryugina & Quigley, 2006). MMP-

1 (collagenase-1) is secreted by the stromal fibroblasts or tumor cells and cleaves collagen type I, II, III, VII, and X, aggrecan, fibronectin, serine peptidase inhibitors (α1-antitrypsin, α1-antichymotrypsin) and other elements of the ECM (Ala-aho & Kähäri, 2005). In addition, the role that MMP-1 plays in tumor cell proliferation and angiogenesis has been proposed (Gialeli et al, 2011). MMP-1 expression is linked with poor prognosis of several malignancies such as esophageal cancer and colorectal cancer (Johansson et al, 2000).

MMP-13 (collagenase-3) promotes invasion of the tumors by ECM degradation. In addition, expression of MMP-13 is associated with epithelial to mesenchymal transition (EMT) resulting in downregulation of cell adhesion, which enables the epithelial cells to migrate and may this way enhance the migration capacity (Gialeli et al, 2011; Polyak & Weinberg, 2009). MMP-13 cleaves collagen I, II, III, IV, IX, X, and XIV, fibronectin, laminin, aggrecan, osteonectin, and versican and other ECM components (Ala-aho & Kähäri, 2005). MMP-13 expression is correlated with the invasive and metastatic phenotype of breast carcinoma (Zhang et al, 2008), melanoma (Airola et al, 1999) and head and neck SCC (Johansson et al, 1997). An elevated risk of recurrence has been observed in prostate cancer, which has a high expression level of MMP-13 (Escaff et al, 2010). In head and neck SCC, MMP-13 is expressed in large and locally invasive tumors (Stokes et al, 2010). Expression of MMP-13 is absent in premalignant skin lesions and normal skin (Airola et al, 1997; Vaalamo et al, 1997).

In contrast to other collagenases, MMP-8 (collagenase-2) has a dual role both as tumor-promoting and tumor-protective in different stages of tumor progression. Overexpression of MMP-8 is associated with decreased metastatic potential of breast cancer cells (Decock et al, 2008). Its protective role has been also identified in SCC of the tongue (Korpi et al, 2008). On the other hand, expression of MMP-8 is associated with the progression of ovarian cancer (Stadlmann et al, 2003).

Based on the key role that MMPs play in tumor progression, many inhibitors have been developed for the treatment of different malignancies. Although several inhibitors targeting MMPs can inhibit the metastatic and invasive potential of cancer cells, in clinical trials, their effect on the patients' survival has not been promising to date (Hadler-Olsen et al, 2013). The main drawback of the first MMP inhibitors developed, such as Batimastat and its derivatives Marimastat was their effect across a broad spectrum, which causes severe side effects (Steward & Thomas, 2000). The development of the inhibitors, which bind to the specific MMPs, is an enormous challenge, because the structure of the active zone of the MMPs is very similar to each other (Hadler-Olsen et al, 2013). In addition, the fluctuating role of MMPs in malignancies, both as tumor suppressor or promoter, could be one of the possible explanations for the failure of MMP inhibitors in the treatment of cancer (Vilen et al, 2013).

2.3.2 Serine proteinase inhibitors in skin cancer

The superfamily of serine protease inhibitors (serpins) is the largest family of protease inhibitors described in humans. With the size of 350-500 amino acids, serpins are considered as large size molecules compared with other protease inhibitors. Serpins are involved in different biological processes e.g., inflammation and complement activation, angiogenesis, apoptosis, ECM maintenance and remodeling, sperm development, renal development, and prohormone conversion. In addition, the key role that many serpins play in fibrinolytic cascades and clotting has been identified (Silverman et al, 2001). Human serpins are classified into two largest clades of the 36 serpins: extracellular molecules 'clade A' and intracellular serpins 'clade B' (Law et al, 2006; Silverman et al, 2001). Most of the serpins have inhibitory functions (27 out of 36) (Table 2) (Law et al, 2006; Rawlings et al, 2014). In clade A, SerpinA1 and SerpinA3 are inflammatory response inhibitory molecules. Other molecules in clade A have different physiological roles such as transportation of the hormones (SerpinA6 and A7) and regulation of blood pressure (SerpinA8) (Table 2) (Law et al, 2006; Silverman et al, 2001). Clade B molecules are inhibitors of activity of cytotoxic apoptotic proteases (SerpinB6 and B9), papain-like cysteine proteases (SerpinB3) (Law et al, 2006) or have a tumor suppressor role (SerpinB5) (Zou et al, 1994) (Table 2).

Serpin peptidase inhibitor clade A member 1 (SerpinA1), also known as α1-proteinase inhibitor or α1-antitrypsin (AAT), is an inhibitor of neutrophil elastase. SerpinA1 also inhibits plasminogen activator, trypsin, chymotrypsin, plasmin and thrombin (Law et al, 2006; Silverman et al, 2001). SerpinA1 protein is produced in the liver and distributed in the body via blood circulation. The main function of the SerpinA1 is to inhibit neutrophil elastase and results in the protection of the lung from damages cause by proteolysis. Mutations in *SERPINA1* cause AAT deficiency, which is an autosomal dominant disease defined as two inherited deficiencies at the locus coding AAT (Silverman & Sandhaus, 2009). AAT deficiency is a fetal genetic disorder particularly in people with European ancestry (Silverman et al, 1989). The mutations alter the structure of the molecule and it is consequently unable to be secreted into the blood. The accumulation of the AAT in the liver causes severe liver damage. On the other hand, lack of AAT in the lung causes alveolar septal destruction, which may progress to emphysema due to the proteolytic damages (Brantly et al, 1988; Silverman et al, 1989).

Serpin peptidase clade A member 3 (SerpinA3), also known as α1-antichymotrypsin (ACT), is another member of the serpin superfamily with clinical importance. SerpinA3 is an inflammatory response molecule that inhibits mast cell chymase and neutrophil cathepsin G (Law et al, 2006; Silverman et al, 2001). The presence of SerpinA3 in brain amyloid deposits of the patients with Alzheimer's disease is well documented. Although the exact role of the SerpinA3 in the pathogenesis of the disease is not clearly known, it

Table 2. Function of human clade A and B serpins.

Clade	Serpin	Protease target or function
A	SerpinA1	Inhibition of neutrophil elastase, cathepsin G, thrombin, plasmin
	SerpinA3	Inhibition of cathepsin G
	SerpinA4	Inhibition of kallikrein
	SerpinA5	inhibition of active protein C
	SerpinA6	Non-inhibitory; cortisol binding globulin
	SerpinA7	Non-inhibitory; thyroxine binding globulin of serum
	SerpinA8	Non-inhibitory; amino-terminal cleavage by the protease renin results in release of the decapeptide angiotensin I
	SerpinA9	Maintenance of naive B cells
	SerpinA10	Inhibition of activated coagulation factor X and XI
	SerpinA12	Insulin-sensitizing adipocytokine
B	SerpinB1	Inhibition of neutrophil elastase, cathepsin G
	SerpinB2	Inhibition of urokinase-type plasminogen activator
	SerpinB3	Inhibition of cathepsins L, G, S and K
	SerpinB4	Inhibition of cathepsins G and chymase
	SerpinB5	Non-inhibitory; inhibition of metastasis through uncharacterized mechanism
	SerpinB6	Inhibition of cathepsin G and thrombin
	SerpinB7	Megakaryocyte maturation
	SerpinB8	Inhibition of furin
	SerpinB9	Inhibition of granzyme B and elastase
	SerpinB10	Inhibition of thrombin and trypsin
	SerpinB12	Inhibition of trypsin and plasmin
	SerpinB13	Inhibition of cathepsins L and K

(Modified from Law et al., 2006 and Rawlings et al., 2014)

seems that SerpinA3 enhances the fibril formation in these patients and may affect the process of the disease (Abraham et al, 1988; Kamboh et al, 2006).

Cumulative evidence has revealed an elevated expression of serpins in certain malignancies. The level of SerpinB3 (squamous cell carcinoma antigen 1, SCCA1) has been used as a serum marker for the advanced stages of the SCC of lung, head and neck, cervix and esophagus for many years (Silverman et al, 2004). In addition, expression of SCCA indicates poor prognosis in cervical carcinoma (Duk et al, 1996). SerpinB5 has a tumor suppressor role, because it inhibits cell motility and angiogenesis and induces apoptosis (Silverman et al, 2004). This notion is supported with the inhibition of invasion of breast cancer cell lines by induction of the SerpinB5 expression (Sheng et al, 1996). Thus, loss of SerpinB5 expression is associated with poor prognosis of patients with breast carcinoma. On the other hand, SerpinB5's role in different tumors seems to be a double-edged sword, because elevated expression of SerpinB5 has been reported in colon (Song et al, 2002) and prostate carcinoma (Zou et al, 2002). The expression of SerpinB6, the other member of the clade B, markedly increases during differentiation

of keratinocytes, but its exact role in epidermis remains undetermined (Silverman et al, 2004).

2.3.3 SerpinA1 and SerpinA3 in cancer

SerpinA1 expression is associated with invasive and the metastatic potential of lung cancer cells (Higashiyama et al, 1992; Zelvyte et al, 2004), colorectal (Karashima et al, 1990), gastric carcinoma (Tahara et al, 1984), papillary thyroid cancer (Jarzab et al, 2005; Poblete et al, 1996) and prostate cancer (El-Akawi et al, 2008). In esophageal squamous dysplasia, SerpinA1 expression is associated with progression of the tumor (Joshi et al, 2006). Another piece of evidence supporting the role that SerpinA1 plays in the progression of SCC has been reported in oral mucosal SCC (Shirasuna et al, 1987). In addition, SerpinA1 has been identified as a biomarker in bladder cancer (Rosser et al, 2014) and insulinoma (de Sa et al, 2007). Furthermore, expression of SerpinA3 has been documented in several malignancies such as gastric cancer (Allgayer et al, 1998), cancer of salivary glands (Chomette et al, 1991) and lung adenocarcinoma (Higashiyama et al, 1995). In rat hepatoma cells, SerpinA3 was found to inhibit apoptosis (Emoto et al, 1998). SerpinA3 has been observed to inversely correlate with survival of melanoma patients (stage III) (Wang et al). In endometrial cancer cells, SerpinA3 promotes tumor cell growth via ERK1/2 and AKT signaling pathways (Yang et al, 2014). In addition, co-expression of SerpinA1 and SerpinA3 has been observed in HLA-positive cervical carcinoma and their expression was linked with poor prognosis (Kloth et al, 2008).

2.4 Eph/ephrin tyrosine kinases family in cSCC

2.4.1 Activation and signaling of Eph/ephrin family

Tyrosine kinases are currently categorized into non-receptor and receptor types based on their structure (Fantl et al, 1993). Receptor tyrosine kinases (RTKs) are transmembrane proteins with a ligand-binding domain and an intracellular part, which have a tyrosine kinase domain. If the ligand is unavailable, the RTKs are inactive and unphosphorylated. Binding of the ligand to the extracellular domain of the receptor results in the clustering of the receptor, activation and autophosphorylation of a regulatory tyrosine and consequently internalization of intracellular domain by endocytosis (Lisabeth et al, 2013; Schlessinger, 2000). The roles of RTKs in many biological processes such as controlling of cell proliferation, apoptosis, survival and differentiation have been well documented (Wilkinson, 2001).

Erythropoietin-producing hepatocellular (Eph) receptors represent the largest family of receptor tyrosine kinases (Pasquale, 2008; Pasquale, 2010). Based on their structures

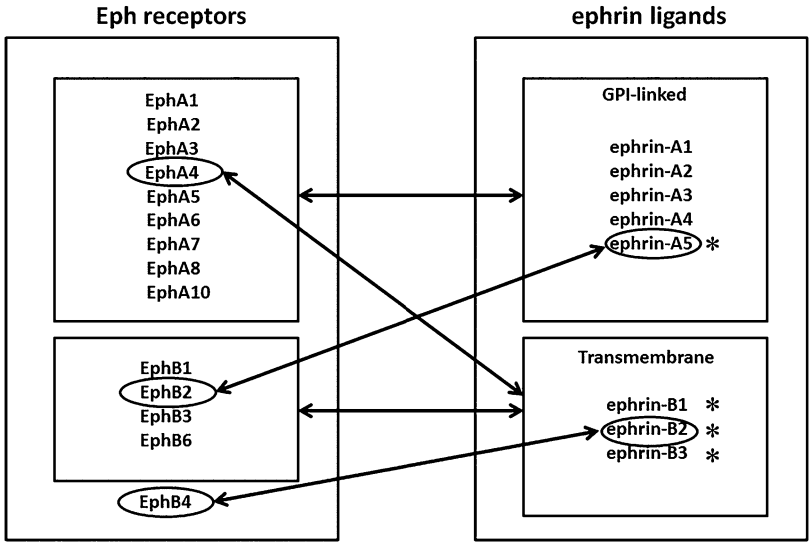


Figure 4. Interactions of Eph receptors and ephrin ligands. (* possible ligands for EphB2 receptor) (Modified from Wilkinson, 2001)

and ligand-binding affinities, the human Eph receptors and ligands (ephrins) are divided into A and B subgroups (Himanen & Nikolov, 2003; Nikolov et al, 2013; Pasquale, 2010). Ephrin-A ligands (5 member), which are glycosylphosphatidylinositol (GPI)-linked, promiscuously bind to the EphA receptors (9 members) and transmembrane ephrin-B ligands (3 members) promiscuously bind to EphB receptors (5 members) (Pasquale, 2005). Exceptionally, ephrin-A5 can interact with EphB2 in addition to A-type receptors and all ephrin-B ligands can bind to EphA4 (Figure 4) (Himanen & Nikolov, 2003; Pasquale, 2004; Pasquale, 2005). The EphB4 receptor has a high affinity to bind to the ephrin-B2 ligand (Pasquale, 2010). Eph receptors have an intracellular and extracellular region. The intracellular part has a kinase and sterile alpha motif (SAM) domain. The extracellular part has an ephrin-binding domain, fibronectin domain and an EGF-like motif (Pasquale, 2005).

Because both receptor and ligand are anchored to the membrane, cell-cell contact is required for their interaction. Upon binding to their cognate receptor, both receptors and ligands can transduce signals into the cell resulting in a bidirectional reverse and forward signaling. Therefore, as a unique characteristic of Eph/ephrin signaling, both Eph receptors and ligands simultaneously act as ligands and receptors between neighbor cells (Figure 5) (Himanen, 2012; Merlos-Suarez & Batlle, 2008; Surawska et al, 2004). The forward signal is due to the activation of the Eph tyrosine kinase domain and the reverse signal is related to the Src tyrosine kinase and other tyrosine kinases (Gu & Park, 2001; Matsuoka et al, 2005; Miao et al, 2005; Pasquale, 2008). In addition, ephrin-B ligands and many of the Eph receptors have a PDZ domain-binding region that is crucial

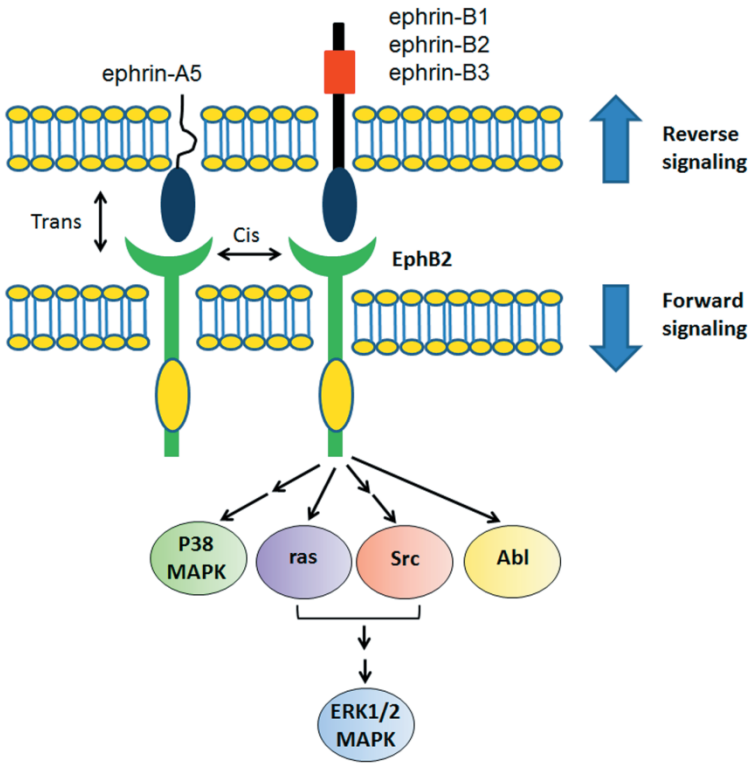


Figure 5. EphB2/ephrin signaling

for their physiological role (Egea & Klein, 2007; Pasquale, 2008). Interaction of the receptor and ligand may take place between the cell membrane of two different cells (in *trans*), which is an activating signal, or it may take place between receptors and ligands located on the same cell (in *cis*), which fails to induce any signaling (Arvanitis & Davy, 2008; Pasquale, 2005). The next stage is tetramerization of the receptors and ligands, which induces activation of the signal and forms large clusters (Himanen et al, 2004; Pasquale, 2005; Smith et al, 2004). Once Eph/ephrin signaling is activated, it induces activation of several downstream signaling pathways. Activation of Eph/ephrin signaling leads to activation of integrins, Src, P21 activated kinase, MAPK pathway, Abl, Rac, G-protein pathway and several other downstream signaling pathways (Gucciardo et al, 2014; Lackmann & Boyd, 2008; Pasquale, 2008; Pitulescu & Adams, 2010; Poliakov et al, 2008).

Eph/ephrins were first discovered as axon guidance molecules in the development of the central nerve system (CNS) (Flanagan & Vanderhaeghen, 1998; Kullander & Klein, 2002) and their roles in other biological process were identified afterwards. Eph receptor and ephrin ligands are expressed in almost all cells of all tissues of the embryo and Eph/ephrin signaling is involved in several embryonic developmental processes such

as skeletal and cardiovascular development (Kullander & Klein, 2002; Palmer & Klein, 2003; Pasquale, 2005). They play an important role in cell-cell interaction and junction, cell movement and migration, cell repulsion, actin cytoskeleton and cell differentiation, proliferation and survival (Egea & Klein, 2007; Pasquale, 2008). Aside from enrollment in the development of CNS, their role in the immune system, stem cells, bone, platelet aggregation and thrombus formation has been documented (Arvanitis & Davy, 2008; Himanen et al, 2007; Prevost et al, 2005). Eph/ephrin signaling contributes to several physiological processes in adults, e.g., memory and learning (Gerlai, 2002) and regulation of the insulin secretion (Konstantinova et al, 2007). Bone remodeling disorders have been reported in the patients with Eph/ephrin signaling deficiency or mutation (Davy et al, 2006). In addition, the role of Eph receptors in the organization of the intestinal villus and crypts in a model of EphB2 knockout mice has been demonstrated (Batlle et al, 2002).

2.4.2 Eph/ephrin signaling in skin

The expression of certain Eph receptor and ephrins is upregulated in normal skin and their role in the regulation of epidermal homeostasis by balancing between proliferation and death of keratinocytes has been documented (Hafner et al, 2006; Lin et al, 2012). In the epidermal layer, ephrin-A1 is expressed in the deeper layer (stratum basale), whereas EphA1, EphA2 and EphA4 are expressed in all epidermal layers particularly suprabasal layer, which are more differentiated (Perez White & Getsios, 2014). The expression of EphA2 receptor in epithelial cells is regulated with E-cadherin (Miura et al, 2009; Orsulic & Kemler, 2000). Ligand targeting of EphA2 receptor promotes differentiation and adhesion of the epidermal keratinocytes via overexpression of desmoglein 1 (Lin et al, 2010). In human epidermal keratinocytes, EphB2, acting as a ligand, triggers reverse signaling *in vitro* and consequently promotes epidermal differentiation (Walsh & Blumenberg, 2012). Eph receptor and ephrins regulate differentiation, migration and adhesion of epidermal keratinocytes, and have been implicated in the pathogenesis of psoriasis (Gordon et al, 2013; Lin et al, 2012; Walsh & Blumenberg, 2012).

2.4.3 The Eph/ephrin family in cancer progression

Eph/ephrins are highly expressed and involved in a variety of embryonic developmental processes. However, the expression of the most of the Eph/ephrins is relatively low in the majority of normal adult tissues (Hafner et al, 2004). Alteration of expression of several Eph receptors (Table 3) and ephrins (Table 4) have been implicated in different malignancies (Ireton & Chen, 2005; Nakada et al, 2004; Noren & Pasquale, 2004). However, the role of Eph/ephrin signaling in cancer progression appears to be complex. There are plenty of mechanisms implicated in the tumorigenic role of the Eph receptor

and ephrins e.g., hypoxia, cytokines and oncogenic signaling pathways (Pasquale, 2008). Depending on the cancer cells, Eph/ephrin signaling recruits different pathways to promote or inhibit tumor progression. Jak/Stat (Lai et al, 2004) and Akt/PI3K (Carpenter & Cantley, 1996) pathways, as downstream pathways of Eph receptors, regulate cell proliferation, growth and migration. In colorectal cancer, EphB expression is regulated through the Wnt/β-catenin/Tcf pathway (Pasquale, 2008). In addition, the Abl-cyclin D1 pathway is the known pathway that EphB2 receptor uses to mediate cell proliferation and invasion in colon cancer cells (Genander et al, 2009). There is overwhelming evidence that the expression of Eph receptors is lost in colorectal cancer suggesting a tumor suppressor role for Eph receptors in these malignancies (Batlle et al, 2005; Guo et al, 2006a; Jubb et al, 2005). The possible mechanism for the downregulation of the Eph receptors in colon cancer is induction of hypoxia. Hypoxia-inducible factor-1 has a high affinity for nuclear β-catenin and can silence target genes by competing with Tcf (Kaidi et al, 2007; Pasquale, 2008). In glioma cells, EphB2/R-Ras signaling is contributed to the invasion of the tumor (Nakada et al, 2005). Eph receptors (EphB4) regulate the migration of the tumor cells through RhoA GTPase in melanoma (Lisle et al, 2013; Parri et al, 2009; Yang et al, 2006).

Table 3. Examples of Eph receptors with prognostic significance in various cancer types.

Expression	Eph receptor	Cancer type	Prognostic indication
Upregulated	EphA2	NSCL	Poor survival
		Hepatocellular carcinoma	Poor survival
		Epithelial ovarian	Poor survival
		Endometrial	Poor survival
		Breast	Poor survival
		Glioma	Poor survival
		Prostate	Poor differentiation
	EphA3	Gastric	Poor survival
		Colorectal	Poor survival
	EphA4	Gastric	Poor survival
	EphA7	Glioblastoma	Poor survival
	EphB3	NSCL	Increased metastasis
	EphB4	Bladder	Poor differentiation
		Ovarian	Poor survival
		Head and neck SCC	Lymph node metastasis
		Prostate	Increased Gleason score
Downregulated	EphA1	Colorectal	Poor survival
	EphA5	Breast	Lymph node metastasis
	EphA7	Prostate	Increased Gleason score
	EphB2	Colorectal	Poor differentiation

(Modified from Lisle et al., 2013)

Upregulation of EphA2 expression has been observed in prostate cancer (Walker-Daniels et al, 1999; Zeng et al, 2003), breast cancer (Zelinski et al, 2001), glioblastoma (Wykosky & Debinski, 2008) and melanoma (Udayakumar et al, 2011), and the receptor has been used as a target for cancer therapy (Tandon et al, 2011). In human breast carcinoma cells, cleavage of EphA2 by MT1-MMP promotes cell invasion and cell-cell repulsion (Sugiyama et al, 2013). Eph receptors, ephrin-A ligands and integrin α3 interact with each other and co-localize on the membrane of glioblastoma cells (Makarov et al, 2013). Expression of EphB4 has been observed in breast cancer (Berclaz et al, 1996). Elevated expression of EphB2 receptor has been previously reported in some malignancies, e.g., hepatocellular carcinoma, colorectal cancer, renal carcinoma (Hafner et al, 2004), neuroblastoma (Tang et al, 2001), gastrointestinal cancers (Lugli et al, 2005), ovarian cancer and lung cancer (Surawska et al, 2004). In glioblastoma, expression of EphB2 receptor and ephrin-B2 ligand is associated with invasive phenotype of the tumor (Nakada et al, 2010; Nakada et al, 2004). In contrast, as mentioned previously, the activation of EphB receptor suppresses growth of colorectal cancer (Batlle et al, 2005) and elevated expression of EphB2 receptor is associated with longer survival (Jubb et al, 2005). Soluble EphA7 has been noted to display tumor suppressor function in follicular lymphoma *in vivo* (Oricchio et al, 2011). In addition, silencing and mutation of Eph receptors have been described in some malignancies (Merlos-Suarez & Batlle, 2008). Loss-of-function mutations, occurring mostly in the A9 track in exon 17, has been identified in prostate cancer (Huusko et al, 2004), colorectal cancer (Alazzouzi et al, 2005) and gastric cancer (Davalos et al, 2007).

Table 4. Examples of ephrin ligands with prognostic significance in various cancer types.

Expression	ephrin ligand	Cancer type	Prognostic indication
Upregulated	ephrin-A1	Melanoma	Poor survival
		Gastric adenocarcinoma	Increased lymph node metastasis
		Hepatocellular carcinoma	Biomarker
		Ovarian	Poor survival
	ephrin-A4	Osteosarcoma	Poor survival
	ephrin-A5	Ovarian	Poor survival
	ephrin-B2	Glioma	Poor survival
		Ovarian	Poor survival
		Uterine cervical	Poor survival
Downregulated	ephrin-A1	Glioma	Poor prognosis
		Prostate	Increased Gleason score
	ephrin-A5	Prostate	Decreased survival
		Chondrosarcoma	Higher clinical grade

(Modified from Lisle et al., 2013)

2.4.4 Eph/ephrin signaling in skin cancer

The role of certain Eph receptors has been examined in skin cancer. Ablation of EphA2 also known as epithelial cell receptor protein kinase (Lindberg & Hunter, 1990) in a homozygous knockout model of chemically induced mouse skin resulted in tumor progression (Guo et al, 2006b). These findings suggest that EphA2 serves as tumor suppressor in epidermal keratinocytes. In human normal skin, the expression level of EphA3, EphA8, EphB2 and ephrin-A2 is very low. Furthermore, downregulation of EphA1 expression has been observed in human non-melanoma skin cancer (Hafner et al, 2006).

2.5 Absent in melanoma 2 (AIM2) in cSCC

2.5.1 The AIM2 inflammasome

There is increasing evidence that chronic inflammation is associated with different types of malignancies (Coussens & Werb, 2002). Besides the role of skin as a barrier, innate and adaptive immune systems work together to protect the body against exogenous organisms. The innate immune system is mainly responsible for initiating the primary response (Lamkanfi & Dixit, 2014). The major issue in the process of immune system is the discrimination between host cells and pathogens. This relies on the sensing of the pathogen-associated molecular patterns (PAMPs) such as part of microbial cell wall, nucleic acids and secretion system by pattern recognition receptors (PRRs) found in innate immune cells (Lamkanfi & Dixit, 2014). PAMPs are unique molecular structures for the microorganisms and absent in host cells. The lack of a PAMP signature protects the host cells from being targeted by immune system activation (Dowling & O'Neill, 2012; Khare et al, 2010). PRRs are categorized into sensor and phagocytic PRRs. Sensor PRRs such as AIM2, membrane-bound Toll-like receptors (TLRs), Nod-like receptors (NLR) and RIG-1-like receptor (RLRs) bind to PAMPs and initiate the signals resulting in activation of inflammatory signaling pathways (Khare et al, 2010). PRRs can also be activated by damage-associated molecular patterns (DAMPS) released from damaged host cells (Kolb et al, 2014; Lamkanfi & Dixit, 2014).

The human IFN-inducible genes comprise *AIM2*, *IFIX* (Interferon (IFN)-inducible protein X), *IFI16* (IFN-inducible protein 16) and *MNDA* (myeloid nuclear differentiation antigen) (Choubey et al, 2010; Ludlow et al, 2005). IFN-inducible family members encode for hematopoietic IFN-inducible nuclear protein with a unique repeat of 200 amino acid (HIN-200) (Choubey et al, 2010; Ludlow et al, 2005). DNA-binding HIN-200 domain of the AIM2 serves as a sensor for cytosolic double-strand DNA (dsDNA) from viruses such as cytomegalovirus and vaccinia, intracellular bacteria and host cells (Lamkanfi & Dixit, 2014; Ponomareva et al, 2013; Rathinam et al, 2010; Schroder & Tschopp,

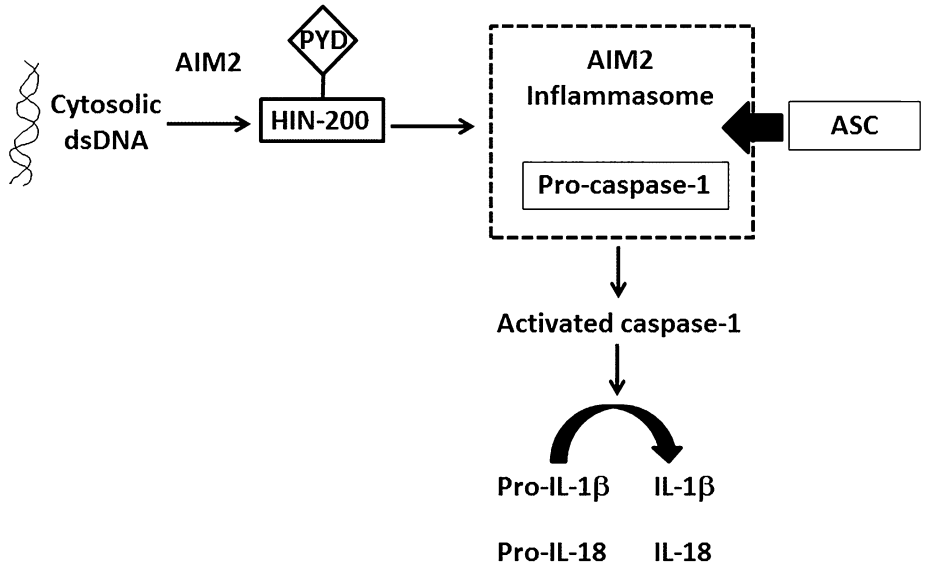


Figure 6. Schematic model of DNA sensing and AIM2 inflammasome activation. ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; HIN, hematopoietic IFN-inducible nuclear protein; PYD, pyrin domain. (Modified from Choubey, 2012; Ponomareva et al., 2013; Vanaja et al., 2015)

2010). After sensing cytosolic dsDNA, AIM2 forms an inflammasome using apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which is necessary for the activation of pro-caspase-1 (Ponomareva et al, 2013). Activation of AIM2 inflammasome in macrophages promotes maturation and secretion of cytokines such as IL-1β and IL-18 through activation of caspase-1 (Figure 6) (Lamkanfi & Dixit, 2014; Schroder & Tschopp, 2010). AIM2 is involved in the pathogenesis of autoimmune disorders such as systemic lupus erythematosus due to the generation of autoantibodies against the host cells (Lamkanfi & Dixit, 2014; Panchanathan et al, 2011). However, no association between AIM2 expression and severity of the disease has been documented (Kimkong et al, 2009).

2.5.2 AIM2 in skin

AIM2 expression is very low in epidermal keratinocytes (de Koning et al, 2012; Dombrowski et al, 2011). However, increased expression of AIM2 has been observed in allergic and inflammatory diseases of the skin such as urticaria, atopic and contact dermatitis (de Koning et al, 2012; Masters, 2013). In addition, in psoriatic lesions, keratinocytes express a high level of AIM2 (de Koning et al, 2012; Dombrowski et al, 2011). Unlike in healthy skin, where cytosolic DNA cannot be detected, in psoriatic lesions AIM2 senses cytosolic DNA in keratinocytes resulting in activation of inflammasome (Dombrowski et al, 2011). Activation of AIM2 inflammasome triggers

activation of caspase-1, which cleaves IL-1β to the active form (Stutz et al, 2009). IL-1β pathway activation is a known phenomenon in the pathogenesis of psoriasis (Nestle et al, 2009; Renne et al, 2010). Furthermore, HPV 16 induces AIM2 inflammasome activation in epidermal keratinocytes (Reinholz et al, 2013).

2.5.3 AIM2 in cancer

In addition to its principal role in the activation of inflammatory responses as part of the innate immunity system, a number of recent studies proposed the role of AIM2 in cancer progression (Kolb et al, 2014). However, depending on the tumor type the activation of inflammasome plays different and sometimes controversial roles (Kolb et al, 2014). Besides cancer cells, the tumor microenvironment includes immune cells responsible for secreting different chemokines and cytokines that are known to play important roles in mediating tumor progression and metastasis (Kolb et al, 2014). AIM2 was first identified in spleen, blood leukocytes and small intestine with a sequence with high similarity to IFI16 and MNDA and was named based on the lack of expression in malignant melanoma cell lines (DeYoung et al, 1997). In a mouse model of breast cancer, AIM2 expression was found to suppress the proliferation of the cancer cells (Chen et al, 2006). Caspase-1-mediated cell death as the result of AIM2 inflammasome activation has been suggested to act as the possible mechanism for the induction of cell senescence by AIM2 (Fernandes-Alnemri et al, 2009; Hornung et al, 2009).

3. AIMS OF THE STUDY

At present, no specific molecular markers for cSCC are available and there is an emerging need to identify novel molecular markers for the progression of cSCC. The general purpose of this study was to identify and characterize novel biomarkers for the progression and invasion of cSCC and to understand their regulation during the progression of the tumor.

The specific aims included:

1. To characterize SerpinA1 (AAT) as a biomarker for the progression of cSCC.

2. To examine the functional roles of EphB2 in cSCC both in cultured cells and in cSCC tumors in a xenograft model.

3. To explore the functional roles of AIM2 in cSCC cells in culture and in cSCC tumors in a xenograft model.

4. MATERIALS AND METHODS

4.1 Study approval

Approval for the use of archival tissue specimens and the collection of normal skin and cSCC tissues was obtained from the Ethics Committee of the Hospital District of Southwest Finland, Turku, Finland (Approval numbers: 187/4/2006; 138/2007). The study was performed in accordance with the Declaration of Helsinki. Prior to the surgery, each patient gave their written informed consent for obtaining normal human epidermal keratinocytes and cSCC cell lines and tumors. The experiments with mice (used in study I, II and III) were approved by the State Provincial Office of Southern Finland and conducted according to institutional guidelines (Approval numbers: ESAVI/8181/04.10.07/2012; ESLH-2007-09159/Ym-23).

4.2 Cell culture

4.2.1 Normal human epidermal keratinocytes (NHEK) (I, II, III)

NHEK cultures (n=9) (NHEK 42, NHEK 45B, NHEK 51, NHEK 52, NHEK 59, NHEK 64, NHEK 65, NHEK 70, NHEK 74) were established from the normal skin of patients undergoing surgery for mammoplasty at Turku University Hospital, Turku, Finland. In addition, one NHEK (NHEK PC) was purchased from PromoCell. Keratinocytes were cultured in Keratinocyte Basal Medium 2 (KBM®-2) supplemented with SingleQuots®.

4.2.2 Human cSCC cell lines (I, II, III)

Primary (n=5) and metastatic (n=3) human cSCC cell lines were established from surgically removed SCCs of skin at the Department of Otorhinolaryngology, Turku University Hospital (Table 5). cSCC cells were cultured in DMEM supplemented with 6 nmol/l glutamine, non-essential amino acids and 10% fetal calf serum (FCS). All cSCC cell lines were sent to DDC Medical (Fairfield, OH) to verify their authenticity by short tandem repeat (STR) profiling.

Table 5. Location and metastatic status of cSCC cell lines.

SCC cell line	Location	Primary/metastatic
UT-SCC12A	Skin of the nose	Primary
UT-SCC91	Skin of the nose	Primary
UT-SCC118	Face	Primary
UT-SCC105	Face	Primary
UT-SCC111	Face	Primary
UT-SCC7	Temporal skin	Metastatic
UT-SCC115	Auricle	Metastatic
UT-SCC59A	Temporal skin,	Metastatic

4.2.3 HaCaT and Ha-ras-transformed tumorigenic HaCaT cell lines (I)

Immortalized nontumorigenic human epidermal keratinocyte-derived cell line, HaCaT (Boukamp et al, 1988) and Ha-*ras*-transformed tumorigenic HaCaT cell lines A5, II4, and RT3 (Boukamp et al, 1990) were a kind gift from Dr. Norbert E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (Table 6). HaCaT and Ha-*ras*-transformed HaCaT cell lines were cultured in DMEM with 10% FCS. G418 (200 µg/ml) was added to the medium of the Ha-*ras*-transformed HaCaT cell lines.

Table 6. Characteristics of HaCaT and Ha-*ras*-transformed tumorigenic HaCaT cell lines (Mueller et al, 2001).

Cell line		Characteristics
HaCaT		Immortalized nontumorigenic human epidermal keratinocyte-derived cell line
Ha-*ras*-transformed HaCaT cell lines	A5	Forms benign tumorigenic tumors *in vivo*
	II4	Forms malignant invasive tumors *in vivo*
	RT3	Forms metastatic tumors *in vivo*

4.3 Expression profiling (I, II, III)

4.3.1 Microarray-based gene expression profiling (Affymetrix) (I, II, III)

Gene expression profiling was performed to compare expression of different genes in NHEKs (n=5) and primary (n=5) and metastatic (n=3) cSCC cell lines using U133 Plus 2.0 GeneChip (Affymetrix Inc) at Finnish Microarray and Sequencing Centre, Turku Center for Biotechnology, Turku, Finland. Normalization of the arrays was performed using the RMA assay, Chipster software. Gene expression profile after EphB2 knockdown

was performed with RNAs derived from cSCC cell lines (n=3) 72 hours after EphB2 or control siRNA transfection using Human Genome U219 Array Plate. Gene expression analysis was then subjected to Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) for pathway analysis. $P<0.05$ and fold change (log2)>0.75 were used as thresholds for analysis.

4.3.2 RNA sequencing (II, III)

Next generation sequencing was performed for RNAs derived from NHEK (n=4) and primary (n=5) and metastatic (n=3) cSCC according to the Whole Transcriptome Analysis Kit procedure (SOLiD™) at the Finnish Microarray and Sequencing Centre, Turku Center for Biotechnology, Turku, Finland. The samples were processed with the SOLiD 3Plus instrument with 35bp read length. The data were normalized using *quantile-to-quantile adjustment* (R/Bioconductor package edgR).

RNA sequencing after AIM2 knockdown was performed with RNAs derived from cSCC cell lines (n=3) 72 hours after AIM2 or control siRNA transfection using Illumina RNA-sequencing at Turku Center for Biotechnology. The samples were sequenced with the HiSeq2500 instrument using single-end sequencing chemistry with 50bp read length and were aligned against the human reference genome (hg19 assembly). Pathway analysis was performed with IPA analysis as mentioned above.

4.4 Quantitative real-time PCR (qRT- PCR) (I, II, III)

Culture media of the cells were changed to serum-free 24 hours before RNA extraction. Total RNA was isolated from cultured cells and tissue samples using RNAeasy kit (Qiagen) by using manufacturer's instructions. cDNA was reverse transcribed from 1 µg RNA using reverse transcriptase M-MLV RNase H minus reverse transcriptase and random hexamers (Promega). qRT- PCR was performed to determine the expression level of human *SERPINA1* and *SERPINA3* and murine *SERPINA1* (study I), human *EPHB2*, *EFNB2* (Study II) and *AIM2* (Study III) using Applied Biosystems 7900HT Fast qRT-PCR System as previously described (Junttila et al, 2007a). The specific primers and probes have been described in study I, II and III.

4.5 Immunofluorescence staining of cSCC cells (II, III)

Immunofluorescence staining was performed to examine the amount of EphB2 receptor and ephrin-B2 ligand on the cell surface of the NHEK and cSCC cell lines. cSCC cell lines and NHEKs were labeled with goat anti-EphB2 antibody (R&D Systems) and rabbit anti-ephrin-B2 antibody (Santa Cruz). As secondary antibodies highly cross-adsorbed

Alexa Fluor® 633 donkey anti-goat IgG (H+L) and Alexa Fluor® 568 goat anti-rabbit IgG (H+L) (both from Invitrogen) were used and nuclei were visualized with Hoechst (H3570; Invitrogen). The cells were mounted in Mowiol-DABCO (Sigma-Aldrich) and observed with Zeiss LSM510 META confocal microscope (Carl Zeiss). For detection of AIM2, cells were permeabilized with 0.1% Triton X-100 and labeled with mouse anti-AIM2 antibody (Abnova).

4.6 Western blot analysis (I, II, III)

Equal amount of heat denatured conditioned media or cell lysates were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membranes were incubated with primary antibodies (Table 7) following a corresponding secondary antibody and visualized using ECL kit (Amersham). Equal protein loading was ensured by reprobing with a monoclonal mouse anti-human anti-tissue inhibitor of metalloproteinase-1 antibody (anti-TIMP1) or monoclonal anti-β-actin (AC-15).

Table 7. Antibodies used in immunoblotting.

Antigen	Product number	Source	Used in
SerpinA1	A0012	DAKO	I
β-actin	A1978	Sigma-Aldrich	I, II, III
p-Creb	9191	Cell Signaling Tech.	I
p-ERK1/2	9101	Cell Signaling Tech.	I
EphB2	AF467	R&D	II
AIM2	H00009447-B01P	Abnova	III
MT1-MMP	IM42L	Calbiochem	II
Anti-p-Tyr	05-321	Millipore	II
MMP-1	AB8105	Chemicon	II, III
MMP-13	IM64L	Calbiochem	II, III
TIMP1	IM32	Calbiochem	II, III

4.7 Analysis of the cell surface proteins (II)

To analyze the expression level of the EphB2 on the cell surface of NHEKs (n=3) and cSCC cell lines (n=4), cell cultures were biotinylated (EZ-Link Sulfo-NHS-LC-Biotin, 21335; Pierce Biotechnology) and biotinylated proteins were immobilized with streptavidin beads (Streptavidin Sepharose HighTM Performance, 17-5113-01; GE Healthcare Life Sciences). After the washing steps, cell surface proteins were eluted from the streptavidin beads and analyzed with immunoblotting using the anti-EphB2 antibody. MT1-MMP was used as marker for equal loading.

4.8 Immunoprecipitation of phosphorylated EphB2 (II)

Activation of the EphB2 receptor with soluble ephrin-B2-Fc ligand was performed as described previously (Chaudhari et al, 2007). Briefly, the conditioned medium of cSCC cells was changed to serum-free 24 hours prior to the experiment. The cells were then incubated with recombinant ephrin-B2-Fc (0.05 µM) (R&D Systems) for 5, 10, 30 and 180 minutes and harvested using lysis buffer (1 mM Tris-HCL pH 7.4, 0.5 mM EDTA, Triton® X-100 Sigma-Aldrich) containing protease inhibitors (Roche Complete; Roche Diagnostic), phosphatase and kinase inhibitors (1 mM Na3VO4, 10 mM Na4P2O7 and 0.5 M NaF). For immunoprecipitation of EphB2, anti-EphB2 antibody (3 µg) was added to the washed protein G agarose bead slurry (Invitrogen). After one hour incubation, EphB2 was precipitated by adding equivalent amounts of cell lysate protein (1500 µg) to the reaction mixture and incubated in room temperature for 1 hour. Precipitates were collected by pulsing the agarose beads and eluted by adding 60 µl of the 2× Laemmli buffer (Tris-HCl buffer 126 mM, Glycerol 20%, SDS 4%, Bromophenol blue 0.02% and 1 M DTT).

4.9 Human tissue samples (I, II, III)

4.9.1 cSCC tumors and normal skin (II, III)

cSCC tumors (n=6) (SCC12, SCC18, SCC19, SCC36, SCC41 and SCC58) were obtained from the Turku University Hospital after surgery of primary tumors. Normal skin samples (n=11) (MAM55, MAM59, MAM62, MAM66, MAM76, Epid3, Epid4, Epid5, skin44, NormE and NormO) were collected after mammoplasty surgery.

4.9.2 Tissue microarrays (TMA) (I, II, III)

All human tissues of normal skin from non-sun-exposed area, AK, cSCCIS and UV-induced cSCC were collected from archives of the Department of Pathology, Turku University Hospital, Turku, Finland. Recessive dystrophic epidermolysis bullosa (RDEB)-associated cSCC tissue samples were obtained by international collaboration (Table 8).

TMAs were generated using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) by making a 1.5-3 mm punched cores from the donor paraffin embedded blocks. Prior to punch each paraffin block, the selected area was reexamined by an expert pathologist (M.K).

4.9.3 Tissues from OTR patients (III)

Whole sections of AK (n=58), cSCCIS (n=59) and cSCC (n=57) of the OTR patients were obtained from organ transplant recipients (Table 8) by collaboration with Leiden University Medical Center, Leiden, the Netherlands.

Table 8. Number of human normal skin, AK, cSCCIS, cSCC and RDEB-cSCC tissue sections used in study I, II, and III.

Study	Type of sections	Normal skin	AK	cSCCIS	cSCC	RDEB-cSCC
I	TMA	-	36	29	71	12
II	TMA	12	69	56	68	-
III	TMA	15	71	60	81	-
III (OTR patients)	Whole section	-	58	59	57	-

4.10 Chemically induced mouse skin SCC (I, II)

Normal (n=5), acetone treated (n=2), hyperplastic skin (n=6) and SCC (n=19) samples were collected from skin of FVB/N HanHsd mice (maintained at the Laboratory Animal Center, University of Oulu). Mouse skin carcinogenesis was induced, as previously described (Brideau et al, 2007). Briefly, one dose of 100 µg of 7,12-dimethylbenz [α] anthracene (DMBA) in 100 µl of acetone was administered topically on the shaved mouse dorsal skin, followed by weekly 12-O-tetradecanoylphorbol-13-acetate (TPA) treatments for 20 weeks period in order to induce cSCC tumors. The skin of the mouse was treated with 5 µg of TPA in 100 µl of acetone four times at two day intervals in order to induce hyperplasia. The control mice were treated the same way with the vehicle (acetone). Mice were sacrificed at week 32 or at an earlier time-point if tumors were large, had features of invasive carcinomas or had the diameter of more than 10 mm. About one out of 10 benign papillomas progressed to malignant SCCs in this model.

4.11 Immunohistochemistry (IHC) (I, II, III)

IHC of the human TMAs (5 µm thick) was performed using an automated immunostaining device (Ventana Medical Systems SA, Illkirch, CEDEX, France). Human sections obtained from OTR patients, mouse cSCC tumors and mouse control tissue samples were analyzed as whole sections. The primary antibodies (Table 9) were detected using the Ventana ultraView Universal DAB detection kit and the Ventana amplification kit (Ventana Medical Systems SA, Illkrich, France). Images were taken from each slide using light microscopy (Olympus BX60) and semiquantitative analysis was performed

independently by two observers. Staining of the normal liver tissue, colorectal carcinoma and normal colon tissue was used as a positive control for strong positive staining for SerpinA1, EphB2 and AIM2, respectively.

Table 9. Antibodies used in IHC.

Antigen	Product number	Source	Used in
SerpinA1	A0012 (polyclonal)	DAKO	I
EphB2	AF467 (polyclonal)	R&D Systems	II
AIM2	HPA031365 (polyclonal)	Sigma	III

4.12 Functional analysis

4.12.1 Knockdown with siRNA (II, III)

In order to knockdown EphB2 or AIM2, cSCC cells were transfected with small interfering RNAs (siRNA) (Qiagen) using the silentFect™ Lipid Reagent (BIO-RAD). The following sequences were targeted:

EPHB2 siRNA: 5'-CCGAGAGGACCTCGTCTACAA-3'

AIM2 siRNA: 5'-CCCGAAGATCAACACGCTTCA-3'

Control siRNA: 5'-AAT TCT CCG AAC GTG TCA CGT-3'

4.12.2 Cell viability assay (II, III)

cSCC cell lines were first cultured on 10 cm dishes and transfected with control siRNA, EphB2 siRNA (study II) or AIM2 siRNA (75nM) (study III) as described above. Six hours after the transfection cells were trypsinized and 10,000 cells/well were seeded on 96-well plates in a final volume of 100 µl of serum-free DMEM. The number of viable cells was determined by CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega) or WST1 assay (Roche Diagnostics) according to the manufactures' instructions.

4.12.3 Cell invasion assay (II, III)

Invasion assay was performed as described previously (Ala-aho et al, 2004). cSCC cell lines were cultured in 6 cm dishes and transfected with EphB2 siRNA (75nM) (study II), AIM2 siRNA (study III) or control siRNA (75nM) as mentioned above. Twenty-four hours after the transfection the cells were seeded on ThinCert™ tissue culture inserts (8.0

mm pore size) coated with collagen type I (Advanced Biomatrix, Fremont, CA, USA) 0.2 M NaOH-HEPES (pH 7.4) and 5×DMEM. After 48 hours, nuclei were visualized by Hoechst 33342 (Invitrogen) and counted.

Cell invasion assay following activation of EphB2 receptor with ephrin-B2-Fc treatment was performed the same way after incubation of cSCC cells with ephrin-B2-Fc (0.05 μM) for 5 minutes.

4.12.4 Cell migration assay (II)

Cell motility assay was performed as described previously (Walsh & Blumenberg, 2012). Briefly, cSCC cells were first transfected with EphB2 siRNA (75nM) as described above, and incubated for 48 hours at 37°C. To inhibit cell proliferation, cells were treated with 2 mM hydroxyurea (Sigma Aldrich) in DMEM with 10% FCS for 6 hours at 37°C (Riihilä et al, 2014). A pipet tip was used to create a scratch of the cell monolayer and incubation was continued in DMEM with 1% FCS and 0.5 mM hydroxyurea for 24 hours. Live cell imaging was performed with Zeiss Axiovert 200M inverted microscope (Carl Zeiss). ORCA 1394 ERG camera with a 10× objective was used to capture images every 10 minutes for 24 hours (Axiovision Release 4.8) and quantitation of the images was performed with Image J (NIH) (Schneider et al, 2012).

4.13 Human cSCC xenograft model (II, III)

To characterize the effects of EphB2 (study II) and AIM2 (study III) knockdown on the growth of the cSCC tumors *in vivo*, cSCC xenograft tumor was established as previously described (Junttila et al, 2007b). cSCC cell lines (UT-SCC7) were first transfected with control and EphB2 or AIM2 siRNA (75nm). Seventy-two hours after the transfection a suspension of the cells (5×10^6) were injected subcutaneously into the back of the SCID mice (n=7-8 for each group). The size of the tumors were measured frequently and tumor volumes were calculated using the formula $V= \pi 4/3((L+W)/4)$. Hematoxylin and eosin (H&E), Ki-67 (Dako) and CD34 (Santa Cruz) stainings were performed after excision of the tumors.

4.14 Statistical analysis (I, II, and III)

Statistical analysis between groups was performed by Student's t-test, and Mann-Whitney *U*-test. Statistical analysis of the IHC results was performed using χ^2-test and Fisher's exact test.

5. RESULTS

5.1 Identification of SerpinA1 as a biomarker for cSCC (I)

5.1.1 Upregulation of SERPINA1 in cSCC cells

Elevated expression level of *SERPINA1* has been previously reported in some malignancies such as cervical carcinoma (Kloth et al, 2008), esophageal squamous dysplasia (Joshi et al, 2006), and squamous carcinoma of the oral cavity (Shirasuna et al, 1987).

Here, microarray-based gene expression profiling of the entire serpin family was performed in NHEKs (n=5) and primary (n=5) and metastatic (n=3) cSCC cells (I, Figure 1A). High expression levels of several serpin family members such as *SERPINA1*, *A3*, *B5*, *B6*, *E1*, *E2* and *H1* were noted in primary and metastatic cSCC cell lines. The results of the analysis showed significant upregulation of *SERPINA1* and *SERPINA3* in cSCC cell lines compared with NHEKs. In addition, qRT-PCR was performed to verify the results of the microarray analysis. The results revealed significant upregulation of *SERPINA1* in cSCC cells compared with NHEKs. In contrast, no significant difference was observed in mRNA expression level of *SERPINA3* between NHEKs and cSCC cells (I, Figure 1B).

To examine SerpinA1 production in cSCC cell lines and NHEKs, western blot analysis of the conditioned media was performed. Specific bands corresponding to SerpinA1 were noted in all cSCC cell lines, whereas only in one out of five NHEKs, production of SerpinA1 was observed (I, Figure 2A).

5.1.2 Regulation of SerpinA1 in cSCC cells

One of the histologic characteristics of the cSCC environment is the infiltration of inflammatory cells and cytokines in the tumor environment (Alam & Ratner, 2001; Madan et al, 2010; Rogers et al, 2010). The effects of different cytokines and growth factors on the expression of SerpinA1 were characterized. The results revealed that SerpinA1 expression is induced by EGF, TNF-α, IFN-γ and IL-1β. This finding was confirmed with western blot analysis (I, Figure 3A and B). Furthermore, the regulation of SerpinA1 expression by p38 MAPKs and ERK1/2 was analyzed. Treatment of the cSCC cell lines with SB203580 (inhibitor of p38α and p38β MAPKs) potently inhibited the expression of SerpinA1. As cSCC cell lines do not express p38β (Junttila et al, 2007a),

it can be concluded that SerpinA1 expression in cSCC cells is regulated by p38α MAPK (I, Figure 3C).

5.1.3 SerpinA1 expression correlates with malignant transformation of the epidermal keratinocytes

To characterize the association of SerpinA1 expression with the malignant transformation of keratinocytes, we analyzed the expression of SerpinA1 in HaCaT, an immortalized nontumorigenic human epidermal keratinocyte-derived cell line and three Ha-*ras*-transformed tumorigenic HaCaT cell lines (A5, II4, and RT3) representing different stages of tumor progression (Boukamp et al, 1988; Boukamp et al, 1990; Mueller et al, 2001). Specifically, A5 is a benign tumorigenic cell line, II4 forms malignant invasive tumors and RT3 cells form metastatic tumors *in vivo* (Mueller et al, 2001). The highest SerpinA1 expression was detected in RT3 and the lowest expression level was noted in non-tumorigenic HaCaT cells, which had inactivation of both alleles of p53 tumor suppressor. The expression of SerpinA1 was noted to increase when the cells were transformed from non-tumorigenic HaCaT cells to A5, II4 and RT3 (I, Figure 2B). Based on these findings, induction of SerpinA1 can be considered as a marker for the malignant transformation of normal epidermal keratinocytes to the aggressive and metastatic cSCC.

5.1.4 Expression of SerpinA1 correlates with tumor progression in vivo

To study the expression of SerpinA1 *in vivo*, IHC analysis of human TMAs consisting of AK lesions (n=36), cSCCIS (n=29) and UV-induced sporadic cSCCs (n=71) was performed (I, Figure 4). Semiquantitative analysis was performed to assess the SerpinA1 staining intensity in AK, cSCCIS and sporadic SCC tumors. Because SerpinA1 protein is produced in the liver and enters the blood circulation, it is abundantly present in tumor stroma. For that reason, only SerpinA1 staining in epidermal layer and tumor cells was considered for the analysis. According to the semiquantitative analysis, the majority of AK lesions (33 out of 36 samples) had negative (-) or weak (+) staining. In cSCCIS, weak or negative SerpinA1 staining was noted in 26 out of 29 tissues. SerpinA1 staining was significantly stronger in sporadic cSCC compared with cSCCIS and AK (P=0.001). Moderate (++) or strong (+++) cytoplasmic staining for SerpinA1 was noted in the tumor cells in 33 of 71 cSCC tumors (I, Table 1). The expression of SerpinA1 was also examined in RDEB-associated cSCC as an aggressive form of cSCC. Significantly stronger SerpinA1 staining was observed in all RDEB-associated cSCCs compared with sporadic cSCCs (P=0.002).

To further characterize the expression of SerpinA1 *in vivo*, a well-characterized model of chemically induced mouse skin carcinogenesis was employed (Abel et al, 2009; Brideau et al, 2007) and stained with an antibody against SerpinA1 (I,

Figure 5). In the epidermal layer of normal skin and vehicle treated mouse skin SerpinA1 staining was negative. In TPA-treated hyperplastic epidermis, only weak SerpinA1 staining was detected. In mouse cSCC, moderate and strong staining was noted in the majority of the tumors (11 out of 17 samples) and the staining was significantly stronger when compared with non-malignant lesions (P=0.002) (I, Table 2). Overexpression of *SERPINA1* in chemically induced mouse skin SCC compared with TPA-treated and untreated mouse skin was verified at the mRNA level by qRT-PCR analysis of the RNAs that were derived from the tumors. These findings indicate that SerpinA1 could serve as a biomarker for the progression of cSCC *in vivo*.

5.2 EphB2 promotes progression of cSCC (II)

5.2.1 Upregulation of EphB2 in cSCC cells and tumors

Elevated expression of EPH receptors and EFN ligands has been previously shown in certain malignancies (Lugli et al, 2005; Tang et al, 2001; Wykosky et al, 2005; Zelinski et al, 2001). Here, the expression of entire EPH receptor and EFN family in primary (n=5) and metastatic (n=3) cSCC cell lines and NHEKs (n=5) was analyzed using microarray-based gene expression profiling.

The results of the microarray analysis revealed upregulation of *EPHB2* and *EPHA4* in primary and metastatic cSCC cell lines compared with NHEKs (II, Figure 1A). In addition, next generation sequencing analysis confirmed specific upregulation of *EPHB2* mRNA in cSCC cells as compared with NHEKs (II, Figure 1B). The results of the microarray analysis and RNA sequencing identified *EPHB2* as the only EPH significantly upregulated (P<0.05) in cSCC cell lines compared to NHEKs. Overexpression of *EPHB2* in cSCC cell lines compared to NHEKs was verified by qRT-PCR (II, Figure 1C). Furthermore, qRT-PCR revealed upregulation of *EPHB2* in cSCC tumors (n=6) compared to normal skin (n=7) (II, Figure 1D).

Western blot analysis was performed to quantify the level of EphB2 production in cSCC cell lines and NHEKs. Specific bands corresponding to EphB2 receptor were noted in cell lysates of all cSCC cell lines, whereas EphB2 protein level was very low in NHEKs (II, Figure 1E).

Because receptor tyrosine kinases are transmembrane proteins (Schlessinger, 2000), the expression of EphB2 receptor on the cell surface of cSCC cell lines and NHEKs was examined using western blot analysis of the biotinylated cell surface proteins pulled down with avidin. Markedly more EphB2 was noted on the cell surface of the cSCC cells when compared with NHEKs (II, Figure 1F). In addition, immunofluorescence staining

revealed more abundant EphB2 on the cell surface of the cSCC cells when compared with NHEKs. Co-localization and clustering of the EphB2 receptor and ephrin-B2 ligand on the cell surface of cSCC was noted, whereas no clustering was noted on the cell surface of the NHEKs (II, Figure 1G).

5.2.2 Overexpression of EphB2 in human cSCC tumors in vivo

To examine the expression of EphB2 *in vivo*, TMAs generated from an archive of normal skin (n=12), AK (n=69), cSCCIS (n=56) and UV-induced cSCCs (n=68) were stained with anti-EphB2 antibody (II, Figure 2).

Positive tumor cell-specific EphB2 staining was noted in cell surface or cytoplasm of invasive cSCC and cSCCIS. The staining intensity was stronger in SCCIS and invasive cSCC compared with AK and normal skin (II, Figure 2A-F). Semiquantitative analysis of the EphB2 staining intensity revealed weak (+) and negative staining in the epidermal layer of the normal skin and AK lesions. Moderate (++) staining intensity was noted in 19 percent of cSCCIS and in invasive cSCC, 24 percent of the samples had moderate or strong (+++) EphB2 staining. Statistical analysis of the staining intensity revealed significantly stronger EphB2 staining in cSCCIS and invasive cSCC compared with AK and normal skin as a group (P<0.001) (II, Figure 2G).

5.2.3 Expression of EphB2 in chemically induced mouse cSCC

To further examine the expression of EphB2 *in vivo*, the paraffin tissues of normal mouse skin (n=5), vehicle-treated (n=2), hyperplastic mouse skin (TPA treated) (n=6) and mouse cSCCs (DMBA-TPA treated) (n=19) were stained with EphB2 antibody (II, Figure 3). Semiquantitative analysis of the staining intensity revealed moderate or strong EphB2 staining in 95 percent of mouse cSCC tumors. In hyperplastic mouse skin induced by TPA treatment, weak EphB2 staining was observed in 83 percent of samples. In vehicle-treated or untreated mouse skin, the EphB2 staining was absent in the epidermal layer of majority of the samples (86%) (II, Figure 3A-F). Staining intensity was significantly stronger in mouse skin SCCs than in nonmalignant lesions as a group (P< 0.001) (II, Figure 3G).

5.2.4 Gene expression profile alteration in cSCC cells after EphB2 knockdown

To explore the molecular mechanisms of EphB2 in cSCC cells, microarray-based gene expression profile analysis was performed after knockdown of EphB2 receptor in cSCC cells using specific siRNA (II, Figure 4A). Gene expression profiling was then subjected to IPA software (Thomas & Bonchev, 2010).

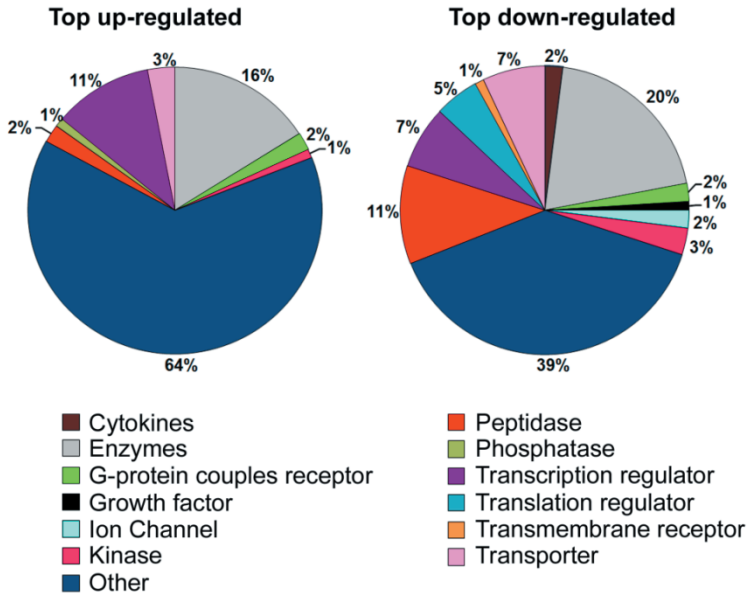


Figure 7. The gene expression profiles of cSCC cells after EphB2 knockdown were compared with control cultures. 100 most upregulated and downregulated genes were classified based on molecular function.

Differentially expressed genes after EphB2 knockdown were significantly associated with the biofunction categories *cell death*, *cellular movement*, *cell-to-cell signaling and interactions* and *cellular growth and proliferation* (II, Figure 4B). *Cell viability*, *invasion of tumor cells*, *migration of tumor cells*, *migration of cells* and *cell movement* were the top biofunctions significantly downregulated after EphB2 knockdown based on regulation *z*-score. Functional classification of the 100 most upregulated and downregulated genes after EphB2 knockdown identified enzymes as the largest specific group (20%) and peptidases as the second largest group (11%) of downregulated genes (Figure 7).

All together 2460 probe sets were differentially regulated after EphB2 knockdown (II, Figure 4C). *MMP1* and *MMP13* were among the most downregulated genes significantly regulated after EphB2 knockdown (II, Figure 4C and D). In addition, analysis of the molecular networks using IPA revealed involvement of MMP-1 and MMP-13 expression in significantly downregulated biofunctions *invasion of tumor cells* (II, Supplementary Figure S1), *migration of tumor cells* (II, Supplementary Figure S2), *migration of cells*, *cell movement* and *cell viability*.

5.2.5 EphB2 knockdown inhibits proliferation and migration of cSCC cells

To elucidate the functional role of EphB2 in the progression of cSCC cells, cell proliferation assays were performed after EphB2 knockdown. EphB2 knockdown significantly reduced the number of viable cSCC cell lines (II, Figure 5A). Migration assay was conducted 48 hours after transfection of the cSCC cells with control or EphB2 siRNA. In the control group, the scratch wound was healed 24 hours after making the scratch, whereas analysis of the cell migration with time-lapse microscopy showed significant inhibition of the directional migration in skin SCC cell lines transfected with EphB2 siRNA (II, Figure 5B and C).

5.2.6 EphB2 regulates invasion, and expression of invasion-related MMPs (MMP-1 and MMP-13) in cSCC cells

Analysis of the invasion of cSCC cells into collagen revealed significantly reduced invasion of the cells through collagen following EphB2 knockdown (II, Figure 5D). As mentioned above, the results of the gene expression profiling revealed downregulation of *MMP1* and *MMP13*, two members of the MMP family promote invasion of the cSCC cells (Ala-aho et al, 2004). Here, the protein expression of MMP-1 and MMP-13 following EphB2 knockdown was examined. EphB2 knockdown was noted to markedly inhibit production of MMP-1 and MMP-13 in cSCC cells lines (II, Figure 5E).

To get further insights into the role that the EphB2 receptor plays in the invasion of cSCC cells and regulation of MMPs, soluble ephrin-B2-Fc was used to induce activation of endogenous EphB2 (Chaudhari et al, 2007). Immunoprecipitation with EphB2 antibody followed by blotting against anti-p-Tyr showed activation of the receptor already 10 minutes after adding the soluble ligand (II, Figure 5F). Activation of EphB2 signaling by a soluble EphB2 ligand, ephrin-B2-Fc, induced invasion of cSCC cells through collagen (II, Figure 5G). In addition, EphB2 receptor activation induced production of MMP-1 and MMP-13 in cSCC cells (II, Figure 5H and Supplementary Figure S3). These findings identify the role that EphB2 plays in invasion, and regulation of the invasion related MMPs, MMP-1 and MMP-13.

5.2.7 EphB2 knockdown inhibits growth of human cSCC xenografts in vivo

To characterize the role of EphB2 receptor in growth of cSCC tumors *in vivo*, cSCC xenograft was established by injecting skin SCC cells into the back of SCID mice subcutaneously. cSCC cells were transfected with control or EphB2 siRNA 72 hours before the injection. Examination of the tumor size at different time-points revealed a significant delay in the growth of the tumors established with EphB2 siRNA transfected cells compared with control tumors (II, Figure 6A). In addition, IHC staining of the extracted tumors revealed significantly lower numbers of proliferating tumor cells (Ki-

67-positive) in EphB2 siRNA transfected tumors compared with tumors established from cells transfected with control siRNA (II, Figure 6B and C). To study the role of EphB2 in tumor vascularization *in vivo,* cSCC xenografts were stained with anti-CD34 antibody, which served as a vascular endothelial cell marker. IHC analysis revealed a significantly reduced number of CD34-positive blood vessels in EphB2 knockdown tumors compared with control xenografts (II, Figure 6B and D). These findings indicate the role of EphB2 in growth and vascularization of the cSCC tumors *in vivo*.

5.3 AIM2 promotes progression of cSCC (III)

5.3.1 Upregulation of AIM2 in cSCC cell lines and tumors

Expression of AIM2 has been reported in certain malignancies (Chen et al, 2006). Analysis of the expression of IFN-inducible family genes using microarray-based gene expression profiling revealed upregulation of *AIM2*, *IFI16* and *IFX* in primary and metastatic cell lines compared with NHEKs (III, Figure 1A). Upregulation of *AIM2* and *IFI16* was also noted with RNA sequencing of the cSCC cell lines and NHEKs (III, Figure 1B). The results of both analyses showed low expression of *AIM2* in NHEKs and overexpression of *AIM2* in cSCC cell lines (III, Figure 1A and B). Significant upregulation of *AIM2* mRNA in cSCC cell lines compared to NHEKs was verified by qRT-PCR (III, Figure 1C). In addition, elevated mRNA expression of *AIM2* was noted in cSCC tumors when compared to normal skin samples (III, Figure 1D). Western blot analysis of the total cell lysates revealed more AIM2 production in cSCC cell lines compared to NHEKs (III, Figure 1E). In addition, immunofluorescence staining showed more AIM2 labeling in cSCC cells when compared with NHEKs (III, Figure 1F).

5.3.2 Tumor cell-specific overexpression of AIM2 in cSCC tumors in vivo

Expression of AIM2 *in vivo* was examined by IHC staining of TMAs consisting of normal skin (n=15), AK (n=71), cSCCIS (n=60) and sporadic cSCCs (n=81) (III, Figure 2A-H). Tumor cell-specific AIM2 staining was detected in the cytoplasm and perinuclear region of cSCC tumor cells. In cSCC tumors, staining intensity was strong (+++) (38%) or moderate (49%). AIM2 staining intensity was mainly moderate (++) in cSCCIS. Most AK samples (69%) were weakly positive (+) for AIM2. In normal skin, staining intensity was absent (-) (67%) or weak (+) (33%). Semiquantitative analysis of the AIM2 staining intensity revealed abundant AIM2 staining in sporadic cSCC compared with cSCCIS, AK and normal skin (III, Figure 2I). These results indicate that AIM2 expression is increased in progression of AK to cSCC *in vivo*.

5.3.3 Tumor cell-specific overexpression of AIM2 in cSCC of OTR patients

Immunosuppressed patients are at a higher risk of developing a more aggressive type of cSCC (Hameetman et al, 2013). Expression of AIM2 was examined in whole sections of AK (n=58), cSCCIS (n=59) and cSCC (n=57) of OTR patients (III, Figure 3A-H). Positive tumor cell-specific AIM2 staining was detected in the cytoplasm and the perinuclear region of all cSCCs tumors of OTR patients examined. Staining intensity was in general stronger in cSCC compared to AK and cSCCIS. Semiquantitative analysis of the AIM2 staining revealed strong (+++) AIM2 staining (51%) in the majority of cSCC tumors. In cSCCIS, most of the samples had moderate (49%) or weak (36%) staining and strong staining was noted in small number of samples (7%). In AK lesions, AIM2 staining was either absent (53%) or weak (38%). In general AIM2 staining intensity was significantly more abundant in cSCC of OTR patients compared to cSCCIS and AK (III, Figure 3I). Interestingly, AIM2 staining was significantly stronger in cSCC of OTR patients compared to sporadic cSCC (P=0.0038).

5.3.4 AIM2 knockdown inhibits proliferation and invasion of cSCC cell lines

To study the functional role of AIM2 in cSCC cell lines, AIM2 was knockeddown using specific siRNA (III, Figure 5A). AIM2 knockdown potently inhibited the viability of cSCC cell lines 24, 48 and 72 hours after the transfection (III, Figure 5B). In addition, AIM2 knockdown significantly inhibited invasion of the cSCC cell lines through collagen (III, Figure 5C). Interestingly, AIM2 knockdown was noted to inhibit production of two invasion related MMPs, MMP-1 and MMP-13 (III, Figure 5D). These findings indicate the role of AIM2 in the regulation of proliferation and invasion of cSCC cells in culture.

5.3.5 Alteration of gene expression profile in cSCC after AIM2 knockdown

To gain an overview of the molecular mechanism of AIM2, RNA-sequencing was performed after knockdown of cSCC cell lines (n=3) with specific AIM2 siRNA and the results of gene expression analysis were subjected to IPA. Analysis of the genes significantly regulated following AIM2 knockdown revealed a significant upregulation of biofunctions related to cell death and apoptosis after AIM2 knockdown, whereas biofunction *M phase* of the cell cycle category was significantly downregulated (III, Supplementary Table S1). Expression of *CDK1* as part of *M phase* biofunction was noted to be markedly decreased following AIM2 knockdown. Interestingly *CDK1*, *cyclin A* and *cyclin B* were significantly downregulated in network *Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair*, as one of the top networks regulated (score=24) after AIM2 knockdown (III, Supplementary Figure S1). In addition, *cyclin A* was among the top genes significantly downregulated after

AIM2 knockdown. Inhibition of viability of cSCC cells after AIM2 knockdown can be explained by downregulation of the *cyclin A* and *B* and *CDK1*.

5.3.6 AIM2 knockdown inhibits growth of human cSCC xenograft tumors in vivo

To characterize the role of AIM2 in growth of cSCC tumors *in vivo*, cSCC xenografts were established by injection of cSCC cell lines subcutaneously into the back of SCID mice. Seventy-two hours before injection, cSCC cells were transfected with control or AIM2 siRNA. Measuring the size of the xenograft tumors revealed a significant delay in growth of the tumors established from cSCC cells transfected with AIM2 siRNA compared to control tumors (III, Figure 6A and B). Staining of the tumors with a Ki-67 proliferation marker showed a significant reduction in number of proliferating cells following AIM2 knockdown (III, Figure 6B and C). Analysis of the blood vessel formation using CD34 as a vascular endothelial marker revealed a significantly reduced density of CD34-positive blood vessels in AIM2 knockdown tumors compared to the control siRNA xenografts (III, Figure 6B and D). The results of this *in vivo* experiment indicate the role of AIM2 in cSCC tumor growth and vascularization in a xenograft model.

6. DISCUSSION

6.1 Biomarkers for cSCC

Non-melanoma skin cancer, including BCC and SCC, is the most common malignancy in the Caucasian population (Diepgen & Mahler, 2002; Kwa et al, 1992). cSCC is known as the second most common skin cancer among Caucasians (Johnson et al, 1992). The incidence of cSCC is increasing due to lifestyle changes such as voluntary and recreational exposure to the sunlight and aging of the population (Alam & Ratner, 2001; Housman et al, 2003; Rogers et al, 2010). Exposure to UV radiation is the greatest risk factor for cSCC (Alam & Ratner, 2001). With a high tendency for metastasis and recurrence, cSCC is the most common metastatic skin cancer (Brantsch et al, 2008; Czarnecki et al, 1994) and this cancer causes a great economic and medical burden on the health system (Geller & Swetter, 2012; Kivisaari & Kähäri, 2013; Rogers et al, 2010; Tinghog et al, 2008). Thus, diagnosis of cSCC at the early stages is potentially lifesaving. In addition, screening is necessary to identify the recurrence of the tumors and the tumors that are more aggressive and require further treatment modalities (Utikal et al, 2007). The anatomic location of the tumor, size of the tumor, invasion depth, rapid growth of the tumor, history of radiotherapy, tumor differentiation, immunosuppression, chronic ulcer and histologic type of tumor are among the known prognostic risk factors for metastasis and recurrence of cSCC (Alam & Ratner, 2001; Kivisaari & Kähäri, 2013; Lohmann & Solomon, 2001; Petter & Haustein, 2000; Stratigos et al, 2014; Utikal et al, 2007).

So far only few biomarkers for the progression of cSCC have been identified. STAT3 is a regulator of cell movement. The phosphorylated form of STAT3 is more prominently expressed in poorly differentiated cSCC than in well-differentiated tumors. In addition, expression of p-STAT3 is associated with tumor invasion and metastasis (Suiqing et al, 2005). The other known biomarker for cSCC is the E-cadherin molecule. Decreased expression of E-cadherin may be a sign of regional lymph node involvement in patients with cSCC (Koseki et al, 1999). Furthermore, the expression of E-cadherin was decreased in well-differentiated cSCC tumors in an IHC analysis (Koseki et al, 1999). Ets-1 is a transcription factor involved in the regulation of different genes linked with angiogenesis and matrix remodeling such as MMPs (Behrens et al, 2001; Liotta & Stetler-Stevenson, 1990; Naito et al, 2002; Westermarck et al, 1997). Ets-1, in turn, is upregulated in poorly differentiated and metastatic cSCCs compared with well-differentiated tumors and has been suggested as a marker for invasive tumors (Keehn et al, 2004). In SCC of the vulva, MMP-7, -9 and -12 are upregulated in less-differentiated tumors (Kerkelä et al, 2002). Expression of MMP-12 in

macrophages has been identified as a prognostic marker for SCC of the vulva (Kerkelä et al, 2002). MMP-13 (collagenase-3) is expressed in SCC of the head and neck (Johansson et al, 1997). In SCC of the skin, MMP-13 has been detected in the epithelial tumor front (Airola et al, 1997) and is associated with the invasive capacity and growth of the tumor (Ala-aho et al, 2004). Epithelial expression of MMP-7, -12 and -13 have been identified as markers for distinguishing benign chronic wounds from cSCCs arising from chronic ulcers (Impola et al, 2005). MMP-19 is expressed in hyperproliferative keratinocytes but disappears in invasive cSCC (Impola et al, 2005). In addition, abundant expression of MMP-12 has been detected in cSCC and the expression has been shown to correlate with tumor aggressiveness (Kerkelä et al, 2000). In an IHC analysis of RDEB-associated cSCC as an aggressive form of SCC (South & O'Toole, 2010), MMP-7 is specifically expressed in RDEB-associated cSCCs with significantly stronger staining intensity compared to sporadic cSCC (Kivisaari et al, 2008). Furthermore, abundant expression of MMP-13 has been noted in RDEB-associated cSCC (Kivisaari et al, 2008).

This current work has mainly focused on the identification and characterization of novel biomarkers for growth and progression of cSCC. State-of-the-art methods were used to identify novel genes that may play an important role in initiation and progression of cSCC. As the first step, microarray-based gene expression profiling and RNA sequencing were performed to identify an array of new genes differentially expressed in cSCC cells as compared to NHEKs. The mRNA expression level of selected genes was validated by qRT-PCR and the protein levels were analyzed by western blot analysis. A large panel that consisted of normal skin, AK, cSCCIS, sporadic cSCC and RDEB-associated cSCC, chemically induced mouse cSCC, as well as AK, cSCCIS and cSCC of OTR patients was created to study the expression of the genes of interest *in vivo*. The role of the selected genes in the progression of cSCC was further elucidated by analysis of the migration, invasion and proliferation of the cSCC cells. Furthermore, a xenograft model of cSCC was established to examine the growth of cSCC tumors *in vivo*.

6.2 SerpinA1 as a biomarker for progression of cSCC

SERPINA1 that codes for AAT belongs to the serpin family, which is the largest and most abundant member of protease inhibitors in humans. Serpins are secreted in plasma and play an important role in regulation of biological activities such as coagulation (anti-thrombin III), complement system (C1-inhibitor), inflammation (α1-antichymotrypsin) and fibrinolytic system (plasminogen activator inhibitor-1) (Kummer et al, 2004; Law et al, 2006; Silverman et al, 2001). The most important function of SerpinA1 is to inhibit neutrophil elastase through an irreversible inhibition by covalent binding (Carrell & Lomas, 2002; Silverman et al, 2001). Besides neutrophil elastase, SerpinA1 can inhibit other proteases such as chymotrypsin and trypsin (Beatty et al, 1980; Kuiperij et al, 2009). High expression of SerpinA1 has recently

been identified in various malignancies. In bladder cancer, the expression of SerpinA1 increases the risk of tumor progression and is associated with late stage bladder cancer (Linden et al, 2013; Zhang et al, 2014). In addition, the urine level of AAT has been identified as a specific and sensitive biomarker for invasive bladder cancer (Linden et al, 2012). In gastric cancer, SerpinA1 expression has emerged as a biomarker for poor prognosis (Kwon et al, 2014). Microarray-based meta-analysis of papillary thyroid carcinomas revealed SerpinA1 as a reliable diagnostic biomarker with a high sensitivity and specificity (Vierlinger et al, 2011). Moreover, SerpinA1 has been previously identified as a biomarker for the diagnosis of insulinoma (de Sa et al, 2007) and thyroid cancer (Griffith et al, 2006).

As mentioned previously, so far there is no reliable biomarker available for the early diagnosis and prevention of cSCC tumor metastasis and morbidity. Therefore, there is a great need for identification of novel diagnostic tools for the progression of cSCC. In study I, in an effort to identify novel biomarker for the progression of cSCC, a gene expression profile of the entire serpin family in primary and metastatic cSCC and NHEKs was conducted. The expression of *SERPINA1* was significantly higher in primary and metastatic cSCC cell lines compared to NHEKs. qRT-PCR was used to validate the overexpression of *SERPINA1* in cSCC cell lines compared to NHEKs. The other member of the serpin superfamily, SerpinA3, has been previously described as a biomarker in some malignancies such as colorectal adenocarcinoma (Dimberg et al, 2011). Although *SERPINA3* expression was upregulated in cSCC cells lines compared to NHEKs by microarray analysis, this notion was not observed with qRT-PCR. Therefore, it was considered to be of value to further examine SerpinA1 production in cSCC cells. Analysis of the SerpinA1 protein level markedly revealed more SerpinA1 production in all cSCC cell lines compared to NHEKs. The expression level of SerpinA1 *in vivo* was examined by IHC analysis of the TMAs generated from paraffin embedded tissue blocks (Idikio, 2011; Kononen et al, 1998; Torhorst et al, 2001). Conventional IHC analysis of the tissue sections is time-consuming and there exists a need for a large section of the tissue and a larger amount of the antibody. In addition, TMA blocks have enabled us to examine a large number of tumors. Thereby, in order to save time and resources, human TMAs were generated from wide variety of normal skin, AK, cSCCIS, sporadic cSCC and RDEB-associated cSCC tumors. The results of the IHC analysis of the TMAs revealed tumor cell-specific SerpinA1 expression in sporadic cSCCs. In UV-independent RDEB-associated cSCC (Fine et al, 2009), as the one of the most aggressive form of cSCC (South & O'Toole, 2010), strong SerpinA1 staining in the tumor area was noted. In AK and cSCCIS, SerpinA1 staining was absent or weak in the majority of the samples. Accordingly, it was hypothesized that SerpinA1 staining intensity is correlated with the malignant transformation of cSCC *in vivo*.

To further elucidate the role of SerpinA1 as a biomarker for progression of cSCC in culture, *SERPINA1* expression was analyzed in HaCaT immortalized nontumorigenic

keratinocyte cell lines and Ha-*ras*-transformed HaCaT cell lines represent different stages of cSCC tumor progression (Mueller et al, 2001). While *SERPINA1* expression was very low in HaCaT cell lines, the highest mRNA expression level of *SERPINA1* was noticed in RT3 cell lines, which form metastatic tumors *in vivo*. A low expression level of *SERPINA1* in HaCaT cell lines with UV-typic mutations in both p53 alleles (Boukamp et al, 1995) and high expression level of *SERPINA1* in Ha-*ras*-transformed HaCaT cell lines implies the fact that besides p53 inactivation, *ras* transformation is essential for expression of *SERPINA1*. This notion was further supported with analysis of SerpinA1 expression in chemically induced mouse cSCC (Abel et al, 2009; Ward et al, 1986). As the primary target for the early stages of the tumor progression, activating *Hras* mutations in the epidermis can be noted in epidermal layer three to four weeks after treatment with DMBA (Nelson et al, 1992). Abundant SerpinA1 staining in the tumor area of chemically induced mouse cSCC and a lack of staining in normal skin and vehicle-treated skin further emphasized the correlation of SerpinA1 expression with malignant transformation of epidermal keratinocytes and its putative role as a biomarker for the progression of cSCC.

The possible role for SerpinA1 in cSCC progression remains to be studied, but inhibition of natural killer cells (Laine et al, 1990), inhibition of caspase-3 activation and consequently an antiapoptotic effect in lung endothelial cells (Petrache et al, 2006) and induction of cancer cell proliferation by C-terminal 26-residue peptide of SerpinA1 (Congote & Temmel, 2004; Zelvyte et al, 2004) are among the proposed mechanisms.

6.3 Upregulation of EphB2 in cSCC cells in culture and *in vivo*

Eph receptors and ephrins play a critical role in many normal biological processes and in the pathogenesis of different diseases (Pasquale, 2010). The role of Eph receptors and ephrin ligands in cancer is complex (Pasquale, 2010). Eph and ephrin are upregulated in some malignancies and their expression is associated with cancer progression and poor prognosis (Brantley-Sieders et al, 2008; Easty & Bennett, 2000; Landen et al, 2005; Li et al, 2014; Wykosky & Debinski, 2008; Zhuang et al, 2010). On the other hand, Eph receptors and ephrins have a tumor suppressor role in certain cancer cell lines and tumors (Batlle et al, 2005; Merlos-Suarez & Batlle, 2008; Noren et al, 2006; Noren & Pasquale, 2007). Furthermore, certain Eph receptors and ephrin ligands such as EphB2 play a dual role both as tumorigenic or tumor suppressor depending on the tumor type (Guo et al, 2006a; Huusko et al, 2004). In chemically induced mouse cSCC, the EphA2 receptor has been recognized to have a tumor suppressor role as loss of EphA2 expression has been shown to increase the proliferation of the tumor cells (Guo et al, 2006b). However, the role of the EphB2 receptor in progression of cSCC has not been analyzed previously.

In study II the expression profile of the entire *EPH* and *EFN* family was examined. *EPHB2* was identified as the only receptor significantly upregulated in primary and metastatic cSCC cell lines compared to NHEKs in both microarray-based gene expression profiling and RNA sequencing. In addition, upregulation of *EPHB2* was observed in cSCC tumors compared to normal skin. Western blot analysis of the cell surface proteins showed markedly more EphB2 receptor on the cell surface of the cSCC cells compared to NHEKs. As mentioned previously, Eph receptors and ligands cluster upon activation of the signal between neighbor cells (Himanen et al, 2004). In addition, independent of the ligand, Eph receptors can form cluster and activate on the cell surface (Nikolov et al, 2013). In Study II, co-localization of the EphB2 receptor and ephrin-B2 ligand in cell-cell contact sites and clustering of the EphB2 receptor was observed in cSCC cells.

6.4 Overexpression of EphB2 by tumor cells *in vivo*

IHC analysis of the human TMAs was performed to examine the expression of EphB2 *in vivo*. Tumor cell-associated staining for EphB2 was noted to be significantly stronger in cSCCIS and sporadic cSCC than in AK and normal skin. As another supporting piece of evidence for the *in vivo* expression of EphB2, analysis of the EphB2 in chemically induced mouse cSCC revealed abundant tumor cell-associated staining in DMBA-TPA induced mouse skin cSCC, whereas the EphB2 staining was weak or absent in control sections. The EphB2 labeling was detected on the cell surface but predominantly in the cytoplasm of tumor cells. Cumulative evidence favors the idea that the full-length Eph/ephrin complex protein is internalized upon activation via an endocytosis process (Mann et al, 2003; Marston et al, 2003; Pasquale, 2005; Zimmer et al, 2003). This could explain the cytoplasmic localization of the EphB2 receptor observed in different tumors such as breast (Wu et al, 2004) and ovarian carcinoma (Wu et al, 2006) *in vivo*.

6.5 EphB2 regulates proliferation, migration and invasion of cSCC cell lines

In study II, EphB2 knockdown was noted to inhibit viability of cSCC cell lines. Inhibition of viability of cSCC cell lines by EphB2 knockdown could be through inhibition of ERK1/2 MAPK known as the downstream signaling of EphB2 (Figure 8) (Pasquale, 2010). EphB2 knockdown was shown to significantly inhibit the directional migration of the cSCC cell lines recorded by time-lapse microscopy. It is known that Eph signaling can mediate cell contact-dependent repulsion that can guide directional migration of the cells (Lin et al, 2008; Poliakov et al, 2004). Accordingly, it can be hypothesized that the inhibition of the cell repulsion due to knockdown of EphB2 is the possible mechanism for the inhibition of the directional migration in cSCC cells. Besides, migration of the tumor cells came up as one of the top biofunctions in pathway analysis performed following EphB2 knockdown.

EphB2 knockdown potently inhibited invasion of cSCC cell lines through collagen. In addition, pathway analysis revealed the association of significantly downregulated genes with the top biofunction *invasion of the tumor cells*. Interestingly, analysis of the expression profile in cSCC cells after EphB2 knockdown revealed *MMP1* and *MMP13* among the most downregulated genes. MMP-13 promotes invasion of cSCC cells and growth of cSCC *in vivo* (Ala-aho et al, 2004) and its expression in head and neck SCCs correlates with local invasion (Stokes et al, 2010). It is known that the expression of MMP-1 (Westermarck et al, 1998) and MMP-13 (Ravanti et al, 1999) is regulated by p38 MAPK. Furthermore, the activation of the EphB/ephrinB signaling induces activation of p38 MAPK signaling (Cao et al, 2008). Accordingly, it could be proposed that EphB2 regulates expression of MMP-1 and MMP-13 and consequently invasion of the cSCC cells through p38 MAPK activation (Figure 8). This notion was further supported by activation of EphB2 receptor with ephrin-B2-Fc ligand (Chaudhari et al, 2007; Salvucci

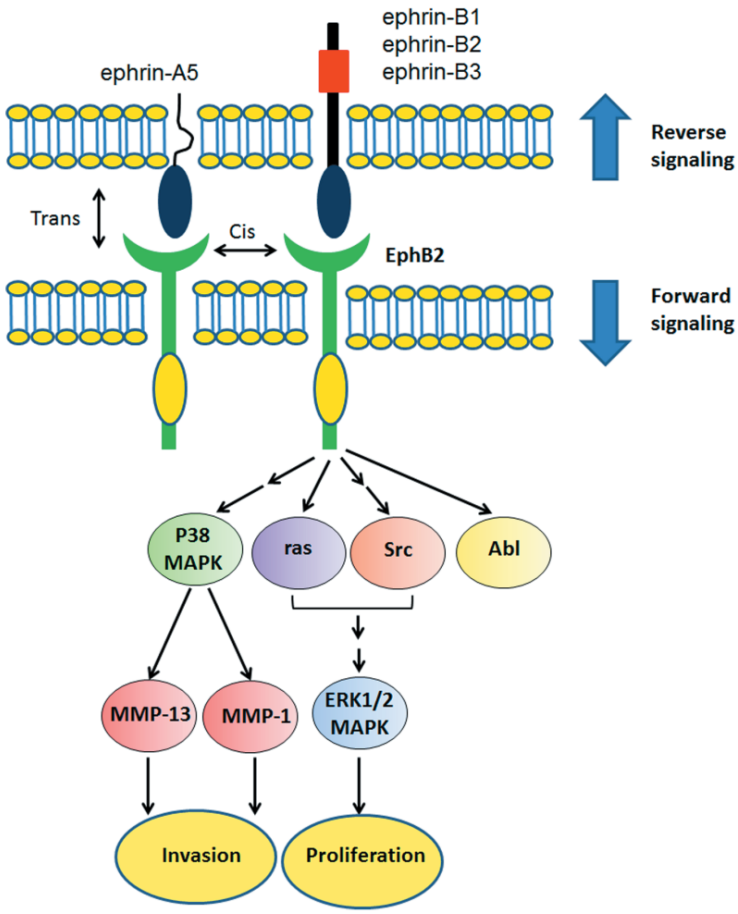


Figure 8. Proposed signaling pathways for the regulation of invasion and proliferation by Eph/ephin signaling in cSCC cells.

et al, 2006), which induced production of MMP-1 and MMP-13 and invasion of the cSCC cells through collagen in study II.

6.6 EphB2 regulates the growth of cSCC tumor in a human cSCC xenograft model

In study II, invasive cSCC cell lines were implanted subcutaneously into the back of SCID mice. SCID mice were first established more than three decades ago with the induction of the homozygous mutation that severely impairs lymphopoiesis in mice (Bosma et al, 1983). Although the immune system, mediated by T cell and B cell lymphocytes, is impaired in SCID mice (Bosma et al, 1983), the complement system activity and natural killer cells are still intact in these animal models (Greiner et al, 1995; Shultz et al, 1995) This model has been widely used to study the growth of human tumor cells in an *in vivo* environment. Knockdown of EphB2 potently delayed the growth of cSCC xenografts. In addition, the number of proliferating cells was significantly reduced in the tumors established from EphB2 knockdown cSCC cell lines compared to control xenografts. Given the fact that blood vessels formation is an essential part of tumor growth, the tumor vasculature of the xenografts was examined with a CD34 vascular endothelial marker (Hirahashi et al, 2009; Schem et al, 2013). EphB2 knockdown potently inhibited vascularization of cSCC tumors which demonstrates the role that EphB2 plays in cSCC tumor angiogenesis.

6.7 Upregulation of AIM2 in cSCC cells and tumors

The association of chronic inflammation and innate immunity and malignancies has been well documented (Aggarwal et al, 2006; Coussens & Werb, 2002). Inflammation is a risk factor for the development of cSCC and patient with chronic inflammatory skin diseases such as lichen sclerosus have higher risk of developing cSCC (Alam & Ratner, 2001; Ratushny et al, 2012). Recently, there has been an increasing interest on studying the role that AIM2 plays as part of innate immunity in different cancers. However, the exact role of AIM2 as a tumor suppressor or oncogene remains unclear. AIM2 has been reported to have a protective role against colorectal cancer progression and a lack of AIM2 expression is associated with the relapse of cancer, progression of the cancer, shorter survival and metastasis (Dihlmann et al, 2014). AIM2 overexpression has been shown to suppress proliferation of breast cancer cells *in vitro* and growth of the tumors in a mouse model (Chen et al, 2006). An elevated expression level of AIM2 in senescent epithelial prostate cells is associated with increased production of IL-1β resulting in development of benign prostatic hyperplasia (Ponomareva et al, 2013). However, the mRNA expression level of AIM2 has been shown to be significantly lower in prostate

cancer cells (Ponomareva et al, 2013). On the other hand, overexpression of AIM2 promotes growth of oral SCC cells in the absence of functional p53 (Kondo et al, 2012) and AIM2 is strongly expressed in primary cSCC and melanoma tumors and weakly present in poorly differentiated and metastatic tumors (de Koning et al, 2014). In study III, overexpression of *AIM2* in cSCC cells was observed when compared to NHEKs. In addition, the mRNA expression level of *AIM2* was shown to be significantly upregulated in cSCC tumors as compared to normal skin.

6.8 Overexpression of AIM2 by tumor cells in sporadic cSCC and cSCC of OTR patients *in vivo*

In study III, examination of AIM2 expression in TMAs of normal skin, AK, cSCCIS and sporadic SCC revealed tumor cell-specific AIM2 expression in sporadic cSCC tumors and AIM2 staining intensity was more abundant in sporadic cSCC compared to cSCCIS, AK and normal skin. In addition, the analysis of AIM2 *in vivo* was extended to the large panel of AK, cSCCIS and cSCC tissue sections obtained from OTR patients. The risk of developing AK (250 times) and cSCC (100 times) is markedly higher in immunosuppressed patients compared to immunocompetent patients (Stockfleth et al, 2011). In addition, cSCCs developed in immunosuppressed patients have more aggressive behavior and a higher metastatic rate (Euvrard et al, 2003; Hameetman et al, 2013). Apart from more abundant AIM2 expression in cSCC of OTR patients compared to cSCCIS and AK lesions, AIM2 staining was significantly stronger in cSCC of OTR patients compared to sporadic cSCCs. These findings suggest the association of AIM2 expression with cSCC tumor progression *in vivo*, particularly in cSCC of OTR patients.

6.9 AIM2 knockdown inhibits proliferation and invasion of cSCC cells

Although the expression of AIM2 in certain malignancies has been recently studied, little is known about the role that AIM2 plays in tumor progression. In study III, AIM2 knockdown with specific siRNA inhibited the viability of cSCC cells. This finding is in accordance with the inhibition of the growth of oral SCC cells following downregulation of AIM2 (Kondo et al, 2012). In addition, the inhibitory effect of AIM2 knockdown on the viability of cSCC cells was supported by pathway analysis of the RNA sequencing data following AIM2 knockdown. Analysis of the gene expression profile following AIM2 knockdown revealed significant downregulation of biofunction *M phase* of the cell cycle category and upregulation of the genes associated with biofunctions related to cell death and apoptosis. In addition, cell cycle-related genes *CDK1*, *cyclin A* and *cyclin B* were significantly downregulated after AIM2 knockdown (Figure 9).

Tumor cells recruit certain innate immune system chemokines and cytokines for the invasion and metastasis (Coussens & Werb, 2002). In colorectal cancer, restoration of AIM2 has been shown to stimulate invasion of the colorectal cancer cells through a matrix-coated membrane (Patsos et al, 2010). In the present study it was shown that AIM2 knockdown inhibited invasion of the cSCC cells through collagen. In addition, these results provide evidence for the first time that AIM2 is involved in the regulation of MMP-1 and -13, two metalloproteinases associated with cancer invasion (Figure 9) (Ala-aho & Kähäri, 2005). IL-1β has been shown to regulate expression of MMP-13 and -1 via the ERK and NFκB pathways (Fan et al, 2006), which could be the possible mechanism for the regulation of the expression of MMP-1 and -13 by AIM2.

6.10 AIM2 knockdown suppresses the growth and vascularization of human cSCC xenografts *in vivo*

In study III, in an attempt to analyze the role of AIM2 in growth of cSCC tumors *in vivo*, cSCC xenografts were established. The previous findings on the role that AIM2 plays in the progression of cSCC in study III was further strengthened by observation of a potent delay in the growth of cSCC tumors established from AIM2 knockdown cSCC cells compared to the tumors established from the cells transfected with control siRNA. Accordingly, the number of proliferating tumor cells was significantly lower in AIM2

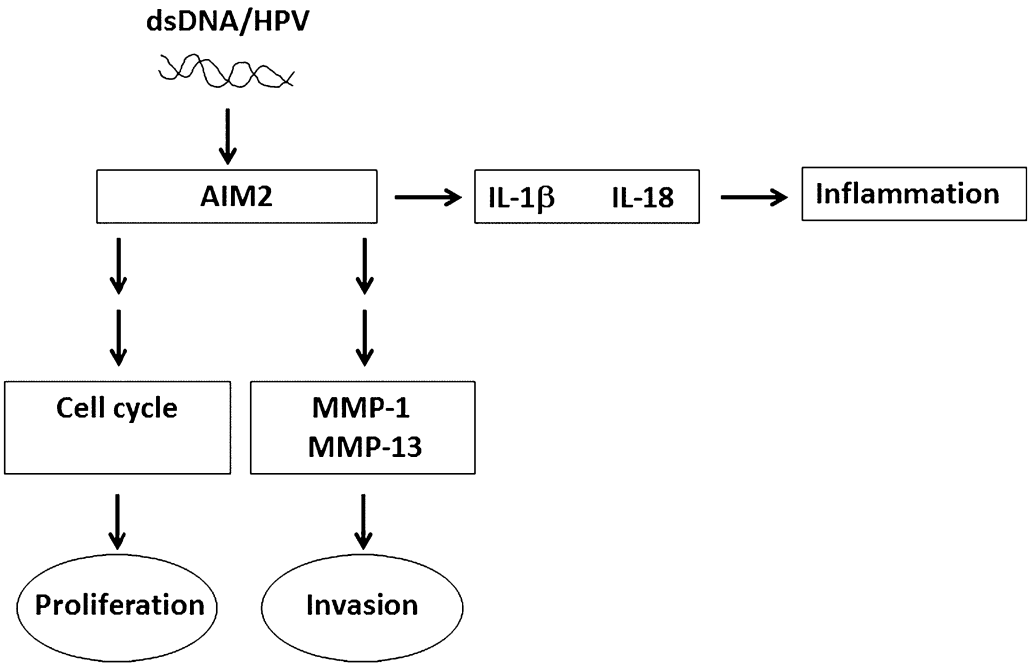


Figure 9. Proposed model for AIM2 activation and regulation of inflammation, proliferation and invasion in cSCC cells.

knockdown tumors than in control tumors. In addition, we found for the first time that AIM2 knockdown inhibits tumor angiogenesis. These results identified the important role of AIM2 in vascularization and growth of cSCC xenografts *in vivo*. On the basis of the knowledge available on the role of chronic inflammation and in particular IL-1, a product of AIM2 inflammasome activation in tumor progression (Drexler et al, 2012), we can hypothesize that AIM2 drives the progression of cSCC by activation of interleukins and cytokines. These findings provide evidence for the role of AIM2 as part of innate immune system in the growth and progression of cSCC.

7. SUMMARY AND CONCLUSIONS

The current work was planned to identify novel biomarkers for the growth and progression of cSCC. As the most common metastatic form of the cutaneous malignancy, cSCC has a risk of metastasis and invasion if left untreated. In addition, early detection of the tumor is invaluable in the patients with high risk of cSCC such as immunosuppressed patients. Furthermore, these biomarkers could serve as therapeutic targets particularly in the patients with metastatic and unresectable lesions. This thesis project was mainly established on the extensive gene expression profiling of the primary and metastatic cSCC cells and NHEKs.

We found that *SERPINA1*, which codes for AAT, is upregulated in cSCC cells compared to NHEKs. IHC analysis of the TMAs, consisting of a large panel of normal skin, AK, cSCCIS, sporadic cSCC and RDEB-associated cSCC, revealed a strong tumor cell-associated labeling of SerpinA1 in sporadic cSCC and RDEB-associated SCC. In addition, the expression of SerpinA1 is correlated with the malignant transformation of epidermal keratinocytes in cell culture and the progression of cSCC *in vivo*.

Given the fact that the role that Eph receptors and ephrin ligands play in different malignancies is complex, part of this thesis work was focused on investigating the role of Eph/ephrin in cSCC. *EPHB2* gained our attention in this study, because it revealed a significant upregulation in cSCC cells compared to NHEKs both in microarray-based gene expression profiling and next generation sequencing. Further analysis by qRT-PCR verified markedly overexpression of *EPHB2* in cSCC cells and tumors. EphB2 staining was significantly stronger in cSCC and cSCCIS compared to AK and normal skin. Furthermore, EphB2 knockdown inhibited growth of the cSCC in a xenograft model. EphB2 knockdown was shown to inhibit proliferation, migration and invasion of cSCC cell lines. Inhibition of invasion related MMPs, MMP-1 and MMP-13 as a result of EphB2 knockdown could be the possible mechanism for the inhibition of the invasion of cSCC cells.

Because inflammation is involved in the development of cSCC, as the third part of this thesis project, the role of AIM2 in the progression of cSCC was analyzed. Overexpression of AIM2 was observed in cSCC cell lines and tumors when compared with NHEKs and normal skin, respectively. Tumor cell-specific AIM2 labeling was detected in sporadic cSCC and cSCC of the OTR patients. In addition, AIM2 knockdown was shown to inhibit proliferation and invasion of cSCC cell lines and delay the growth and vascularization of cSCC tumors in a xenograft model, which indicates the role that innate immunity and inflammation plays in the progression of cSCC.

In conclusion, the current work revealed novel biomarkers for the progression of cSCC. SerpinA1 level can be used as a simple way to diagnose cSCC in early stages. The findings on the role of EphB2 and AIM2 in the progression of cSCC identified them as attractive therapeutic targets for cSCC. This may open new horizons for the treatment of cSCC, especially metastatic and unresectable tumors. AIM2, in particular, could be a novel therapeutic target for cSCC in immunosuppressed patients.

8. ACKNOWLEDGEMENTS

This work was carried out at the MediCity research laboratory and Department of Dermatology, University of Turku and Turku University Hospital. I am grateful for everyone who has been there to provide an inspiring environment and support my journey towards completing this PhD thesis. Professor Sirpa Jalkanen, the director of MediCity Research laboratory, is thanked for providing all the facilities for the research.

Foremost, I would like to express my deepest thanks to my supervisor Professor Veli-Matti Kähäri for his advice, tenacity and constant support throughout this thesis project. His immense knowledge, encouragement and patience were key motivators during my PhD work. I am very grateful and fortunate to have you as my mentor both in science and life.

This thesis would not have been possible without the financial and practical support of The National Graduate School of Clinical Investigation (CLIGS) and University of Turku Doctoral Programme of Clinical Investigation (CLIDP) for that I am very grateful.

I warmly thank my thesis committee advisors Docent Sirkku Peltonen and Docent Liisa Nissinen for their valuable advice and discussion on my thesis.

Professor Jorma Keski-Oja and Professor Tuula Salo are acknowledged for reviewing the thesis manuscript and for their thorough and excellent feedback.

I am grateful to all co-authors Liisa Nissinen, Atte Kivisaari, Elina Siljamäki, Pilvi Riihilä, Mervi Toriseva, Markku Kallajoki, Risto Ala-aho, Esko Veräjänkorva, Hanne-Kaisa Honkanen, Ritva Heljasvaara, Taina Pihlajaniemi, Reidar Grénman, Juha Peltonen, Sirkku Peltonen, Koen D. Quint and Jan Nico Bouwes Bavinck for their contribution to this thesis.

To the current and former members of the Skin cancer and proteinases (SkiCap) group: Liisa Nissinen, Atte Kivisaari, Pilvi Riihilä, Elina Siljamäki, Mervi Toriseva, Minna Piipponen, Risto Ala-aho, Niina Hieta, Janne Kallio, Sari Pitkänen, Johanna Markola, Esko Veräjänkorva, Kiira Houtsonen and Lea Toikka, thanks for making the lab a wonderful place to be and for all cheerful moments we spent together.

I want to thank Docent Leena Koulu and Docent Sirkku Peltonen and all the other doctors from the Departement of Dermatology for their encourangment and support.

I would also like to thank members of Professor Heino and Professor Elenius group, Kari Kurppa, Maria Salmela, Maria Tuominen, Johanna Jokinen and Anna Knittle for

all their technical support and joyful moments and discussions we had together. Sinikka Kollanus is acknowledged for the technical assistance.

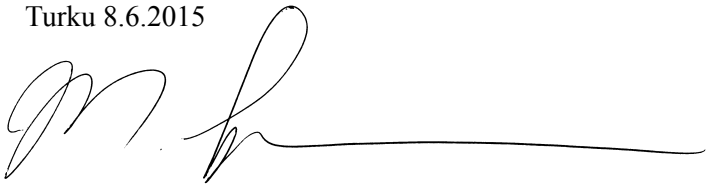
I would like to thank Dr. Robert M. Badeau and Aura Professional English Consulting for the language checking of the thesis.

I am indebted to my uncle Professor Mahmood Farshchian. Thank you for encouraging me, supporting me and giving me the passion for Dermatological research. To my family, particularly my parents and my only brother Dr. Ali Farshchian, thank you for your support and unwavering belief in me. Without you, I would not be the person I am today.

My warmest thanks to my friends Reza, Saeed, Mehdi, Amir and Arash and especially Shohreh for the continued friendship and support during these years. I am also grateful for my old friends from the medical school Mehdi, Babak, Maryam and Sima for the encouragements and long friendship.

The Academy of Finland, Sigrid Jusélius Foundation, Cancer Research Foundation of Finland, Turku University Foundation, the Finnish Cultural Foundation, the Cancer Society of Finland, the Cancer Society of Southwestern Finland, the EVO-Government Grant of Turku University Hospital and University of Turku, Orion-Pharmos Research Foundation, the Maud Kuistila Foundation, the K. Albin Johansson Foundation, and the Instrumentarium Foundation are greatly acknowledged for the financial support of this work.

Turku 8.6.2015



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