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COMPLEMENT SYSTEM IN CUTANEOUS SQUAMOUS CELL CARCINOMA

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To Petri, Lumia and Elia

ABSTRACT

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Complement system in cutaneous squamous cell carcinoma

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Cutaneous squamous cell carcinoma (cSCC) consists 20% of keratinocyte-derived non-melanoma skin cancers (NMSC), the incidence of which is increasing globally. cSCC is the most common metastatic skin cancer and it causes approximately 20% of skin cancer-related deaths. At present, there are no molecular markers for predicting which cSCC lesions are aggressive or metastasize rapidly. UV radiation is the most important risk factor for cSCC. During the development of cSCC, normal epidermal keratinocytes are transformed and form actinic keratosis (AK), which progresses to cSCC *in situ* (cSCCIS, Bowen's disease) and finally to invasive and metastatic cSCC. Inflammatory factors and cells are a part of cancer microenvironment and cSCC can develop in the chronically irritated skin or in the context of chronic inflammation. The complement system is a central part of innate immunity and it regulates normal immunological and inflammatory processes.

In this study, the role of complement system components and inhibitors were studied in the progression of cSCC in culture and *in vivo*. Elevated expression of complement factor H (CFH), complement factor I (CFI), complement component C3 and complement factor B (CFB) was noted in cSCC cells in culture. The analysis with immunohistochemistry (IHC) revealed that the expression of CFH, CFI, C3 and CFB was specifically noted in tumor cells *in vivo*. The staining intensity of CFH, CFI, C3 and CFB was also stronger in invasive cSCC than in AK or cSCCIS samples. The knockdown of CFH, CFI and CFB with specific siRNAs decreased cSCC cell viability and migration, whereas the knockdown of C3 reduced only cSCC cell migration. Moreover, the knockdown of CFI, C3 and CFB inhibited growth of cSCC xenograft tumors established in SCID mice *in vivo*. In these tumors, CFI, C3 and CFB knockdown decreased the number of proliferating cells. Moreover, the knockdown of CFI increased local inflammation and complement activation.

This study provides evidence for the roles of CFH, CFI, C3 and CFB in the tumor progression indicating these as molecular biomarkers and putative therapeutic targets of cSCC.

Keywords: cutaneous squamous cell carcinoma, complement factor H, complement factor I, complement component C3, complement factor B

TIIVISTELMÄ

Pilvi Riihilä

Komplementtijärjestelmä ihon okasolusyövässä

Iho- ja sukupuolitautioppi, Kliininen laitos, Lääketieteellinen tiedekunta, Turun yliopisto, MediCity Tutkimuslaboratorio, Turun yliopisto, Turun Yliopiston Tutkijakoulu, Kliininen Tohtoriohjelma, Turun yliopisto ja Valtakunnallinen kliininen tutkijakoulu.

Keratinosyyttiperäiset ei-melanoottiset syövät lisääntyvät maailmanlaajuisesti ja näistä syövistä 20% on ihon okasolusyöpää. Ihon okasolusyöpä on yleisimmin metastasoiva ihosyöpä ja se aiheuttaa 20% ihosyöpäkuolemista. Tällä hetkellä ihon okasolusyövän aggressiivisuutta tai nopeaa metastasointia ennustavia merkkitekijöitä ei tunneta. UV-säteily on tärkein riskitekijä ihon okasolusyövän kehittymiselle. Normaalit ihon keratinosyytit voivat muuttua ja muodostaa aktiinisia keratooseja, mitkä etenevät *in situ* muotoiseksi okasolusyöväksi (Bowenin tauti) ja lopulta ihon okasolusyöväksi. Tulehdussolut ja -tekijät kuuluvat syöpien mikroympäristöön. Myös ihon okasolusyöpä voi kehittyä kroonisesti ärsyyntyneelle tai tulehtuneelle ihoalueelle. Komplementtijärjestelmä on tärkeä osa ihmisen synnynnäistä immuniteettia ja se säätelee tulehduksellisia tapahtumasarjoja.

Tässä työssä tutkittiin komplementtijärjestelmän komponenttien ja inhibiittoreiden osuutta ihon okasolusyövän kehittymisessä soluviljelmässä ja *in vivo*. Komplementin tekijä H:ta (CFH), komplementin tekijä I:tä (CFI), komplementin komponenttia C3:a ja komplementin tekjä B:tä (CFB) havaittiin kohonneina pitoisuuksina soluviljelmässä ihon okasolusyöpäsoluissa. Immunohistokemiallisessa analyysissä paljastui, että erityisesti kasvainsolut ilmensivät CFH:ta, CFI:tä, C3:a ja CFB:tä *in vivo* ja värjäysintensiteetti oli vahvempi okasolusyöpäsoluissa kuin aktiinisessa keratoosissa tai Bowenin taudissa. Kun spesifistä siRNA:ta käytettiin hiljentämään CFH:n, CFI:n ja CFB:n ilmaantuvuus, solujen elinkykyisyys ja liikkuminen väheni. C3 geenin hiljentäminen vähensi solujen liikkumista. Lisäksi CFI-, C3- ja CFB-geenin hiljentäminen vähensi okasolusyövän kasvua *in vivo* SCID-hiirissä kasvatetuissa ksenograftikasvaimissa. CFI-, C3- ja CFB-geenin hiljentäminen vähensi solujen jakautumista. CFI-geenin hiljentäminen lisäsi lisäksi paikallista tulehdusta ja komplementin aktivaatiota näissä kasvaimissa.

Tässä tutkimuksessa osoitetaan CFH:n, CFI:n, C3:n ja CFB:n rooli okasolusyöpäkasvaimen kehittymisessä. CFH, CFI, C3 tai CFB voisivat myös toimia merkkitekijöinä ja hoitokohteina ihon okasolusyövälle.

Avainsanat: ihon okasolusyöpä, komplementin tekijä H, komplementin tekijä I, komplementin komponentti C3, komplementin tekijä B

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ABBREVIATIONS

AK	Actinic keratosis
AKT	Protein kinase B (PKB)
BCC	Basal cell carcinoma
CCP	Complement control protein domain (SCR)
CD35	Complement receptor type 1 (CR1)
CD46	Membrane cofactor protein (MCP)
CD55	Decay-accelerating factor (DAF)
CDCC	Complement-dependent cellular cytotoxity
CFB	Complement factor B (Factor B)
CFD	Complement factor D (Factor D)
CFH	Complement factor H (Factor H)
CFI	Complement factor I (Factor I)
CR1	Complement receptor type 1 (CD35)
cSCC	Cutaneous squamous cell carcinoma
cSCCIS	Cutaneous squamous cell carcinoma *in situ*
DAF	Decay-accelerating factor (CD55)
EB	Epidermolysis bullosa
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
FHL-1	Factor H-like protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gs-RDEB	Generalised severe recessive dystrophic epidermolysis bullosa
HaCaT	Non-tumorigenic immortalized human keratinocyte cell line
HE	Hematoxylin and eosin
iC3b	Enzymatically inactivated C3b
IHC	Immunohistochemistry
mAb	Monoclonal antibody
MAC	Membrane attack complex of complement
MASP	Mannose binding lectin associated serine protease
MBL	Mannose binding lectin
MCP	Membrane cofactor protein (CD46)
MEK	Mitogen-activated protein kinase kinase

MMP	Matrix metalloproteinase
NHEK	Normal human epidermal keratinocytes
NMSC	Non-melanoma skin cancer
p53	Tumor protein 53
PI3K	Phosphoinositide 3-kinase
PDT	Photodynamic therapy
qRT-PCR	Quantitative real-time polymerase chain reaction
RDEB	Recessive dystrophic epidermolysis bullosa
SCID	Severe combined immunodeficiency
SCR	Short consensus repeats (CCP)
SFK	Src-family tyrosine kinase
siRNA	Small interfering RNA
SK	Seborrheic keratosis
TLR	Toll-like receptor
TMA	Tissue microarray
TPA	12-*0*-tetradecanoylphorbol-13-acetate
UT-SCC	Human cutaneous squamous cell carcinoma cell line
UV	Ultraviolet

LIST OF ORIGINAL PUBLICATIONS

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

I. Riihilä PM, Nissinen LM, Ala-aho R, Kallajoki M, Grénman R, Meri S, Peltonen S, Peltonen J, Kähäri VM. Complement factor H: a biomarker for progression of cutaneous squamous cell carcinoma, J Invest Dermatol. 134:498-506, 2014

II. Riihilä P, Nissinen L, Farshchian M, Kivisaari A, Ala-aho R, Kallajoki M, Grénman R, Meri S, Peltonen S, Peltonen J, Kähäri VM. Complement Factor I promotes progression of cutaneous squamous cell carcinoma, J Invest Dermatol. 135:579-588, 2015

III. Riihilä P, Nissinen L, Farshchian M, Kallajoki M, Kivisaari A, Meri S, Grénman R, Peltonen S, Peltonen J, Pihlajaniemi T, Heljasvaara R, Kähäri VM. C3 and Complement Factor B regulate growth of cutaneous squamous cell carcinoma (manuscript)

1 INTRODUCTION

The incidence of skin cancers is rising worldwide while the human lifespan expectancy has increased. Recreational sun exposure followed by increased ultraviolet (UV) radiation exposure, is the most important risk factor for the cancers of the skin. The skin cancers are divided into melanocyte-derived melanoma and keratinocyte-derived non-melanotic skin cancers (NMSC). NMSCs are divided into cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC). In this study, the focus is on cSCC. The incidence of cSCC increases annually 3-8%. cSCC is the second most common skin cancer in the white population worldwide and it is the most common metastatic skin cancer. cSCC is the reason for the majority of the NMSC related deaths, and approximately 20% of skin cancer related deaths are caused by cSCC.

Progression of cSCC takes place from actinic keratosis (AK) *via* cSCC carcinoma *in situ* (cSCCIS, Bowen's disease) to invasive cSCC. In addition to UV radiation, the other two important risk factors for cSCC are chronic cutaneous ulcers and immunosuppression. Inflammatory cells are typically present in the microenvironment of cSCC and chronic inflammation is known to be associated with many types of cancer. In addition, cSCCs developed in the presence of chronic inflammation or in chronic ulcers tend to be more aggressive than UV-induced cSCCs. The complement system is an important part in the innate immune system and it has a role in the regulation of inflammation.

The treatment of choice for cSCC is excision but relapses and metastases can occur. Thus, it is important to recognize the high-risk types of cSCCs and identify molecular markers to predict cSCC aggressiveness and metastasis. Here, the role of complement system inhibitors, complement factor H (CFH) and complement factor I (CFI), and components C3 and complement factor B (CFB) in the progression of cSCC has been studied. The role of the complement system in the cSCC tumor growth *in vivo* was also studied.

2 REVIEW OF THE LITERATURE

2.1 Cutaneous squamous cell carcinoma (cSCC)

2.1.1 Overview

The skin cancers are divided into the melanocyte-derived melanoma and keratinocyte-derived non-melanoma skin cancers (NMSC). NMSC is mainly divided into basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC). (Madan et al, 2010) The incidence of NMSC is increasing at the rate 3-8%/ year in the United States and it is the most common cancer worldwide (Rogers et al, 2010; Tufaro et al, 2011). BCC comprises about 80% and cSCC *ca* 20% of NMSCs (Alam & Ratner, 2001). The other malignancies grouped to NMSCs are rare malignancies *i.e.* cutaneous lymphomas, adnexal tumors and Merkel cell carcinomas (Madan et al, 2010). BCC grows locally and rarely metastasizes (in 0.003-0.1% of cases). The metastasis of BCC occurs in large ulcerated and recurring tumors with perineural or blood vessel invasion. (Reifenberger & Ruzicka, 2009) In the white population, invasive cSCC is the second most frequent cutaneous malignancy and it is the most common metastatic skin cancer causing the majority of NMSC deaths. Approximately 20% of the skin cancer related deaths are caused by cSCC. The estimation for a 5-year metastasis rate of cSCC is 5%. (Alam & Ratner, 2001; Rogers et al, 2010; Tufaro et al, 2011) A 5-year survival rate of the metastatic cSCC is less than 35%, (Tufaro et al, 2011) and the death rate of cSCC is estimated to be 2% (Parikh et al, 2014). Moreover, the risk of a recurrence or a metastasis of cSCC cannot be underestimated, because the risk of cSCC metastasis is up to 40% in the case of cSCC having developed in the injured or chronically diseased skin (Königová & Rychterová, 2000; Novick et al, 1977).

In Finland cSCC is the fifth most common cancer in men (5.7 % of all cancers) and fourth most common in women (5.3 % of all cancers). There were 1681 cases in 2012; 884 men and 797 women. The incidence of cSCC is growing also in Finland annually and is comparable to the incidence of cSCC worldwide. cSCC was the cause of death in 47 cases in Finland in 2012. The men's 5-year survival rate of cSCCs was 89% and women's 91% in the years 2007-2009. (Finnish Cancer Registry, 2014)

2.1.2 Progression of cSCC

Actinic keratosis (AK) is the premalignant form of cSCC. AKs can be also classified as *in situ* cSCCs (Ackerman, 2003; Heaphy & Ackerman, 2000). cSCC carcinoma *in situ* (cSCCIS, Bowen's disease) is also a form of cSCC. The change from AK to cSCC is gradual. AK will progress to cSCC in 0.025%-20% of the cases in over 10 years, and the risk increases with multiple lesions. When the patient has less than 5 lesions, the risk of cSCC progression is less than 1%. Whereas, when the number of lesions is 20 the risk of cSCC is up to 20%. Usually patients have 6-8 lesions at the time of the diagnosis. (Ratushny et al, 2012) In addition, the incidence of AK and cSCCIS has increased dramatically worldwide (Rogers et al, 2010). Clinically AK appears as a red or brown scaly patch (Alam & Ratner, 2001). It has been estimated that approximately 26% of the AK lesions regress within one year (Ratushny et al, 2012). AK is clinically classified as mild moderate or severe on the basis of the thickness of the AK lesion. Mild lesion is slight palpable, moderate lesion is moderately thick and easily felt and seen, and severe lesion is very thick. (Olsen et al, 1991)

The histological picture of AK typically consists of enlarged, irregular and hyperchromatic nuclei, disorganized growth of keratinocytes, thickened stratum corneum and hyper- and parakeratosis. Solar elastosis and inflammatory infiltrate are also seen in dermis. (Ratushny et al, 2012; Sterry & Stockfleth, 2009) (**Figure 1**) The severity of AK lesion is classified histologically by the extend of the atypical keratinocytes in the epidermis. In mild dysplastic AK (grade I) the atypical keratinocytes locate in the lower third of the epidermis *i.e.* in the basal and suprabasal layers. In moderate AK (grade II) the atypical keratinocytes are found in the lower two-thirds of the epidermis, and in severe AK lesion (grade III) the atypical cells extend to the full thickness of the epidermis. Severe dysplastic AK equal with cSCCIS. (Cockerell, 2000; Roewert-Huber et al, 2007)

cSCCIS appears as a form of cSCC that is restricted to epidermis and does not penetrate the basement membrane. The histopathological analysis of cSCCIS reveals atypical keratinocytes at all levels of epidermis. Acanthosis and several mitoses are also seen in epidermis and the rete ridges are large. (**Figure 1**) Clinically cSCCIS presents as a slowly enlarging plaque which resembles AK but the hyperkeratosis over cSCCIS is thicker and it is seen as a crust. (Sterry & Stockfleth, 2009) In estimation, 3-5% of the cSCCIS plaques will progress to cSCC, but the estimation does not include patients who have not requested medical care or who have been treated in primary care. (Morton et al, 2014)

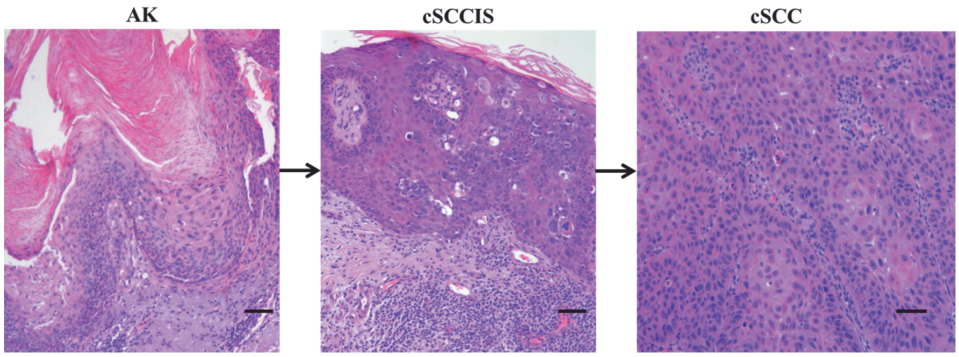

Figure 1. A histologic presentation of AK, cSCCIS and cSCC. Scale bar= 200µm. Abbreviations are listed in pp. 8-9 (Photographs by P. Riihilä)

2.1.3 Molecular pathogenesis of cSCC

The main risk factor for cSCC is solar UV radiation and as a consequence cSCC usually arises in the sun exposed areas of the elderly people (Kivisaari & Kähäri, 2013). Main risk factors for cSCC are listed in **Table 1**. Despite the growing public awareness, changing sun exposure behavior of adults and children is a big challenge for physicians. For example, several sunburns during childhood are in association with cSCC progression. (de Vries et al, 2012; Traianou et al, 2012) Sunburn minimization during the UV exposure is the most important issue in preventing cSCC formation (Mahé et al, 2011). UV radiation causes immunosuppression and inhibits macrophage migration (Kivisaari & Kähäri, 2013). Moreover, UV radiation is also able to generate DNA damage and the subsequent cellular responses are dependent on wavelength. UVA (400nm-320 nm) causes indirect DNA damage through photo-oxidative stress, (Madan et al, 2010; Ridley et al, 2009) and UVB (320 nm-280 nm) causes direct DNA and RNA damage by forming pyrimidine dimers (Madan et al, 2010). UV radiation also causes mutations in the tumor suppressor protein p53 (Brash, 2006). The mutations in the p53 gene are detected in about 90% of all the cSCCs (Kivisaari & Kähäri, 2013). In addition, the p53 mutation is common in AKs, and the p53 mutation is found in 40% of the cSCC *in situ* (cSCCIS) lesions indicating it as an initial genetic mutation towards cSCC. The mRNA and protein levels of p53 are regulated by EGFR and Fyn, a Src-family tyrosine kinase (SFK). Both in the cSCC and in AK lesions the activation levels of EGFR and SFK are increased. (Ratushny et al, 2012) In recent studies the Notch signaling has been noted to have a role in the normal epidermal development and in the pathogenesis of cSCC. Stem cell maintenance, cell fate decision, apoptosis, proliferation and differentiation are regulated by the Notch signaling. Notch1 is expressed in all

the layers of epidermis, whereas Notch2 only in the basal layer. The mutations in both genes have been noted in cSCC. In human keratinocytes p53 regulates Notch1. Thus, the p53 mutation may cause Notch1 down-regulation. As a consequence EGFR and Fyn regulate also Notch1 through the inhibition of p53. (Kraft & Granter, 2014; Ratushny et al, 2012; South et al, 2014; Wang et al, 2011) (**Table 2**)

Table 1. Risk factors for cSCC.

Ultraviolet radiation (UVB and UVA)
Methoxalen and UVA therapy
Chronic ulcers
Chronically injured or diseased skin
Organ transplantation
Immunosuppressive medication
Immunosuppression (HIV/AIDS)
Genodermatosis (dystrophic epidermolysis bullosa, xenoderma pigmentosum, oculocutanous albinism)
Human papilloma virus infection (HPV types 6, 11, 16 and 18)
Ionizing radiation
Radiation dermatitis
Radiation induced keratosis
Leukemia and lymphoma
Chemical carcinogens
Sinus tracts
Osteomyelitis
Arsenical keratoses
cSCC *in situ* (actinic keratoses; AK)
cSCC *in situ* (cSCCIS; Bowen's disease and Erythroplasia of Queyrat)

(Modified from Kivisaari and Kähäri, 2013)

In general, four to six genetic changes are required for the transition of benign epithelium to metastatic carcinoma. In the AKs the activation levels of inositol polyphosphate 5'-phosphatase are decreased indicating increased PI3K/Akt signaling (Ratushny et al, 2012; Sekulic et al, 2010), whereas the activation levels of Myc and ATF-3 are increased in the AK formation (Ratushny et al, 2012). Furthermore, *Ras* activation is noted both in the AK and cSCC lesions. (Pierceall et al, 1991; Spencer et al, 1995) *Ras* activates Raf/Mek/Erk1/Erk2 kinase pathway and the mutation in this signaling pathway is also a result of UVB exposure (Ratushny et al, 2012). Moreover, the loss of p16 function, a cell cycle regulator or tumor suppressor, is more frequently seen in the primary cSCC than in the AK (Mortier et al, 2002; Ratushny et al, 2012). The genetic changes in cSCC are summarized in **Table 2**.

Table 2. Molecular alterations in AK and cSCC.

	AK	cSCC
p53	↓	↓
EGFR	↑	↑
Fyn/SFKs	↑	↑
Inositol polyphosphate 5'-phosphatase	↓	
Myc	↑	
ATF-3	↑	
Ras	↑	↑
p16		↓
Notch		↓

(p53= tumor protein p53, EGFR= epidermal growth factor receptor, Fyn= tyrosine-protein kinase Fyn, SFK= Src-family tyrosine kinase, Myc=transcription factor, ATF-3= cyclic AMP-dependent transcription factor, p16=cyclin-dependent kinase inhibitor 2A (CDKN2A), Notch= signaling pathway controlling cell-fate)

2.1.4 Clinical signs and histopathology

cSCC presents clinically as a painless and inflamed exophytic nodule which has grown rapidly and has developed in the sites exposed to the sun. In the cSCC nodule typical findings can also be bleeding, ulceration or induration. There is usually a crust over the lesion and the edges are ill-defined. The metastases of cSCC in regional lymph nodes are commonly firm, matted and are often adhered to the overlying skin. (Madan et al, 2010; Sterry & Stockfleth, 2009)

The diagnosis of cSCC is based on the histopathological examination of the biopsy or excised tumor. In addition, the regional lymph nodes should be palpated and if a metastasis is suspected, imaging performed (Sterry & Stockfleth, 2009).

The histopathological picture of cSCC is characterized by atypical proliferating keratinocytes which have penetrated the basement membrane and are located in the epidermis and dermis. The tumor cells are usually large, unusually shaped and also abnormal mitoses are seen. The tumors are classified into well, moderately or poorly differentiated tumors on the basis of their histopathology. For example, keratinization is visible in the well differentiated tumors. In the poorly differentiated lesion there is no keratinization visible and the inflammatory cells are prominent on the tumor site. (Sterry & Stockfleth, 2009) A histological picture of cSCC is seen in **Figure 1**.

2.1.5 Prognosis of cSCC

Tumor thickness, the degree of the differentiation and localization of the primary tumor affect the prognosis of cSCC. The tumors thicker than 4-6 mm are more likely to recur locally. Large lesions, diameter >2 cm, recur twice more often than smaller lesions. cSCCs on the lip or on the ear are aggressive and recur and metastasize in 10-25% of the cases. A poor prognosis is also typical of the cSCC tumors penetrating the subcutaneous fat or when a perineural invasion is detected. Approximately 5-16% of the invasive cSCC recur and 5% metastasize within a 5-year period from the diagnosis. (Alam & Ratner, 2001; Kivisaari & Kähäri, 2013; Madan et al, 2010; Sterry & Stockfleth, 2009) In addition, the risk of a metastasis is high (up to 40%) in the tumors arising in chronic ulcers (Königová & Rychterová, 2000; Novick et al, 1977). The 10-year survival rate is <20% when regional lymph nodes are involved and <10% when distal metastases are detected (Alam & Ratner, 2001).

2.1.6 Treatment of cSCC

cSCC is usually curable by a simple excision. In the low-risk tumors <2 cm in diameter the recommended excision margins are 4-5 mm. The margins should be wider than 6 mm when located in the high-risk locations such as scalp, ears, eyelids, lips or nose or when the diameters is > 2cm. Mohs micrographic surgery is also one option and is recommended for high-risk cases (Alam & Ratner, 2001; Bonerandi et al, 2011; Ferrandiz et al, 2012; Kivisaari & Kähäri, 2013; Madan et al, 2010). Radiation therapy has also been used as adjuvant therapy in high-risk cSCCs (Bonerandi et al, 2011; Parikh et al, 2014). In some cases, however, cSCC recurs or metastasizes after the surgical removal. In the low-risk tumors the recurrence rate after the surgery is 5-8% and in the high-risk tumors approximately 16%. The poorly differentiated tumors recur in approximately 25% of the cases and the well-differentiated recur in approximately 13% of the cases after the surgery. (Alam & Ratner, 2001) Currently, there are no generally accepted molecular markers for the detection of high-risk cSCC in clinical use. Consequently, there is a demand for predictive biomarkers which could be used to identify the high-risk cases (Dooley et al, 2003; Farshchian et al, 2011).

For AK and cSCCIS there are many different treatment options. They are mainly non-invasive. The choice of the treatment depends on the site and size of the lesion and the number of the lesions found. The treatment choices are cryotherapy and photodynamic therapy with ALA or methyl ALA and topical agents such as topical 5-fluorouracil and imiquimod. AK can also be treated topically with diclophenac or ingenol mebutate. Topical retinoids can be used as

a prevention of new lesions. (de Berker et al, 2007; Ferrandiz et al, 2012; Siller et al, 2009; Stockfleth et al, 2008)

In the metastatic nodal disease the treatment is the dissection of the lymph nodes or if not operable, the irradiation of the lymph nodes. The treatment of a metastatic cSCC consists of the local excision of the metastasis with 6 mm margin with the irradiation or dissection of the regional lymph nodes. If surgery is not possible then radiation therapy alone or in the combination with chemotherapy is another option of the treatment. Systemic chemotherapies or biologic response modifiers are also used, but there are no established standard regiments. In addition, monoclonal EGFR inhibitors such as cetuximab or panitumab and also small molecule tyrosine kinase inhibitors such as erlotinib and gefitinib have been used as a treatment. (Alam & Ratner, 2001; Parikh et al, 2014)

2.2 Immunosuppression and cSCC

The other most important risk factors of cSCC in addition to UV exposure are the chronic ulceration of the skin and immunosuppression (Madan et al, 2010). The risk of cSCC is 65-250 fold in the white organ transplant patients and cSCCs occur more frequently than BCCs unlike in the non-transplanted population (Madan et al, 2010). In the organ transplant recipients, the biology of cSCC is changed. The cSCC tumors recur or metastasize more often and the tumors are numerous. HPV DNA infection is often involved in the cSCC lesions of the organ transplant patients (in 80% of the cases). (Tufaro et al, 2011) As a consequence, cSCC will develop 65 times more likely to the organ transplant patients than in the age-matched control subjects. The lesions appear approximately in a 2-4 -year period after the transplantation and the incidence grows over time. The rate of a metastasis is up to 10%. The locally recurred tumors metastasize in 25% of the cases and if the tumor is on an ear or on a lip the rate of metastasizing is 30-45%. (Alam & Ratner, 2001)

2.3 Inflammation and cSCC

The relationship between inflammation and cancer is well recognized, and inflammation plays an important role in cSCC tumor development (Coussens & Werb, 2002; Ratushny et al, 2012). cSCC is also known to develop in the sites of chronic inflammation, including chronic ulcers, osteomyelitis, radiation dermatitis or sinus tracts. Moreover, certain chronic inflammatory disorders, such as discoid lupus erythematosus, lichen planus, lichen sclerosus and cutaneous

tuberculosis (lupus vulgaris) are risk factors for cSCC. (Alam & Ratner, 2001) Inflammatory cells are also typically noted in the microenvironment of cSCC progression (Ratushny et al, 2012). In general, tumor cells express the same inflammatory molecules as normal cells and these molecules, such as chemokines and selectins, are used for invasion, migration and metastasis. Moreover, the frequency of the p53 mutations in the chronic inflammatory diseases is similar to that in tumors. (Coussens & Werb, 2002) cSCCs arising at the site of chronic inflammation are more aggressive and 40% will metastasize (Tufaro et al, 2011).

2.4 Epidermolysis bullosa (EB) and cSCC

2.4.1 Overview

The epidermolysis bullosa (EB) is an inheritable disease which is characterized by blisters, erosions and chronic wounds arising spontaneously or after a minor trauma to the skin or mucosa. (Pearson, 1962) EB is divided into four different major types. Namely, EB simplex (intraepidermal), junctional EB (dermo-epidermal junction), dystrophic EB (dermolytic) and Kindler syndrome (mixed). In the dystrophic EB the blisters are formed in the deep parts, namely the sublamina densa and in the upper dermis. As a result of that, the blisters often heal with extensive scars and can result in chronic ulcers. (Bruckner-Tuderman et al, 1989) The dystrophic EB is divided into the recessive or dominant forms. The most severe subtype of the dystrophic EB is generalized severe RDEB (gs-RDEB, Hallopeau-Siemens). In general, the recessive dystrophic EB (RDEB) is more severe than the dominant dystrophic EB. (Fine et al, 2014)

The invasive cSCCs which form on the skin of the patients suffering from gs-RDEB are more aggressive than the spontaneous UV-induced cSCCs (Fine et al, 2009). The difference is that the cSCC develops in the site of the chronic skin ulceration in the gs-RDEB patients (Reed et al, 1974). The cumulative risk of cSCC on the skin of the gs-RDEB patients is 90% and the risk of death is 78% by the age of 55 (Fine et al, 2009). Furthermore, a sporadic UV-induced cSCC diagnosis is usually made in the elderly individuals whereas in the gs-RDEBSCC patients, the diagnosis is made at the age of 30-40 (Mallipeddi, 2002). The risk of cSCC is also increased on the skin of junctional EB patients whereas in EB simplex and dominant dystrophic EB patients the risk is similar than in normal population (Fine et al, 2009). In dystrophic EB, mutations are identified in the COL7A1 gene which encodes the type VII collagen protein (COL7) (Uitto & Christiano, 1994).

2.5 Complement system

The complement system is a part of the innate immune system and acts as a first line of the host defense against different pathogens. Its function is to destroy foreign structures and defend the host against the microbial attack. The complement system also promotes the clearance of immune complexes and apoptotic, damaged or altered host cells from blood and other tissues. Furthermore, the complement system plays an important role in the enhancing of humoral immune responses and has a role in the adaptive immune system through the receptor-ligand interactions. (Ricklin et al, 2010; Rutkowski et al, 2010a)

The complement system comprises a large number of protein components. As a total, about 40 complement system proteins are present on cell membranes, in human plasma and in lower concentrations in the other body fluids. The complement system components can act as precursor enzymes, effector molecules, receptors or regulators. Approximately 5% of the total protein content of the human plasma consists of the soluble members of the complement system. The primary source of the complement proteins is the liver. Moreover, the other tissues are known to produce complement proteins locally. The locally produced complement components are believed to perform some important functions at the tissue level. Some complement proteins also act as serine proteinases, such as complement components C1r, C1s, C2, C3, C4, C5, complement factor B (CFB), complement factor D (CFD), MASP1-3, and complement factor I (CFI). (Forneris et al, 2012; Merops database, 2014; Ricklin et al, 2010; Sim & Laich, 2000; Sim & Tsiftsoglou, 2004)

The activation of the complement system is strictly regulated to prevent damage to the host cells. Most complement components are produced and they circulate as precursor enzymes, but multiple regulatory proteins are also involved in the cascade. Firstly, the activation of the complement cascade is initiated by the interaction of a foreign target structure and the complement proteins. Secondly, the complement system precursors are activated and after that, a second component is activated in a sequential manner allowing a considerable amplification loop to start. The activation trigger defines which proteins are activated. The complement cascade can be activated *via* three distinct pathways, **classical**, **lectin** or **alternative pathways** which all lead to lytic pathway activation. Finally, the activation of complement results in three main types of biological responses. Firstly, a pore like structure, by the membrane attack complex (MAC) is formed on the target cell membrane. As a consequence, the target cell is destroyed. Secondly, the fragments C3b and iC3b serve as cell surface receptors for phagocytes to induce the complement dependent cellular

cytotoxicity (CDCC). And thirdly, cleavage fragments of the complement activation (C3a, C4a and C5a) act as the anaphylatoxins and chemotactic activators of the leukocytes. (Janssen & Gros, 2006; Sim & Tsiftsoglou, 2004) (**Figure 2**)

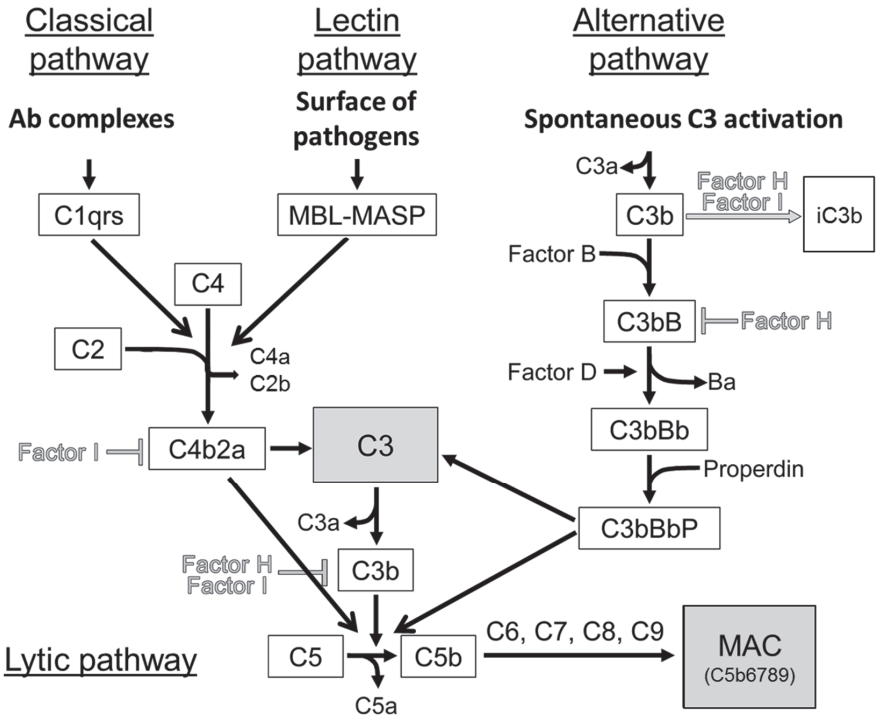

Figure 2. The schematic representation of complement pathways activation. The classical pathway is activated by antigen-antibody complexes (IgG or IgM). The lectin pathway is activated by microbic surfaces containing mannose or residues of N-acetylglucosamine. The alternative pathway is activated by the activating surfaces of microbes. All the three pathways will lead to C3 breakdown and the lytic pathway initiation. Finally the membrane attack complex (MAC) is formed and the target cell destroyed. The sites of the complement factor H and the complement factor I functions as the inhibitors of the complement system are presented in the cascade. Ab= antibody. The other abbreviations are listed in pp. 8-9. (Modified from I, suppl. figure)

2.5.1 Complement component C3

All the complement pathways will lead to C3 activation (Zipfel & Skerka, 2009). C3 is synthesized as a single-chain precursor (185 kDa) composed of two polypeptide chains (α-chain; 115 kDa and β-chain; 75 kDa) connected with two disulfide bonds (Gros et al, 2008). (**Figure 3**) The active C3 is generated by C3 convertases cleave the α-chain of C3 to the fragments C3a (9 kDa) and C3b (175 kDa). C3a is an anaphylatoxin, which enhances inflammation and C3b is a central component of the complement system (Sim & Tsiftsoglou, 2004). The conformation of C3b changes rapidly and the active internal thiolester bond is accessible only for a short time (60 μs) for covalent binding of the target cell (Isenman & Cooper, 1981; Nilsson & Nilsson, 1985; Nilsson et al, 1987; Sim & Tsiftsoglou, 2004).

On the host cell membrane the fragment C3b is inactivated by different soluble and membrane bound inhibitors *i.e.* CFH, CFI, complement receptor 1 (CR1; CD35), membrane cofactor protein (MCP; CD46) and decay accelerating factor (DAF/CD55) (Gros et al, 2008). The inactive forms are named as iC3b, $iC3b_1$, $iC3b_2$, C3c, C3dg and C3f (Rodriguez de Cordoba et al, 2004; Sahu & Lambris, 2001). The inactivation sites in C3 are shown in **Figure 3**.

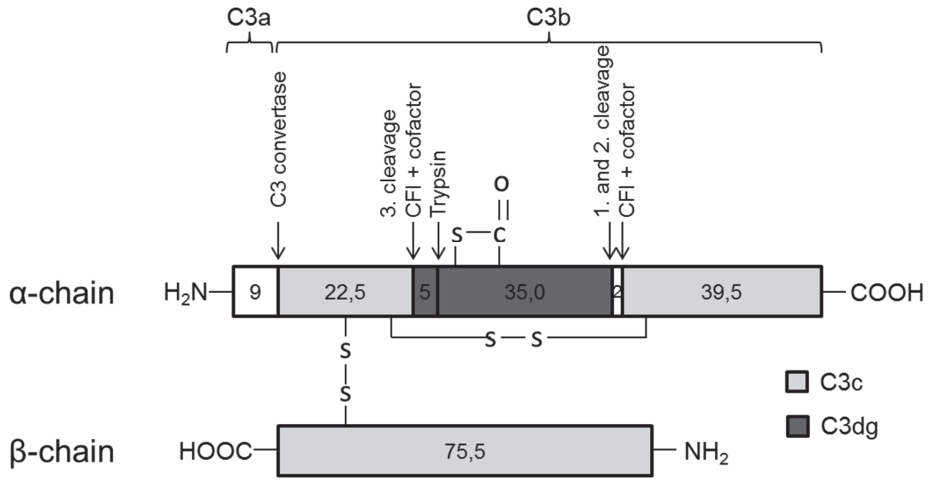

Figure 3. The schematic structure of C3 shows the cleavage sites of the complement factor I (CFI) and cofactors in the molecule C3. First, the C3 convertase cleaves C3, and C3a (9 kDa) and C3b (176 kDa) are formed. C3b is inactivated by CFI in three steps. After the first and second cleavage $iC3b_1$ and $iC3b_2$ are formed, respectively, and also the 2 kDa fragment (C3f) is cleaved away. The third cleavage of C3b by CFI and the cofactor liberates C3dg (40 kDa) and as a consequence an inactive form of C3, C3c (137 kDa) is formed.

2.5.2 Classical and lectin pathways

The classical pathway is activated by at least two surface-bound Fc regions of antigen-bound IgMs or IgGs, serum amyloid P component (Hicks et al, 1992), membrane blebs on apoptotic cells (Navratil et al, 2001) or C-reactive protein (Agrawal et al, 2001). First, the C1 subcomponent C1q, is activated resulting in alteration in the tertiary structure of C1q in the complex $C1qr_2s_2$. The changing structure of C1q is in the C-terminal globular head and six collagenous stems are arranged a bouquet-like. The binding of the pathogens induces a conformational change in C1q that drives the activation of the zymogen proteinases C1r and C1s in stepwise fashion. C1r is first auto-activated and then C1r activates C1s. No proteolytic fragment is released when C1r or C1s is activated, only the conformation is changed. (Venkatraman Girija et al, 2013) Then C1s cleaves C4 to the fragments C4a and C4b. C4b attaches on the target cell membrane covalently. C1s cleaves C2 to C2a and C2b. C2a is attached to C4b on the target cell membrane to form the complex C4b2a, a classical pathway C3 convertase. The C4b2a cleaves C3 and the lytic pathway is initiated. (Ricklin et al, 2010) (**Figure 2**)

The lectin pathway is activated by the mannose binding lectin (MBL). MBL binds to a specific mannose and the N-acetylglucosamine structures on the microbial cell surfaces. (Gros et al, 2008; Zipfel & Skerka, 2009) MBL is a C1q-like molecule and it forms a similar complex as C1qrs with the serine proteinases MASP1-3. MBL activates MASPs and that activation causes the cleavage of C4 and C2 as in the classical pathway. After this activation of lectin pathway, the lytic pathway continues as in the classical pathway. (Lu et al, 1990) (**Figure 2**)

2.5.3 Alternative pathway

The alternative pathway is the most important pathway of the complement system, because it is continuously activated at a low rate in the human plasma, seen as a spontaneous and slow hydrolysis reaction and breakdown of C3. As a consequence, the complex $C3b(H_2O)$ (also known as iC3) is formed. CFB binds to $C3b(H_2O)$. CFD cleaves CFB to the fragments Ba and Bb. The fragment Bb remains attached to C3b and the initial C3 convertase, $C3b(H_20)Bb$ is formed. C3 convertase cleaves the fluid phase C3 to C3b and C3a. The cascade is continued and C3b attaches covalently to hydroxyl groups (-OH) with an ester bond or to the amino groups ($-NH_2$) with an amide bond on the target cell membranes within a short period. CFB binds to C3b in the presence of the catalyst Mg^{2+} and CFB is cleaved to Ba and Bb by CFD. Finally, C3bBb, the actual C3 convertase is formed. Thus, $C3b(H_20)Bb$ promotes deposition of C3b on the cell surfaces,

formation of new C3 convertases (C3bBb) and activation of the amplification loop (Gros et al, 2008). The half-life of the C3bBb convertase is very short (about 90s) and Bb is released from the complex. Bb cannot rebind to C3b. (Pangburn & Muller-Eberhard, 1986) Properdin (complement factor P) is the only positive regulator of the complement system and it can stabilize the complex C3bBb up to 5-10 fold (Fearon & Austen, 1975; Kemper et al, 2010). In the end, the amplification cascade causes plenty of C3b accumulation on the target cell surface. This leads to effective opsonization with C3b and lytic pathway activation. (Schreiber & Müller-Eberhard, 1978) (**Figure 2**)

2.5.3.1 Complement factor B (CFB)

CFB is a single-chain polypeptide (93 kDa) (Gros et al, 2008). (**Figure 4**) CFB is susceptible to cleavage by CFD when bound to C3b. As a result of the cleavage, two chains, the non-catalytic Ba (30 kDa) and the catalytic serine proteinase Bb (63 kDa) are formed. Ba is cleaved away and Bb remains associated with C3b to form the C3 convertase. Ba is known to inhibit the proliferation of B lymphocytes, whereas Bb can enhance the proliferation of the preactivated B lymphocytes (Ambrus et al, 1990; Peters et al, 1988). The serine proteinase Bb is known to cleave C3, C5, plasminogen and certain other proteins (Medicus et al, 1976; Sundsmo & Wood, 1981).

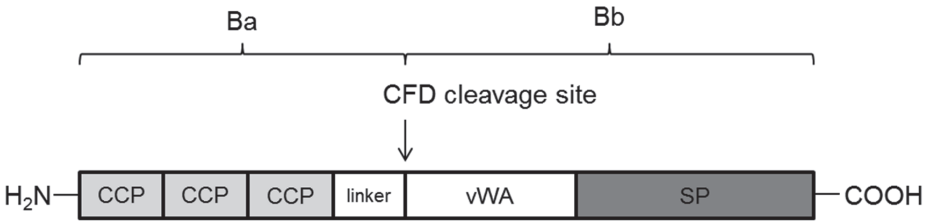

Figure 4. The schematic structure of CFB. CFB is a single chain glycoprotein which consists of three CCP domains, a linker, von Willebrand factor type A domain (vWA) and serine proteinase (SP) domain. The complement factor D (CFD) activates CFB by cleaving it between the linker region and vWA into Ba and Bb. The abbreviations are explained in pp. 8-9.

2.5.4 Lytic pathway

The activation of the lytic pathway is initiated when C3b is activated and the complex C3bBb formed. First, C5 is attached to C3b. Bb cleaves C5 to C5a and C5b. C5a serves as an anaphylatoxin and a chemotaxin and promotes inflammation. C5b activates C6, C7, C8 and C9. C9 polymerizes and 12-18 subunits form together a pore-like structure called MAC on the target cell membrane (Müller-Eberhard, 1986; Tschopp et al, 1984). As a result, the adenine nucleotides ATP, ADP and AMP leak out of the target cell, intracellular Ca^{2+} leaks and other ions, proteins and intracellular molecules pass through the membrane and also the mitochondrial membrane potential is lost (Papadimitriou et al, 1991).

2.5.5 Complement regulation

The complement cascade is strictly controlled by soluble and membrane-bound inhibitors to maintain the homeostasis or to prevent the excessive consumption of the components. Soluble regulators are present in the plasma.

2.5.5.1 Membrane-bound regulators

At least four membrane-bound regulators are present on the host cell membranes to protect them from an inappropriate cell lysis.

The complement receptor 1 (CR1, CD35) inhibits C3Bb and C4b2a and functions as a cofactor for CFI in the proteolytic inactivation of C3b and C4b. The cofactor activity of CR1 in the inactivation of the surface bound C3b functions in all the three different cleaving sites of C3b. Moreover, CR1 functions as a receptor for C3b, C4b, iC3b and C1q on the surfaces of phagocytes. CR1 is expressed on the surface of monocytes, B lymphocytes, neutrophils, erythrocytes, and eosinophils. (Fearon, 1980)

Like CR1, the membrane cofactor protein (MCP, CD46) also serves as a cofactor for CFI in the inactivation of C3b and C4c to iC3b and iC4b, respectively (Seya & Atkinson, 1989; Seya et al, 1986). MCP is a cofactor for CFI in the first two cleavage steps in the proteolytic C3b inactivation (Seya & Atkinson, 1989). It is expressed on the membranes of all the cells of human origin except for erythrocytes (Seya et al, 1986). The decay accelerating factor (DAF, CD55) dissociates the C3 convertases and also releases Bb or C2a from the C3 convertase complexes, but does not act as a cofactor for CFI. (Fujita et al,

1987) DAF is expressed on most cells in contact with the complement components, including platelets, neutrophils, monocytes, lymphocytes and erythrocytes. (Kinoshita et al, 1985; Nicholson-Weller et al, 1982)

Protectin (CD59) prevents the activation of the lytic pathway and the formation of MAC by binding to C8 and C9. It also prevents C9 polymerization into lipid bilayers. (Meri et al, 1990; Rollins & Sims, 1990) CD59 is expressed on the surfaces of all the human membranes (Meri et al, 1991).

2.5.5.2 Complement factor H (CFH) and factor H-like protein 1 (FHL-1)

CFH is a soluble inhibitor of the alternative pathway of complement (de Córdoba & de Jorge, 2008; Zipfel & Skerka, 1999). It consists of a single polypeptide chain composed of 20 N-terminal complement control protein domains (CCP). CCP is also called short consensus repeats (SCR) domain, and it contains several glycosylation sites. (de Córdoba & de Jorge, 2008) The molecular weight is 150 kDa (**Figure 5.**). The factor H-like protein 1 (FHL-1, 45 kDa) is a splicing variant of the primary transcript, coded by the same gene as CFH (Zipfel & Skerka, 1999). FHL-1 contains the same first seven CCP domains as CFH and in addition four additional unique amino acid residues at the carboxy-terminal end (Hellwage et al, 1997; Rodriguez de Cordoba et al, 2004; Zipfel & Skerka, 1999) (**Figure 5**). The concentration of FHL-1 is only about 10% of the concentration of CFH in human plasma (Fontaine et al, 1989; Misasi et al, 1989; Schwaeble et al, 1987). FHL-1 may have other important functions in body compartments where the concentration of CFH is low (Zipfel & Skerka, 1994).

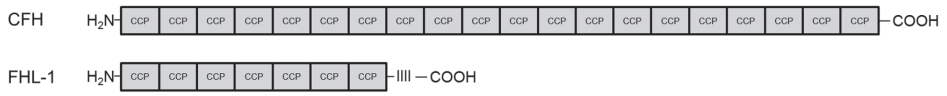

Figure 5. The schematic structure of CFH and FHL-1. CFH is composed of 20 CCP domains. FHL-1 is formed of the same 7 first CCP domains as CFH and at the carboxy terminus, there is a hydrophobic tail (IIII) that is specific for FHL-1 molecule. The abbreviations are listed in pp. 8-9.

CFH is the main soluble regulator of the alternative pathway. CFH competes with CFB for binding to the activated C3b. It also functions as a cofactor for CFI in the proteolytic cleavage of C3b to the inactive form iC3b, and it dissociates the convertase C3 by displacing Bb from the complex C3bBb (Schulze et al, 1993). (**Figure 3**)

2.5.5.3 Complement factor I (CFI)

CFI (88 kDa) is a soluble serine proteinase composed of two polypeptide chains (50 kDa heavy and 38 kDa light chains) connected covalently with a disulfide bond (Goldberger et al, 1984; Lachmann & Müller-Eberhard, 1968; Nilsson et al, 2009; Ruddy & Austen, 1969). (**Figure 6**) It is synthesized as a single polypeptide mainly in the liver and cleaved by furin to form a mature, proteolytically active protein. CFI is also produced by keratinocytes (Timar et al, 2007b), skin fibroblasts (Vyse et al, 1996), endothelial cells (Julen et al, 1992), monocytes (Whaley, 1980) and myoblasts (Schlaf et al, 2001).

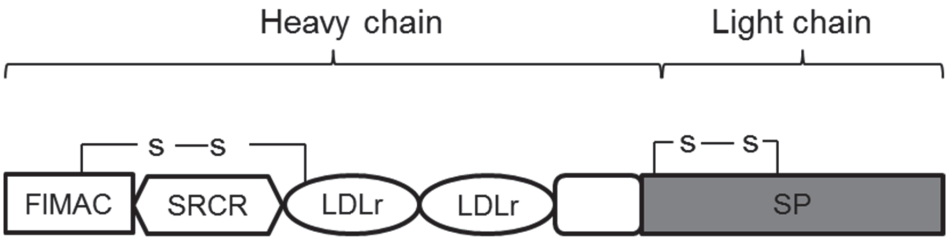

Figure 6. The schematic structure of CFI. CFI is composed of membrane attack complex domain of FI (FIMAC), scavenger receptor cysteine-rich protein domain (SRCR), two low-density lipoprotein receptor (LDLr) domains the homology of which is not known and serine proteinase domain (SP). The heavy chain comprises the first five domains and the light chain is consisted of the domain SP.

CFI plays an important role in the regulation of the complement activation by inhibiting classical, lectin and alternative pathways. It has a highly restricted substrate specificity and it has no endogenous inhibitors. The activity of CFI is regulated by the supply of substrates *in vivo*. (Nilsson et al, 2011) CFI inactivates activated C3b proteolytically by cleaving it at three distinct sites. (**Figure 3**) It needs a cofactor to function properly. These cofactors are CFH (Schulze et al, 1993), CR1 (Medicus et al, 1983; Medof et al, 1982; Ross et al, 1982) and MCP (Seya & Atkinson, 1989; Seya et al, 1986). All three cofactors function in the first two steps of a cleavage of the activated α'-chain of C3b to generate iC3b and C3f (Harrison, 1983; Nilsson et al, 2011). The third cleavage step of iC3b to C3dg and C3c is performed by CFI and CR1. (Ross et al, 1982; Ross et al, 1983) In addition, CFI cleaves the activated α'-chain of C4b with the cofactors MCP, C4b binding protein or CR1 (Nagasawa & Stroud, 1977).

2.5.5.4 Other soluble regulators

There are also four other soluble inhibitors besides CFH and CFI. The inhibitor of the plasma protein C1 (C1 INH) blocks the serine proteinase activities of C1r and C1s in the complex C1rC1s preventing the cleavage of C4 and C2. (Ziccardi, 1982) C4b binding protein (C4bp) prevents the C4b2a complex function and it functions as a cofactor for CFI in the inactivation of C4b preventing a new convertase formation (Gigli et al, 1979). In addition, clusterin (SP40) (Davies et al, 1989; Jenne & Tschopp, 1989) and vitronectin (S-protein) (Podack & Müller-Eberhard, 1979) inhibit the complement lytic pathway by binding to the forming C5b-9 complex.

2.5.6 Mouse complement system

In general, the complement system in a mouse is quite similar to that in humans but certain differences remain. CDCC is more potent in the immune system of a human than a mouse and that correlates with the lower antigen and complement concentration in the plasma of a mouse. (Bergman et al, 2000; Ong & Mattes, 1989) Some differences in the number of transcript variants can be found between humans and mice. For example, there are two isoforms of CFB in the circulation of a mouse whereas in humans there is only one. The isoforms of the CFB of a mouse are a result of an alternative site of transcriptional initiation. (Nonaka et al, 1989) The most important complement component C3, resembles the human C3, although the cleavage of C3 of a mouse is slower than that in humans (Pepys et al, 1977). Murine CFI resembles human one (Minta et al, 1996).

Different models of complement component deficient mice have been generated to test the function of the complement system. A multi-stage epithelial carcinogenesis model was used to test the function of C3 in transgenic HPV16 mice (Coussens et al, 1996). Surprisingly, HPV16/C3$^{-/-}$ mice had the same effects on mast cell activation at each neoplastic stage examined and also there were no vascular architecture changes compared to HPV16 mice (de Visser et al, 2004). However, the deficiency of C3 is associated with reduced airway reactivity in C3 deficient mice compared to wild-type mice indicating that C3 plays a role in the promotion of the hypersensitivity reaction in asthma (Walters et al, 2002). Furthermore, mice deficient of C3 and C4 were more susceptible to an endotoxin shock than wild-type controls (Fischer et al, 1997). The function of the complement system in skin diseases was studied with mice deficient of CFB. The deficiency of CFB was associated with delayed and less intense bullous pemphigoid (Nelson et al, 2006). Deficiency of CFH is associated with

membrano-proliferative glomerulonephritis type II. In $CFH^{-/-}$ mice, C3 was deposited on the glomerular basement membrane and the mice developed membrano-proliferative glomerulonephritis type II (Pickering et al, 2002). In contrast, the disease did not develop in $CFI^{-/-}$ mice. However, in the combined deficiency of CFI and CFH, no deposition of C3 in the glomerular basement membrane was noted. As a conclusion, CFI is required for the development of the membrano-proliferative glomerulonephritis type II combined with the deficiency of CFH. (Rose et al, 2008)

2.5.7 Complement system in skin

One of the major functions of the skin is to serve as a barrier against pathogens. The complement system plays an important role in the defense of a human host to protect the human body against pathogens. Several complement system components and inhibitors are expressed in the human skin. C3, CFB, DAF, CR1 and C3d receptor (CR2) are expressed by the epidermal keratinocytes. (Dovezenski et al, 1992; Terui et al, 1997; Yancey et al, 1992) The soluble inhibitors of the alternative pathway are also expressed by the keratinocytes *i.e.* CFH (Timar et al, 2006) and CFI (Timar et al, 2007b). Furthermore, the keratinocytes express mRNA of the terminal complement components, C5, C7, C8γ and C9 (Timar et al, 2007a). The expression of the complement components in keratinocytes is regulated by inflammatory cytokines. The expression of both CFH and CFI is up-regulated by the proinflammatory cytokine IFN-γ and the expression of C9 by TNF-α (Timar et al, 2007b; Timar et al, 2006) IFN-γ also up-regulates C3 and CFB but TNF-α up-regulates only C3 in keratinocytes (Pasch et al, 2000).

2.5.8 Complement system in cancer

Chronic inflammation is a typical feature in many types of the microenvironments of cancer. It also enhances tumor progression. (Rutkowski et al, 2010a) Complement components are known to be part of the microenvironment of a tumor (Baatrup et al, 1994; Niculescu et al, 1992). As discussed above, skin cancers also often arise at the sites of chronic inflammation. The activation of the complement system has also a role in chronic inflammation. One survival strategy of cancer cells is to escape the attack of the immune system. The immune evasion of a cancer cell can be achieved by utilizing complement inhibitors. (Gorter & Meri, 1999) Complement components and inhibitors are associated with many types of cancers. CFH and FHL-1 have

a role in the escape of cancer cells from the complement –mediated cytotoxicity in bladder cancer (Cheng et al, 2005), glioblastoma (Junnikkala et al, 2000), colon (Wilczek et al, 2008), the lung (Ajona et al, 2004; Ajona et al, 2007) and ovarian cancer (Junnikkala et al, 2002). In the same way, the expression of CFI has been documented in non-small-cell lung cancer (Okroj et al, 2008), glioma (Gasque et al, 1992) and rhabdomyosarcoma (Legoedec et al, 1995). Similarly, CFB has been proposed as a novel biomarker for pancreatic ductal adenocarcinoma (Lee et al, 2014) or for rhabdomyosarcoma (Legoedec et al, 1995). In addition, glioma cells express C3 and CFB (Gasque et al, 1992; Hanahan & Weinberg, 2000).

Certain complement components have also been documented to have additional functions besides complement activation or inhibition and those functions may have a role in the progression of a tumor. As mentioned above, certain complement system components have an indirect effects on tumor growth by altering the immune response of the host. (Corrales et al, 2012; Gunn et al, 2012; Rutkowski et al, 2010b). For example, some complement components act as serine proteinases. In the classical and lectin pathways these proteinases are C1r, C1s, C2, C4, MASP1, MASP2, and MASP3 (Forneris et al, 2012; Sim & Laich, 2000), and in the alternative pathway CFB (Bb part of this complex) and CFD. Moreover, CFI and C5 are also serine proteinases (Merops database, 2014; Sim & Laich, 2000).

3 AIMS OF THE STUDY

1. To investigate the expression of the complement system components in normal human epidermal keratinocytes (NHEK) and cSCC cells. (I-III)
2. To examine the role of the specific complement system components as biomarkers in the progression of cSCC *in vivo*. (I-III)
3. To elucidate the functional role of the complement inhibitors CFH and CFI, and also the complement components C3 and CFB in cSCC cells in culture and *in vivo* in a xenograft model. (I-III)

4 MATERIALS AND METHODS

4.1 Ethical issues (I-III)

The use of cSCC cell lines, NHEKs, cSCC tumor samples, and normal human skin samples were approved by the Ethics Committee of the Hospital District of Southwest Finland. All the patients gave their written informed consent before the surgery. The study was conducted according to the Declaration of Helsinki (I-III). The animal experiments were carried out according to the institutional guidelines with the approval of the animal test review board of Southern Finland (II, III).

4.2 Antibodies

The use of primary antibodies is described in the original publications (I-III) in detail, as indicated. The primary antibodies used in IHC are presented in **Table 3**, and the primary antibodies used in the Western blot are presented in **Table 4**.

Table 3. Antibodies used in immunohistochemistry (IHC)

Antigen	Catalog No.	Supplier	Conc.	Used in
CFH (Clone OX-24)	#MCA509G	AbD Serotec	1:850	I
Factor I (Clone OX-21)	NBP1-02915	Novus Biologicals	1:150	II
Complement C3	204869	Calbiochem	1:2000	I-III
Factor B	341272	Calbiochem	1:1400	III
Ki-67 (Clone MIB-1)	M7240	Dako	1:200	II
TNF-α	sc-8301	Santa Cruz Biotech.	1:200	II

Table 4. Antibodies used in immunoblotting.

Antigen	Catalog No.	Supplier	Conc.	Used in
Factor H	341276	Calbiochem	1:1000	I
Factor I (Clone OX-21)	NBP1-02915	Novus Biologicals	1:500	II
Complement C3	204869	Calbiochem	1:500, 1:1000	I, III
Factor B	341272	Calbiochem	1:500	III
ERK	#9102	Cell Signaling Tech.	1:1000	I-III
pERK1/2	#9101	Cell Signaling Tech.	1:1000	I-III
p38	#9212	Cell Signaling Tech.	1:1000	I, II
p-p38	#9211	Cell Signaling Tech.	1:1000	I, II
β-Actin	A-1978	Sigma-Aldrich	1:10000	I-III

4.3 Cells and tumor samples

4.3.1 Human cell cultures (I-III)

Human primary (n=5; UT-SCC-12A, -91, -105, -111 and -118) and metastatic (n=3; UT-SCC-7, -59A and -115) cSCC cell lines (**Table 5**) were established at the time of the operation from the surgically removed cSCC tumors in Turku University Hospital. The SCC cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, nonessential amino acids, 100 IU penicillin G and 100 µg/ml streptomycin.

Table 5. cSCC cell lines used in original publications

UT-SCC	Location	Type of lesion
12A	Skin of nose	Primary
105	Face	Primary
111	Face	Primary
118	Face	Primary
91A	Skin of nose vestibulum	Recurrent
7	Temporal skin	Metastasis in neck
59A	Temporal skin	Metastasis
115	Face	Metastasis

NHEK cells (n=10; NHEK-42, -45, -51, -54, -59, -65, -70, -74, -78 and -84) were obtained from normal skin samples during the mammoplasty operation in Turku University Hospital and prepared in the method described previously (Boyce & Ham, 1983). The NHEK-PC cells were purchased from PromoCell. The NHEK cells were cultured in KGM-2 with supplement mix (PromoCell) and 0.5M calcium chloride.

The cell lines representing different stages of the progression of the cSCC tumor were used to create an *in vitro* model. The HaCaT cells are benign spontaneously immortalized non-tumorigenic keratinocyte-derived cells, whereas A5, II-4 and RT3 cell lines are Ha-*ras* -transformed tumorigenic HaCaT-derived cell lines (Boukamp et al, 1988). A5 cells form benign, II-4 cells form low grade and RT3 cells form high grade metastatic tumors in the nude mice *in vivo*. The cell lines mentioned above were kindly provided by Dr. Nobert Fusenig (Deutshes Krebsforschungscentrum, Heidelberg, Germany). The HaCaT cells were cultured as cSCC cell lines and in addition G418 (200 µg/ml) was added in the A5, II-4, and RT3 cell medium.

4.3.2 Effect of the growth factor and MAPK signaling (I-III)

To study the effects of the cytokines and growth factors the cell cultures of the cSCC cell lines (UT-SCC-7, -118, -12A and -105) and the HaCaT cells were treated with IL-1β, IFN-γ, TGF-β1, TNF-α or TGF-α for 24 hours. (I-III)

The role of MAPK signaling was studied with UT-SCC-118 cell cultures treated with MEK1/2 inhibitor PD98059, or with p38α/p38β inhibitor SB203580, for 24 h or with p38α/p38β/p38γ/p38δ inhibitor BIRB796 for 48 hours. (I)

4.3.3 Tissue specimens (I-III)

Normal skin samples (n=11) were obtained from the upper arm of healthy volunteers and during a mammoplasty operation in Turku University Hospital. Primary cSCC tumor samples (n=6) were collected from the surgically removed tumors in Turku University Hospital (Farshchian et al, 2011). The total RNA was isolated from the tissue samples and analyzed by qRT-PCR (Stokes et al, 2010). (I-III)

The tissue sections of normal skin, AK, cSCCIS, cSCC, seborrheic keratosis (SK) and aggressive form of cSCC, RDEBSCC embedded in paraffin and fixed with formalin (FFPE) were used as clinical material. (I-III) The RDEBSCC samples consisted of tissue samples from 17 individuals and of a total of 25

tumors (mean age 33 years, range 12–56 years). (Kivisaari et al, 2008; Weber et al, 2001) The tissue samples were collected during the surgical excision by an international collaboration between the years 1988-2004. (II, III)

All the other clinical tissue material (n=260) was obtained from the archives of the Department of Pathology, Turku University Hospital. It consisted of sporadic probably UV-induced cSCC, AK, cSCCIS, SK and normal skin. (**Table 6**) All the tissue samples were collected into tissue microarray (TMA) blocks (Kononen et al, 1998). The tissue sections stained with hematoxylin-eosin were checked for the appropriate location and the area of the target of interest was marked. The selected point was punched from the original block and transferred to the TMA block with a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, U.S.A.). The tissue samples of liver, cecum, renal cell carcinoma and ovary clear cell adenocarcinoma were added to the TMA blocks as control tissues. The individual TMA blocks consisted of 19-47 different samples. (I-III)

Table 6. Clinical tissue material used in original publications

Tissue material	Number of samples	Mean age (years)	Age range (years)
Normal skin	9	62	41-84
SK	39	66	26-95
AK	64	78	58-95
cSCCIS	65	79	59-95
cSCC	83	79	45-102
RDEBSCC	33	33	12-56

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

An RNeasy Mini Kit was used to isolate the total RNA from the cultured cells. Next, cDNA was synthesized from the total RNA using 1 mg of RQ1 DNase–treated RNA, Random Primers, M-MLV Reverse Transcriptase and RNase H minus polymerase. The qRT-PCR analysis of cDNA was performed in duplicate with specific primers and fluorescent probes. The sequences of the primers and probes used are presented in the original publications (I-III). In each measurement, the range of the threshold cycle values were <5 % of the mean. The results were corrected for the levels of the reference gene (β-Actin or glyceraldehyde-3-phosphate dehydrogenase; GAPDH) mRNA in the same samples (Zhong et al, 2011).

4.5 Western blot analysis (I-III)

Equal aliquots of the cultured cell lysates and media were fractionated using 10% SDS-PAGE gel, followed by transferring the proteins to a Hybond ECL filter, (Amersham, U.K.) and labeling them with the primary antibodies listed in **Table 4**. To detect the specifically bound primary antibodies, corresponding peroxidase-conjugated secondary antibodies were used and binding was visualized by enhanced chemiluminesence (ECL) Western Blotting kit (GE Healthcare Life Sciences, Amersham, U.K.)

4.6 Immunohistochemistry (IHC) (I-III)

The FFPE sections of the whole and TMA tumors were sectioned to 5-µm-thick slices for the IHC analysis. The sections were deparaffinized, rehydrated and processed with EDTA (pH=9) buffer in a microwave oven. Specific immunostaining was performed as described in the original publications (I-III) using an avidin–biotin–peroxidase complex technique (VectaStain ABC Kit; Vector Laboratories, Burlingame, CA) (Wood & Warnke, 1981). As a negative control, sections were stained with PBS. Mayer's hematoxylin (Sigma-Aldrich Chemie, Steinheim, Germany) was used as the nuclear counterstain and diaminobenzidine as the chromogen (Kivisaari et al, 2008).

For the detection of TNF-α, the automated slide stainer was used (Lab Vision™ PT Module, Thermo Fisher Scientific Inc., Waltham, MA, USA) on study II. The slides were pretreated in the buffer (pH=9) for 2x 7min in a microwave oven and the primary TNF-α antibody was detected with the Bright Vision poly-HRP anti-rabbit IgG secondary antibody (ImmunoLogic, Duiven, The Netherlands) and the system of Liquid DAB+Substrate Chromogen (DAKO K3468, Dako, Glostrup, Denmark).

The IHC stainings were analyzed with an Olympus BX60 microscope (Olympus Optical, Tokyo, Japan), photographed digitally and examined by a cellD program (Olympus Soft Imaging System). For a semiquantitative analysis, the staining intensity was scored as negative (-), weak (+), moderate (++) and strong (+++) independently by two observers (P. Riihilä and M. Kallajoki). (I-III)

4.7 Analysis of cell proliferation (I-III)

To study cell viability, specific small interfering RNAs (siRNA) of CFH/FHL-1 (120 nM), CFI (120 nM), C3 (75 nM), CFB (75 nM) or negative control was

used for cSCC cell transfection. CFH/FHL-1 siRNA knocked down both CFH and FHL-1 genes. cSCC cells (1.0 x 10^4 cells/well; UT-SCC-59A, -118, -91, -12A and -7) were seeded on 96-well plates 24 hours after the transfection as described in the original publications. The WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim, Germany) was used to determine the number of viable cells at the time points 48 and 72 hours after the siRNA transfection.

4.8 Analysis of cell migration (I-III)

The cell migration potential of cSCC cells (UT-SCC-59A, -118, -91 and -105) was determined 48 hours after the transfection with specific CFH/FHL-1 (120 nM), CFI (120 nM), C3 (75 nM), CFB (75 nM) or the negative control siRNA. A scratch was created in the monolayer of the cells with a pipet tip. The division of the cell was inhibited with hydroxyurea (Sigma Aldrich) as described in the original publications. The migration of the cells was analyzed with an inverted microscope Olympus IX70 (Olympus Optical, Tokyo, Japan) and measured with software ImageJ (Schneider et al, 2012).

4.9 Gene expression profiling

4.9.1 Microarray-based expression profiling (I, II)

The gene expression profile of complement components in NHEKs (n=5), primary (n=5) and metastatic SCCs (n=3) was determined with Affymetrix Human Genome U133 Plus 2.0 array (Affymetrix Inc., Santa Clara, CA) at the Finnish Microarray and Sequencing Centre, Turku Center for Biotechnology. The mean signal level of NHEKs was used as a control for signal levels for different SCC cell lines. For normalizing the arrays Robust Multichip Average assay (Chipster software, CSC, Espoo, Finland) was used.

4.9.2 Whole transcriptome expression profiling (II)

The RNA expression profiling based on next-generation sequencing was used to verify the findings of the gene expression profiling of the expression of the complement components. Whole transcriptome libraries of NHEK (n=4) and cSCC cell lines (3 primary and 5 metastatic) were prepared with the SOLiD Whole Transcriptome Analysis Kit (Applied Biosystems, Foster City, CA) at the

Finnish Microarray and Sequencing Centre, Turku Center for Biotechnology according to the manufacturer's instructions. The samples were processed with the SOLiD 3Plus instrument (read length 35 bp). The colorspace reads were obtained aligning against the human reference genome (GRCh37 assembly for NHEKs and SCCs) using the standard whole transcriptome pipeline and the colorspace alignment tool (Applied Biosystems). To normalize the data, the quantile-to-quantile adjustment (R/Bioconductor package edgR) was used (Robinson et al, 2010)

4.10 Mouse skin chemical carcinogenesis (III)

Normal (n = 8), acetone-treated (n = 2), hyperplastic 12-O-tetradecanoylphorbol-13-acetate (TPA) -treated (n=6), papillomas; (n = 17) and SCC (n = 17) murine skin samples were collected from the FVB/N HanHsd mice (maintained at the Laboratory Animal Centre, University of Oulu). The carcinogenesis of the mouse skin was carried out as previously described (Brideau et al, 2007). The formation of the skin tumor was induced by the topical administration of a single dose of 7,12-dimethylbenz[α]anthracene (100 µg; DMBA, Sigma-Aldrich, St. Louis, MO, USA) in acetone (100 µl) on the shaved dorsal skin and then the skin was treated with TPA (5 µg; Sigma-Aldrich, St. Louis, MO, USA) in acetone (100 µl) once a week during a 20-week period. The appearance of the SCC tumors was monitored weekly and the mice were sacrificed at week 32 or earlier if invasive carcinomas appeared and the tumor load was excessive, or the size of the tumor exceeded 10 mm. The dorsal skin of the control mice was shaved and treated four times at 2-day intervals with TPA (5 µg) in acetone, (100 µl) to induce hyperplasia. Other control mice were treated similarly four times at 2-day intervals with acetone. Approximately 10% of the hyperplastic skin progressed to malignant SCCs in this model. The skin modifications were removed and fixed in fresh phosphate-buffered 4% paraformaldehyde for 24 hours at 4°C and paraffin-embedded. The sections were stained with HE and classified in a blinded manner as hyperplasia or cSCC.

4.11 Xenograft model (II, III)

UT-SCC-7 or -91 cells were transfected with specific CFI (II), C3 (III), CFB (III) or control siRNA. Incubation was continued for 72 hours and the cells were detached. In CFI and C3 siRNA experiment the cSCC cells ($5x10^6$) and in CFB siRNA experiment the cSCC cells ($7x10^6$) in 100 µl of PBS were injected into the back of 6-8 -week old female mice (n=8 for each experimental group) having

severe combined immunodeficiency (SCID/SCID). The size of the tumor was measured once or twice a week and calculated as π4/3((length+width)/4)3 (Leivonen et al, 2006; Watt et al, 2011). The mice were sacrificed at week 17-18 and the tumors were removed, prepared and fixed in fresh phosphate-buffered 4% paraformaldehyde at 4°C for 24 hours. After that, the tumors were embedded in paraffin and sectioned for 5-µm-thick slices (Junttila et al, 2007). The slices were immunostained as described in the original publications.

4.12 Statistical analysis (I-III)

The statistical analysis was performed with SPSS software (IBM SPSS Armonk, New York, USA). The significance between two non-normally distributed independent sample groups was analyzed with Mann–Whitney U-test or with Student's *t*-test for qRT-PCR, oligonucleotide array, the profiling of the whole transcription, Ki-67 or xenograft analysis of the size of the tumor as well as assays of proliferation and migration. The χ^2-test was selected to compare the staining intensities of IHC.

5 RESULTS

5.1 Increased expression of the complement components in the cSCC cells (I, II)

First, microarray-based whole-genome-wide expression profiling (GeneChip®) of cSCC (five primary and three metastatic) and NHEK (n=8) cell lines were performed to screen for novel biomarkers of the progression of cSCC with oligonucleotide array-based device. The analysis revealed that specific complement system components were clearly up-regulated in the cSCC cells compared with the NHEK cell lines. The mRNA expression level of most of the complement components was low in NHEKs, except for C1qBP and C3. The expression of C1r, C1rl, C1s, C3, CFB, FHL-1, CFH/CFHR1, and CFI was up-regulated in the cSCC cell lines. (I, Figure 1a; II, Figure 1a).

The expression of the complement components and inhibitors was also analyzed with the next generation RNA sequencing based whole transcriptome expression profiling (SOLiD). The findings with this method verified the results of microarray-based profiling. The complement component and inhibitor (C1rl, C1r, C1s, C3, CFB, CFH and CFI) mRNA levels were found to be elevated in the cSCC cell lines as compared with NHEKs. (II, Figure 1b)

The analysis revealed that the mRNA expression of the alternative pathway components (CFB and C3) and inhibitors (CFH, FHL-1 and CFI) was up-regulated in the cSCC cell lines and those genes were chosen for further investigation.

5.2 Overexpression of inhibitors CFH, FHL-1 and CFI and components CFB and C3 at the mRNA and protein level in cSCCs (I-III)

The results of the gene microarray and the expression profiling based on RNA sequencing were verified with qRT-PCR. The mean levels of the mRNA expression of CFH, FHL-1 (I, Figure 1b), and CFI (II, Figure 1b) and also C3 and CFB (III, Figure 1a) were significantly up-regulated in the cSCC cells (n=8) compared with NHEKs (n=10). In addition, the expression level of CFB mRNA was mainly higher in the primary cSCC cell lines compared with the metastatic

cell lines. In the cSCC tumors (n=6) the mean mRNA expression levels of CFH (I, Figure 1d), FHL-1 (I, Figure 1d), C3 (III, Figure 1a) and CFB (III, Figure 1a) were significantly higher than in the normal skin (n=11). In addition, to investigate the activation of the alternative pathway, the mRNA expression of CFD was verified by qRT-PCR. The mRNA expression of CFD was noted in the cSCC cells and tumors. (III, Figure 1d)

The expression analysis of the inhibitors CFH, FHL-1 and CFI and also the complement components C3 and CFB was continued by the detection of the protein levels with Western blotting. Specific bands for CFH (155 kDa) and FHL-1 (45 kDa) were noted in the conditioned media of the cSCC cell lines, but not of NHEKs (I, Figure 1c). The expression of CFI (88 kDa) was noted in the cSCC cell lysates most abundantly under non-reducing conditions (II, Figure 1d). Similar results were seen with C3 and CFB. Under non-reducing conditions, C3 (185 kDa) was detected in the total cell lysates and CFB (86 kDa) in the conditioned media of the cSCC cell lines (III, Figure 1b) and NHEKs. β-Actin was used as a loading control for all molecules.

The function of CFH, FHL-1 and CFI in the inactivation of the alternative pathway by promoting the proteolytic cleavage of C3 to C3b was analyzed at the protein level by determining the C3 cleavage products by Western blotting. The inactivation of C3b is a stepwise cascade in which smaller cleavage fragments are formed. The cleavage products of C3 (C3α'43 and C3β'+α'67) were detected in the conditioned media of UT-SCC-7, UT-SCC-59A and UT-SCC-91 cell cultures. (I, Figure 4b)

5.3 Overexpression of CFH, FHL-1, CFI, C3 and CFB in cSCCs *in vivo* (I-III)

The TMA sections were analyzed by IHC to investigate the expression of CFH, FHL-1, CFI, C3 and CFB *in vivo* during the progression of cSCC from AK to cSCCIS and finally to an invasive cSCC. As a control, normal skin (I-III) and benign epidermal papillomas *i.e.* SKs (II) were used. To broaden the view of cSCC, tissue samples of an aggressive form of cSCC, RDEBSCCs were used (II, III).

5.3.1 Tumor cell -specific overexpression of CFH and FHL-1 in cSCC tumors in vivo (I)

The antibody used for CFH recognized both CFH and FHL-1. The intensity of the IHC staining for CFH and FHL-1 was stronger in the sporadic cSCC tumors (n=65) than in cSCCIS (n=38) or AKs (n=37). There was no difference in the intensity of the staining between the inflamed and noninflamed cSCCs. In the AK and cSCCIS sections, the intensity of immunostaining for CFH and FHL-1 was in general weaker. The semiquantitative analysis of the immunostainings revealed that the proportion of the tissue samples with strong staining was significantly higher in the cSCC tumors than in cSCCISs and AKs (P=0.035) (I, Figure 2).

5.3.2 Tumor cell -specific overexpression of CFI in the cSCC tumors in vivo (II)

Marked tumor cell -specific IHC staining of CFI in the sporadic cSCCs (n=83) and RDEBSCCs (n=7) was noted. The expression of CFI was generally weaker in cSCCIS (n=65) and AK (n=64). In the normal skin and SK samples the intensity of staining was mainly negative or weakly positive. The semiquantitative analysis showed that the percentage of the tumors with a strong staining was significantly higher in cSCCs and RDEBSCCs than in the normal skin, SK, AK, or cSCCIS sections (for all $P<0.001$) (II, Figure 2)

5.3.3 Tumor cell -specific overexpression of C3 in cSCCs in vivo (III)

In the C3 IHC stainings, the results showed remarkable tumor cell -specific staining intensity in cSCCs (n=68). The staining was either cytoplasmic or on the surface of the cell. The staining intensity of C3 was strong in RDEBSCCs (n=11). The staining for C3 in cSCCIS (n= 59) and AK (n=63) tissue samples was clearly weaker than in cSCCs. (III, Figure 2) The semiquantitative analysis revealed that strong or moderate staining was detected in most tumor samples in the cSCC and RDEBSCC groups. On the other hand, in the AK and cSCCIS groups, the proportion of negative and weak staining was significantly higher than in the cSCC or RDEBSCC samples. Interestingly, also in the group of the normal skin (n=5), samples were negative or moderately stained. As a result, a correlation between the cSCC progression and the expression of C3 *in vivo* was seen. In RDEBSCC the expression of C3 was the most intense. (III, Figure 2)

5.3.4 Tumor cell -specific overexpression of CFB in cSCC tumors in vivo (III)

The staining of CFB in the cSCC (n=71) tumor cells was specifically seen in the tumor cell cytoplasm and was classified mainly as moderate or strong in the semiquantitative analysis. As for C3, also for CFB the staining intensity was clearly stronger in RDEBSCC (n=10). In AK (n=56) and cSCCIS (n=69) the staining was negative or weak in the majority of the samples. In the normal skin (n=5), the staining was negative in most cases. No clear difference was detected between the inflamed and non-inflamed regions. In the semiquantitative IHC analysis, the staining intensity was stronger in the cSCC and RDEBSCC groups than in AK and cSCCIS or the normal skin groups. As a total, when the clinical diagnosis progressed towards an invasive and aggressive cSCC, the staining intensity for CFB increased. (III, Figure 3)

5.4 Expression of CFH, FHL-1, CFI, C3 and CFB in the Ha-*ras* -transformed tumorigenic HaCaT cells (I-III)

To gain a further insight into the significance of CFH, FHL-1, CFI, C3 and CFB in the epidermal carcinogenesis, the expression of CFH, FHL-1, CFI, C3 and CFB mRNA was studied in the immortalized non-tumorigenic keratinocyte-derived cell line (HaCaT) lacking a functional p53, and in three of the Ha-*ras* -transformed tumorigenic HaCaT-derived cell lines (A5, II-4 and RT3) (Mueller et al, 2001).

The mRNA levels of CFH and FHL-1 were low in the parental HaCaT cells, whereas in Ha-*ras* -transformed HaCaT cell lines, the expression of both CFH and FHL-1 mRNAs was abundant. Accordingly, at the protein level the expression of CFH and FHL-1 was clearly up-regulated in the Ha-*ras* -transformed HaCaT cells. (I, Figure 3) On the contrary, the expression of the other inhibitor, CFI, at the mRNA and protein level was low in HaCaT, A5, and II-4 cells and the expression was higher in the most aggressive *ras*-transformed tumorigenic HaCaT-derived cell line, RT3. (II, Figure 3a, b)

The production of C3 and CFB was higher in the HaCaT cells and in all the Ha-*ras* -transformed cell lines compared to NHEK-PC at the protein level. Furthermore, there was a correlation between the aggressiveness of the cell line and the expression level of C3 and CFB. Similarly, at the mRNA level the expression of C3 and CFB was low in the HaCaT and A5 cell line whereas the expression of both C3 and CFB was markedly up-regulated in the metastatic RT3 cell line. The cleavage product of C3, C3b (175 kDa), was also seen in the media

of the RT3 cell line, and to some extent in II-4 cell line as a sign of C3 activation. (III, Figure 5a, c) As shown in the previous experiments, the C3 inhibitors CFH and CFI are expressed by cSCC cells. The function of these inhibitors is seen by the presence of the C3 cleavage products (iC3b, 182 kDa and C3c, 142 kDa) in the RT3 cell line (III, Figure 5a).

5.5 C3 and CFB expression in chemically induced mouse cSCC (III)

To broaden the view of the C3 and CFB role in the cSCC progression, the mouse model of the chemically induced cSCC was used (Brideau et al, 2007; Farshchian et al, 2011). The RNA samples from normal skin (n=8), acetone-treated skin (n=2), TPA-treated hyperplastic skin (n=6), papilloma (n= 17) and DMPA-TPA -induced cSCC (n= 27) were used to analyze the expression of the two variants of the mouse CFB; variant 1 and 2, and C3. (III, Figure 4a-c) In the mouse cSCCs the expression varied and the variation was much broader than in the normal mouse skin. There was also a trend that the expression was higher in cSCCs than in the other groups. Interestingly, in the comparison between benign papillomas and cSCCs, the expression values of C3, CFB variant 1 and variant 2, gained statistical difference ($P=0.002$, $P<0.0001$ and $P<0.0001$, respectively). (III, Figure 4c)

5.6 Regulation of the expression of complement components by inflammatory cytokines (I-III)

The expression studies of CFH, FHL-1, CFI, C3 and CFB in cSCC were followed by regulation experiments. The regulation was studied in cSCC, HaCaT and Ha-*ras* -transformed HaCaT cell lines.

The production of CFH and FHL-1 was up-regulated most potently by IFN-γ, IL-1β and TGF-α and less potently by TNF-α and TGF-β1 at the protein level in the metastatic cell line UT-SCC-7 (I, Figure 4a). The expression of CFI mRNA was up-regulated by IFN-γ and IL-1β in the primary UT-SCC-118 and UT-SCC-12A cell cultures (II, Figure 3d). The regulation of CFI was also examined in HaCaT and the Ha-*ras* -transformed HaCaT cell lines at the mRNA level. IFN-γ and IL-1β also up-regulated the low basal expression level in HaCaT cells, A5 and II-4 cells. When the basal expression level of CFI mRNA was high, as in the RT3 cells, the expression level was not further up-regulated, but was down-regulated by TGF-β1. (II, Figure 3c)

To examine the role of cytokines and growth factors on the regulation of C3 and CFB expression, HaCaT and Ha-*ras* -transformed HaCaT cell lines and primary cSCC cell line were treated with IFN-γ or TNF-α. In the primary cSCC cell line UT-SCC-105, the mRNA expression of C3 was up-regulated by IFN-γ (III, Figure 5b). The low expression level of C3 in HaCaT and A5 cell line was up-regulated by IFN-γ and TNF-α at the mRNA level. In the II-4 cell line the expression of C3 was up-regulated only by TNF-α, and in the RT3 cell lines no up-regulation was detected. (III, Figure 5c) As a result, when the expression level of C3 was high at the basal level, there was no up-regulation by any of the cytokines. In the primary cSCC cell line UT-SCC-118, HaCaT, A5 and II-4 cells, the mRNA expression of CFB was up-regulated by IFN-γ and TNF-α. In contrast, in the RT3 cells the high basal mRNA expression of CFB was up-regulated only by IFN-γ. (III, Figure 5b, c)

5.7 Extracellular signal–regulated kinase (ERK) 1/2 and p38 MAPK regulate CFH and FHL-1 expression (I)

The regulation of CFH and FHL-1 by ERK1/2 and p38 MAPK signaling was studied using specific inhibitors. The primary cSCC cell line UT-SCC-118 was treated with the inhibitor of MEK1/2 mitogen-activated protein kinases (MAPK/ERK kinase ½) PD98059, or the inhibitor of p38α and p38β mitogen-activated protein kinases (SB203580) or the inhibitor of all p38 isoforms α, β, γ and δ (BIRB796) (Johansson et al, 2000; Junttila et al, 2007; Siljamäki et al, 2014). The basal levels CFH and FHL-1 mRNA were potently down-regulated by PD98059 as compared to the untreated control cells. Moreover, SB203580 and BIRB796 inhibited the CFH and FHL-1 expression. (I, Figure 4c) The finding indicates that ERK1/2 and p38 MAPKs have an important role in regulating the basal CFH and FHL-1 expression in cSCC.

5.8 Knockdown of CFH, CFI and CFB inhibit cSCC cell proliferation (I-III)

The functional role of CFH, FHL-1, CFI, C3 and CFB was elucidated in the cSCC cells using siRNAs for knocking down the target genes (I, Figure 5a; II, Figure 4a; III, Figure 6a, b). A significant reduction in the number of viable cells was detected at the time points 48 and 72 hours after the CFH/FHL-1 (I, Figure 5b) or CFI (II, Figure 4b) siRNA transfection as compared with the control siRNA-transfected cultures. The knockdown of the CFB expression decreased the number of viable cells at the 72-hour time point after the siRNA transfection.

The knockdown of C3 had no effect on the cell viability. (III, Figure 6c). The inhibition of the cell proliferation was associated with a potent inhibition of the ERK1/2 activation. (III, Figure 6d)

5.9 Knockdown of CFH, CFI, C3 and CFB inhibit cSCC cell migration (I-III)

The functional studies were continued and the role of CFH, FHL-1, CFI, C3 and CFB was studied in the migration with the specific siRNAs. The migration of the cSCC cells was significantly inhibited in CFH/FHL-1 (I, Figure 5d) or C3 and CFB (III, Figure 6f, g) siRNA-transfected cultures at the time point 24 hours after the wounding. In addition, the knockdown of CFI (II, Figure 4d) inhibited significantly the migration of the cSCC cells at the time points 16 hours and 24 hours after the wounding.

5.10 CFI, C3 and CFB promote the growth of the cSCC xenograft tumors *in vivo* (II, III)

The role of CFI, C3 or CFB in the growth of cSCC was examined *in vivo* in a xenograft model. For CFI and C3, metastatic cSCC cell line (UT-SCC-7) and for CFB, primary cSCC cell line (UT-SCC-91) were cultured and transfected with CFI, C3 or CFB and the control siRNA in parallel. The transfected cells were incubated for 72 h and injected ($5x10^6$ or $7x10^6$) subcutaneously into the back of the SCID/SCID mice. The size of the tumors was measured once or twice a week. Subsequently, the tumors were collected after 17-18 days for a histological analysis and IHC, as previously described (Junttila et al, 2007). The function of siRNAs used was measured both at the mRNA and protein level. For all the specific siRNAs, an efficient gene knockdown was accomplished. The knockdown effect of the CFI siRNA at the mRNA level was analyzed also at longer time points, *i.e.* after 6, 10 and 15 days. The knockdown effect was noted remaining up to 15 days in the siRNA-transfected UT-SCC-7 cell culture.

First, the expression of CFI, C3 or CFB was verified in the cSCC cells *in vivo* with IHC in the xenograft tumors. The expression of CFI was detected specifically in the cSCC tumor cells which were partially differentiated. (II, Figure 1e). The expression of C3 and CFB was also noted in the cSCC cells. (III, Figure 2h and 3h, respectively)

As a result, the growth of the cSCC xenograft tumors with the CFI, C3 or CFB knockdown was significantly reduced at all time points as compared to the

control siRNA tumors (II, Figure 5 a; III, Figure 7a and 8a, respectively). Moreover, a histological analysis of the xenografts revealed that the CFI and C3 knockdown tumors were less cellular than the control tumors (II, Figure 5b and III, Figure 6b accordingly). In addition, the analysis of the Ki-67 staining revealed that the CFI, C3 and CFB knockdown had an effect on the proliferation. Significantly more proliferating cells were seen in the tumor edge in the control tumors compared to the CFI, C3 and CFB knockdown cSCC xenograft tumors (II, Figure 5b, c; III, Figure 7b, c; and Figure 8b, c, respectively). The histological analysis of the CFB knockdown tumors revealed that the morphology of the CFB knockdown tumors was different from the control tumors. The tumor cells appeared smaller and they were arranged in more dense groups in the CFB knockdown tumors. (III, Figure 8b)

Furthermore, the effect of the CFI knockdown on complement activation was examined. The cSCC xenografts were stained with IHC with an antibody against C3/C3b. A positive staining for C3b was detected on the surface of the tumor cells in the invasive edge of the xenografts in cell clusters. Additionally, the number of positive cells and the staining intensity was stronger in the CFI knockdown tumors, as a marker for C3b accumulation providing further evidence for complement activation. Moreover, the IHC staining with an antibody against the proinflammatory cytokine, TNF-α, showed more positive cells and stronger staining intensity in the CFI siRNA tumors as compared with the xenografts of the control siRNA indicating complement activation and increased inflammation in the CFI knockdown tumors. (II, Figure 5b)

6 DISCUSSION

6.1 Molecular markers of the cSCC carcinogenesis

UV-radiation is the main risk factor for cSCC, and it also causes immunosuppression. The level of immunosuppression is in correlation with the development of cSCC in a dose-dependent manner. In immunosuppressed patients, such as organ transplant recipients, the incidence of cSCC is increased up to 65-fold. (Alam & Ratner, 2001; Yu et al, 2014) Chronic inflammation is one trigger for carcinogenesis. On the other hand, inflammation is an important part of cancer progression. For example, the up-regulation of the inflammatory cytokine TNF-α in the ovarian and renal cell cancer cells is associated with a poor prognosis. The up-regulation in the cancer microenvironment may be a result of local hypoxia. (Balkwill & Mantovani, 2001; Mantovani et al, 2008) Moreover, TNF-α has an important role in the early stages of skin carcinogenesis. The TNF-α -deficient mice treated with DMBA-TPA to induce carcinogenesis were more resistant to the development of a benign and malignant skin tumor. (Moore et al, 1999) A sign of cancer-related inflammation is the presence of inflammatory mediators and inflammatory cells in the tumor microenvironment. (Balkwill & Mantovani, 2001; Mantovani et al, 2008) The complement system is a central part of the innate immune system and it serves as a first line of defense against non-self cells. The complement system also has a role in the cell-mediated cytotoxicity and the disposal of apoptotic cells. Complement is activated *via* the classical, lectin or alternative pathways (**Figure 2**). In the alternative pathway, continuous spontaneous activation of complement takes place. That makes the alternative pathway very important. As a result, the complement cascade activation has to undergo a strict control to avoid damage to the host cells. (Zipfel & Skerka, 2009) Interestingly, the complement system activation, especially the expression of C3 and CFB, has been shown to be in association with UVA radiation and may lead to systemic immunosuppression (Stapelberg et al, 2009).

Although cSCC is, in most cases, curable by a local excision, a portion of the tumors recur and metastasize with a high possibility of mortality. For that reason, early detection and excision of cSCC are lifesaving for the patients. The cSCCs developed in the sites of chronic infection or ulcers tend to be more aggressive than the UV-induced cSCCs, and they also metastasize more often (Tufaro et al, 2011). Small lesions recur at a rate of 8% and large lesions at a rate of 15%

within a 5-year period. The recurred lesions metastasize in 25-45% of the cases. The rate of metastases depends on the location of the tumor. (Alam & Ratner, 2001) For now, only a few specific cSCC biomarkers have been identified, such as STAT3 (Suiqing et al, 2005), E-cadherin (Koseki et al, 1999), SerpinA1 (Farshchian et al, 2011) and matrix metalloproteinase (MMP)-13 (Airola et al, 1997), MMP-12 (Kerkelä et al, 2000), and MMP-7 (Impola et al, 2005; Kivisaari et al, 2008). However, there are no biological markers in clinical use which could predict the aggressive behavior of the tumor and its precancerous forms.

In this study, the aim was to identify and characterize new molecular biomarkers for the cSCC progression. Here, the focus was on the complement inhibitors CFH, FHL-1 and CFI and also the complement components of the alternative pathway, C3 and CFB. The role and expression of complement components in cSCC have not been studied before.

6.2 Expression of CFH, FHL-1, CFI, C3 and CFB in normal tissues

CFH and its splicing variant FHL-1 are the most important soluble inhibitors of the alternative pathway. They promote the proteolytic inactivation of C3b and inhibit the activity of C3bBb. CFI is another important inhibitor of the alternative pathway, but it also inhibits the classical and lectin pathways. (Zipfel & Skerka, 1994) CFI is a serine proteinase which requires a cofactor to function properly. CFI and the cofactors together cleave the α'-chain of the activated C3b (Nilsson et al, 2009) and the α'-chain of C4b (Nagasawa & Stroud, 1977). CFH and CFI are mainly synthesized in the liver, but they are also produced by other tissues and cells. (Ricklin et al, 2010; Sim & Tsiftsoglou, 2004) In the previous studies, normal epidermal keratinocytes have been shown to express CFH and CFI (Timar et al, 2007b; Timar et al, 2006).

CFB is a component of the alternative pathway and it plays an important role in C3 activation. CFB is cleaved proteolytically to Ba and Bb. Bb will, in turn, cleave C3, a key component in the complement activation, to C3b and C3a. Certain proteolytic enzymes also cleave C3 such as monomeric β-tryptase (Fukuoka et al, 2008), cathepsin G (Maison et al, 1991) and granzyme B (Perl et al, 2012; Strik et al, 2007) produced by human mast cells. Similarly to CFH and CFI, C3 and CFB are mainly synthesized in the liver, but also other tissues and cells can produce C3 and CFB. For example human skin mast cells produce C3 (Fukuoka et al, 2013). C3 is synthesized as a single-chain precursor (185 kDa) composed of two polypeptide chains (α-chain; 115 kDa and β-chain; 75kDa) connected with two disulfide bonds. Similarly, CFB is a single-chain polypeptide (93 kDa). (Gros et al, 2008) Normal keratinocytes have been shown to express

C3 and CFB (Terui et al, 1997) and also produce CFB protein (Yancey et al, 1992)

6.3 CFH, FHL-1, CFI, C3 and CFB in cancer

Complement activation has been detected in cancer patients' plasma, and it has been considered a host protection mechanism against cancer (Pio et al, 2014). The activation of complement has also been detected in the microenvironment of tumors (Gorter & Meri, 1999). Complement components are known to have other functions besides being part of the complement pathways. Interestingly, complement components can promote initiation and growth of tumors in the environment of chronic inflammation. Inflammation promotes the proliferation and survival of cancer cells, increases metastasis and promotes angiogenesis, and it also reduces response to the chemotherapeutic agents. (Mantovani et al, 2008)

CFH, FHL-1 and CFI mainly inhibit complement activation, but they also have other functions which may be related to carcinogenesis. The mechanistic role of CFH and CFI in tumorigenesis is not fully known, but previous studies have revealed certain tumorigenic properties of CFH and CFI. CFH binds to osteopontin and bone sialoprotein, which can sequester CFH to the cell surface to inactivate the alternative pathway (Fedarko et al, 2004). Furthermore, CFH interacts with three small integrin-binding ligand N-linked glycoproteins (SIBLINGs), namely osteopontin, bone sialoprotein, and dentin matrix protein and thus regulates the activation of MMP-2 and 3 (Fedarko et al, 2004). FHL-1 also promotes cell adhesion (Hellwage et al, 1997; Zipfel & Skerka, 1999) and thereby may, have a specific role in the invasion of cancer cells. Interestingly, the concentration of FHL-1 is lower in the serum of healthy individuals than the concentration of CFH whereas the expression of the FHL-1 gene has been shown to be higher than that of the gene CFH in ovarian cancers and glioblastomas. (Junnikkala et al, 2002; Junnikkala et al, 2000). Furthermore, an inactivation of CFI has been shown to lead to the inhibition of tumor cell mitosis. The exact mechanism is not known, but it may involve a generation of a mitogenic fragment from C3 by CFH and CFI. (Větvicka et al, 1993)

For example, the complement system also has an indirect effect on tumor growth, e.g. by altering the host' immune response, by generating growth factors. (Corrales et al, 2012; Gunn et al, 2012; Rutkowski et al, 2010b) For example, C3 has a role in angiogenesis, extracellular matrix remodeling, migration, invasion and extracellular disintegration in different types of cancers. CFB has a role in extracellular matrix disintegration, migration and invasion. Consequently, the complement activation can be one part in the enhancement of tumorigenesis.

(Hanahan & Weinberg, 2000; Rutkowski et al, 2010a) Moreover, in a mouse model, the C3 inactivation by MMP-1 protected tumor cells from the cytotoxic effects of complement activation *in vivo* (Rozanov et al, 2006). C3 also enhances the production of VEGF in the laser-induced choroidal neovascularization (Hanahan & Weinberg, 2000; Rutkowski et al, 2010a). To sum up, the complement system participates in the cell-cell communication and has a role in the regeneration and angiogenesis of organs.

The expression of complement inhibitors and components in cancer cells has been documented in a great number of studies (Baatrup et al, 1994; Bjørge et al, 2005; Niculescu et al, 1992). The expression of CFH and FHL-1 has been detected in the cancer of colon (Wilczek et al, 2008), lung (Ajona et al, 2004; Ajona et al, 2007), ovary (Junnikkala et al, 2002), bladder (Cheng et al, 2005) and glioblastoma (Junnikkala et al, 2000). The expression of CFI has been shown in quite similar types of cancers as CFH, such as in glioma (Gasque et al, 1992), non-small-cell lung cancer (Okroj et al, 2008), and rhabdomyosarcoma (Legoedec et al, 1995). IL-6 has also been shown to up-regulate the expression of CFI in hepatocellular carcinoma cells (Minta et al, 1998).

The expression of C3 and CFB has been noted in glioma cells able to produce their own growth factors and eventually this will result in a continuous autocrine stimulation of cell growth (Gasque et al, 1992; Hanahan & Weinberg, 2000). The expression of C3 and CFB has also been documented in rhabdomyosarcoma (Legoedec et al, 1995) and the expression of CFB has been found in pancreatic ductal adenocarcinoma (Lee et al, 2014). Interestingly, the production of C3 was in correlation with a better prognosis in non-small cell lung carcinoma (Liu et al, 2007). In contrast, the expression of C3 in ovarian cancer was associated with a poor prognosis, suggesting that the tumor-derived complement proteins promote tumor growth (Cho et al, 2014).

6.4 CFH, FHL-1, CFI, C3 and CFB in the cSCC cells in culture

The first two of the different types of gene expression profiling of the cSCC and NHEK cells were performed. Microarray-based expression profiling and whole transcriptome expression profiling revealed differences in the profiles of the complement components and inhibitors between NHEKs and the cSCC cell lines. The results obtained with the two different types of methods were similar showing that certain complement components and inhibitors were up-regulated in the cSCC cell lines compared to NHEKs. The genes which were up-regulated in the microarray-based expression profiling*, i.e. C1r, C1s, C3, CFB, CFH* and *CFI* were also up-regulated in whole transcriptome expression profiling. For the gene

C1rl, a significant difference was detected between the cSCC cells and NHEKs in the microarray-based expression profiling, but the difference was not as obvious in the whole transcriptome expression profiling. Certain differences between the methods may be explained by the different techniques used in the gene detection. In the microarray-based expression profiling the probe set recognizes different sequences which can be nonspecific or specific for a lot of different genes, whereas in the whole transcriptome expression profiling the specific target gene sequences are recognized. By using the gene expression profiling with two different types of methods, the results are more reliable than using only one method.

The next step was to analyze the mRNA levels in NHEK and the cSCC cells by qRT-PCR. This method verified the results obtained with expression profiling that CFH, FHL-1, CFI, C3 and CFB were up-regulated in cSCCs, as compared with NHEKs. The variation of the mRNA expression in cSCC was wider than in NHEKs with all the components and inhibitors studied, but the mean expression levels of all target molecules were higher in cSCC than in NHEKs. All the samples were run in duplicate and some of the assays were repeated to verify the reliability of the results.

The enhanced production of CFH, FHL-1, CFI, C3 and CFB was also detected by Western immunoblotting at the protein level in the cSCC cell cultures in general. The findings suggest that the cSCC cells promote inflammation in their own microenvironment by producing CFH, FHL-1, CFI, C3 and CFB. The hypothesis is that the inhibitors can protect the tumor cells from the cell killing mediated by complement. The complement components could also have other cancer promoting functions. For example, CFI and the activated CFB (Bb) are serine proteinases and C3a serves as an anaphylatoxin.

A comparison of the primary and metastatic cell lines revealed no marked differences in the mRNA expression levels of CFH, FHL-1 or C3 between the primary and metastatic cSCC cell lines. However, the expression level of CFI in the metastatic cSCC cell lines was higher than in primary cSCC cell lines, whereas the expression level of C3 in the primary cSCC cell lines was, in general, higher than in the metastatic cSCC cell lines. The results suggest that CFI can have a role in the metastatic cSCCs whereas the role of C3, most probably, is more important in the primary cSCCs. The metastatic cSCC cell line material was small (n=3). Thus it would be important to expand the cell line material to verify the results achieved and make more definitive conclusions. In addition, the cell population in culture may be different from that in tumors *in vivo* and may not present the tumor cell population as a whole.

6.5 CFH, FHL-1, CFI, C3 and CFB in cSCC cells *in vivo* and in Ha-*ras* -transformed HaCaT cells

The tissue material was used to broaden the findings *in vivo*. For that purpose, a large panel of tissue sections presenting the whole range of cSCC progression was collected and used to generate the TMA blocks allowing the staining of a number of sections under the same conditions and at the same time (Kononen et al, 1998).

The expression of CFH and FHL-1 was especially noted in the tumor cells. In addition, in AKs and cSCCISs the staining was classified as moderate or strong in a portion of cases indicating that CFH and FHL-1 have a role in the early cSCC progression. The CFI expression was also seen specifically in the cSCC cells and the staining was even stronger in the aggressive form of cSCC, the RDEBSCC, confirming the observation that the CFI mRNA expression is higher in the metastatic cSCC cell line. All samples were positively stained for CFI in the cSCC and RDEBSCC groups revealing that the CFI expression was present in every tumor studied. The staining intensity was stronger in the cSCC than in the AK or cSCCIS sections, unlike in stainings of CFH and FHL-1. The portion of moderate and strong staining for CFH, FHL-1 and CFI is summarized in **Table 7**.

An analysis of the C3 and CFB expression *in vivo* in IHC revealed specific staining for C3 and CFB in the cSCC cell surface and cytoplasm. As for the CFI stainings, the staining for C3 and CFB in the cSCCs and RDEBSCCs was mainly strong and all the samples were stained positive. In the RDEBSCC samples, even stronger staining for C3 and CFB was noted than in cSCCs. This is, to some extent, in conflict with the cell line results, where the expression of C3 was lower in the metastatic cell line. However, the number of the metastatic cell lines was low and the results obtained with the cell lines are not fully comparable to the *in vivo* results. On the other hand, C3 is normally circulating in the plasma. The blood vessels in the skin localize in the dermis and that has an influence on some results of the positive staining of the expression of C3. In the samples of AK and cSCCIS, positive staining for C3 and CFB was also detected, but it was weaker than in the cSCC tumors. In addition, the subpopulation of normal skin, AK and cSCCIS was not stained positively for C3 and CFB. The portion of moderate and strong staining for C3 and CFB is summarized in **Table 7**. Interestingly, when non-small-cell lung cancer cells were C3 positive it was in correlation with a better prognosis (Lin et al, 2014). A question is whether the C3 expression in the cSCC tumors can be in correlation with a better prognosis and whether C3 can be used as a biological marker of a better prognosis? This could be studied with the follow-up and survival data of the patients whose cSCC samples were used.

Table 7. The portion of moderate and strong staining for CFH, FHL-1, CFI, C3 and CFB in normal skin, AK, SK, cSCCIS, cSCC and REDBSCC

Antigen	Tissue	The portion of moderate and strong staining			
		0-25%	26-50%	51-75%	76-100%
CFH and FHL-1	AK		x		
	cSCCIS		x		
	cSCC			x	
CFI	Normal skin	x			
	SK	x			
	AK		x		
	cSCCIS		x		
	cSCC			x	
	RDEBSCC			x	
C3	Normal skin			x	
	AK and cSCCIS			x	
	cSCC				x
	RDEBSCC				x
CFB	Normal skin	x			
	AK and cSCCIS		x		
	cSCC			x	
	RDEBSCC				x

To further explore the role of CFH, FHL-1, CFI, C3 and CFB in the cSCC tumorigenesis, different Ha-*ras* -transformed HaCaT cell lines were examined (Mueller et al, 2001). The results verified that the expression of CFI, was high in the most aggressive form of the Ha-*ras* -transformed HaCaT cell line, RT3. The basal expression level of C3 and CFB was low in the parental HaCaT cell line and in the benign tumorigenic, A5 Ha-*ras* -transformed cell line. The results indicate that an early event in cSCC carcinogenesis, p53 inactivation and also activation of *ras*-signaling are not sufficient for the induction of the expression of CFI, C3 and CFB, but other genomic alterations are required, too. On the other hand, the CFH and FHL-1 expression was low in the parental HaCaT cells and the expression of CFH and FHL-1 was high in the *ras*-transformed benign A5 cell line. The result obtained was in accordance with the *in vivo* data of the CFH and FHL-1 expression. p53 mutation is an early event of cSCC tumor progression and is seen in AK. Ha-*ras* -mutation is detected in 21% of the cSCC cases (Kraft & Granter, 2014). For example, *in vivo* -expression of CFH in the normal skin was, in general, negative and in AK, the staining for CFH was weaker than in cSCC. In summary, CFH, FHL-1, CFI, C3 and CFB seem to have distinct roles in the cSCC tumorigenesis and different genetic alterations are required for the induction of CFH, FHL-1, CFI, C3 and CFB expression.

For C3 and CFB, the model of carcinogenesis was extended to the the use of the DMBA-TPA -induced mouse-skin SCC model. In this model, there is a mutation in the p53 gene and an activating mutation in the H-ras1 gene (Abel et al, 2009). There was a trend towards higher expression levels of C3 and CFB in cSCC as compared to benign papillomas and towards the expression level of CFB in cSCC compared to the normal or TPA-treated skin. As a summary, the results provide further evidence for C3 and CFB as biomarkers for cSCC.

In the conclusion of the *in vivo* data, the hypothesis is that the cSCC cells can protect themselves from complement-mediated lysis by producing CFH and CFI, the two main soluble inhibitors of the complement system. Moreover, the cSCC tumor cells produce functionally active CFH and FHL-1 in culture and they are also expressed *in vivo*. Based on the findings, CFH, CFI, C3 or CFB can be proposed as a biological marker for the progression of cSCC. The most promising results are noted with CFI and CFB, whereas the results indicate that C3 cannot be regarded as a potent specific marker for cSCC alone. The clinical follow-up data of the cSCC cases used would be important to obtain further knowledge about the aggressive behavior of cSCCs. All the biomarkers could be used as a combination to predict the aggressiveness of cSCCs. The combination of biomarkes may be beneficial in the prediction of an individual cSCC because there is variation in the expression levels between different patients. (Rodríguez-Enríquez et al, 2011) The biomarkers could be used at the time of histopathological diagnosis from the biopsy or tumor excision. The biomarker results could be used to determine possible adjuvant therapy after the cSCC excision in the most aggressive cases and the results could also be used for the follow-up of the patients.

6.6 Regulation of CFH, FHL-1, CFI, C3 and CFB

The regulation of the expression levels of CFH, FHL-1, CFI, C3 and CFB was studied with the growth factors and proinflammatory cytokines expressed in the cSCC microenvironment. The expression of CFH and FHL-1 was up-regulated in the Ha-*ras* -transformed HaCaT cell lines. Thus, the regulation of CFH and FHL-1 was also studied with the activation of the ERK1/2 signaling pathway. It is shown that in the cSCC cells, constitutive *ras*-signaling activation results in the permanent activation of the ERK1/2 signaling (Toriseva et al, 2012). Furthermore, p38 MAPK signaling has been shown to play a role in the growth and invasion of cSCCs (Johansson et al, 2000; Junttila et al, 2007). The results were in accordance with the previous studies. The expression of CFH and FHL-1 was down-regulated by the ERK1/2 pathway inhibitor, PD98059. In addition, the inhibition of p38 MAPK signaling down-regulated the CFH and FHL-1

expression in the cSCC cells. The results show that CFH and FHL-1 are regulated *via* ERK1/2 and p38 MAPK signaling cascades in the cSCC cells.

Certain cancers have been shown to regulate local immune responses by producing proinflammatory cytokines (Kim et al, 1995; Lee et al, 1998). cSCC invasion may be promoted by the cytokines released by inflammatory cells in the tumor microenvironment, and complement activation can stimulate the secretion of inflammatory mediators (Ricklin et al, 2010). In the present studies, the CFH expression was up-regulated by the inflammatory cytokines. The basal expression level of CFH and CFI was up-regulated by IFN-γ and IL-1β. In addition, TNF-α up-regulated the CFH expression and TGF-β1 up-regulated the CFI expression. In previous studies, IFN-γ has been shown to up-regulate the CFH and CFI expression in epidermal keratinocytes (Timar et al, 2006). Interestingly, in this study, when the basal expression level of CFI was high, *i.e*. in the RT3 cells, the expression was no further up-regulated.

The basal expression level of C3 and CFB was also up-regulated by IFN-γ and TNF-α. The C3 expression was up-regulated when the basal expression level was low. This is in accordance with the previous observations with keratinocytes where the expression of C3 is up-regulated by IFN-γ and TNF-α and the expression of CFB is up-regulated by IFN-γ (Pasch et al, 2000). In addition, the expression of CFB in macrophages is induced by both IFN-γ and TNF-α (Huang et al, 2002; Huang et al, 2001). Furthermore, CFB has been shown to function as a downstream effector of the TLR signaling and to enhance the production of TNF-α and angiogenesis (Zou et al, 2013).

6.7 Functional roles of CFH, FHL-1, CFI, C3 and CFB in cSCC

In recent years, the relationship between complement and cancer has received increased interest. Several components of the complement system have been shown to regulate tumor growth. As a summary, MAC, C3a and C5a have been shown to increase the production of the tumor-associated growth factors, enhance angiogenesis, prevent apoptosis, induce proliferation, and increase cancer-cell invasion and migration. Moreover, C5a suppresses antitumor immunity. Several components of the complement system *i.e.* C1q, C1s, CFB, C3, C5 and C9, increase cancer invasiveness and migration. The complement component C4 has also been shown to induce cell proliferation and prevent apoptosis. (Hanahan & Weinberg, 2000; Rutkowski et al, 2010b) Those results provide evidence for the functional roles of the complement inhibitors CFH and CFI, and the complement components C3 and CFB in cSCC. CFH, FHL-1, CFI, and CFB showed to regulate cell proliferation and migration in culture and therefore may have a role

in tumor growth and invasion. The proliferative effect of both inhibitors was dependent on ERK1/2 activation. C3 regulated the migration of the cSCC cells only.

The CFI knockdown had a decreasing effect on the proliferation and growth in the xenograft tumors generated in the SCID mice. A similar result was seen with C3 and CFB. Moreover, the accumulation of C3b on the cSCC tumor cells was noted when CFI was knocked down in the xenograft tumors. The accumulation of the inflammatory cytokine, TNF-α, in the CFI knockdown xenograft tumors also indicates complement activation and increased inflammation. Complement activation will also activate inflammatory responses, and TNF-α is secreted in the inflammation site. TNF-α has been known to have a role in the initiation and progression of cancer in mice (Landskron et al, 2014).

The complement system of humans and mice can interact (Cheng et al, 2006). When CFI is knocked down in the xenograft model, C3 inactivation is decreased in tumor cells. As a consequence, the human C3b is deposited onto the membranes of the cSCC cells. The complement system of mice can then be activated and, finally, the destruction of the human cSCC cells occurs. In addition, TNF-α is secreted when complement is activated and this may enhance the destruction reaction by the recruiting inflammatory cells. As a consequence, it is possible that, at least to some extent, the complement-mediated destruction is responsible for the reduced growth of the CFI siRNA xenografts. The similar effect of the complement components, C3 and CFB can be confusing, but it is likely that the tumor growth promoting effect of C3 and CFB can be totally distinct from the complement activation. For example, C3 has been shown to have a role in tumor cell proliferation, migration, invasion and angiogenesis. CFB is known to have a role in tumor cell migration and invasion. (Rutkowski et al, 2010b) The results for CFB are in accordance with the previous findings.

The C3 cleavage product, C3a, is known to activate mitogenic signaling pathways (PI3K/AKT), increase cytokines involved in tumorigenesis (TNF-α, TGF- β and IL-6) and activate the cell cycle (Cho et al, 2014). Moreover, C3a is shown to induce transient ERK phosphorylation in mast-cells by the activation of the C3 receptor, C3R, on mast-cell surfaces (Ali et al, 2000). In this study, the role of C3 in migration can be mediated *via* C3a action.

In summary, the results of this study indicate that the inhibition of the alternative pathway increases tumor cell survival and proliferation. However, it is also possible that CFI as a serine proteinase may have additional functions beyond the regulation of complement activation (Rutkowski et al, 2010a). For example, therapeutic mAbs against cancer function in two different ways, direct and indirect. In the direct function, the aim is to induce anti-proliferative or pro-

apoptotic signals or to prevent the growth factors or cytokines to bind to their receptors. The indirect effect of the therapeutic mAbs is mediated by Fc parts and they are activating, for example, the classical pathway of complement, and consequently the target cell is destroyed. (Meyer et al, 2014) Moreover, the activation of complement advances tumor therapy by mobilizing the cellular inflammatory responses and less likely causes a direct tumor cell lysis, because most of the cells express complement inhibitors on their membranes (Greenwood et al, 1993; Junghans, 1990). Nevertheless, CFH, CFI, C3 and CFB can be considered a potential therapeutic target in cSCCs therapy.

All the three complement activation pathways lead to the activation of C3. The results showed C3 expression in NHEKs but the expression level was significantly higher in cSCC cells. The finding in combination with the CFB expression in cSCC cells provides a possibility for complement activation and, therby, C3 and CFB up-regulation may promote inflammation in the cSCC microenvironment.

The functional roles of CFI, C3 or CFB in cSCC *in vivo* have not been studied previously. In this study, the functional roles of CFH and FHL-1 were not studied for the cSCC tumor growth and progression like the role of CFI, CFB and C3. The functional roles of CFH and FHL-1 could be studied next *in vivo*. The synergistic role of CFH and CFI or CFB and C3 could also be engrossing to study.

As a result, the findings of this study provide evidence that CFH and CFI allow a cSCC escape from complement-mediated destruction and thus have an important role in cSCC progression. CFH and CFI promote the proliferation and migration of the cSCC cells and, interestingly, the effect was similar with CFB. C3 promoted the migration of cSCC cells only. CFI, C3 and CFB also promoted cSCC tumor growth *in vivo,* although the effect of C3 was not as obvious as with other proteins. As a summary, the inhibitors CFH and CFI have important and similar roles together in the cSCC tumor progression. Moreover, the complement components C3 and CFB also have certain similar functions in the cSCC progression. In addition, CFI and CFB are serine proteinases, and consequently, they could also serve as targets for small molecule inhibitors.

7 SUMMARY AND CONCLUSION

In this study the expression of the complement system components was investigated in cSCCs in culture and *in vivo*. The genes which were up-regulated in cSCC cells were selected for further investigation and the role of the complement inhibitors and alternative pathway components was studied in the progression of cSCC.

The expression of complement system inhibitors CFH and its gene splicing variant FHL-1, and CFI, were up-regulated in cSCC cells compared to NHEKs at the mRNA and protein level. Interestingly, the cSCC cells also produced elevated levels the complement components C3 and CFB in cell culture. Moreover, the IHC analysis revealed that CFH/FHL-1, CFI, C3 and CFB were specifically expressed by the tumor cells in cSCC *in vivo*, and the staining intensity of CFH/FHL-1, CFI, C3 and CFB was stronger in cSCCs than in AK or cSCCIS. Moreover, the staining was stronger in the aggressive form of cSCC, in RDEBSCC than in the sporadic cSCCs.

The functional role of the five complement components was studied by the knockdown with specific siRNAs. The knockdown of CFH/FHL-1, CFI and CFB inhibited cell proliferation and this was in association with a potent inhibition of ERK1/2 activation. Furthermore, knockdown of CFH/FHL-1, CFI, CFB and C3 inhibited the migration of cSCC cells.

To obtain a deeper insight into the role of CFI, C3 and CFB in the tumor growth, the cSCC xenograft tumors established in the SCID mice were used. The CFI or CFB knockdown significantly reduced the growth of tumors. The C3 knockdown also reduced tumor growth, although the effect was not as potent as with the CFI or CFB knockdown. In addition, the CFI knockdown decreased the number of proliferating cells, increased local inflammation and induced C3b accumulation on the tumor cell surfaces as a sign of complement activation. The C3 and CFB knockdown also decreased the number of proliferating cells in the cSCC xenograft tumors.

Taken together, the results of this study provide evidence for the roles of CFH, CFI, C3 and CFB in cSCC tumor progression and, indeed, indicate that CFH, CFI, C3 and CFB may act as putative molecular biomarkers for the aggressive or rapidly progressing cSCC. In addition, they could be used as putative therapeutic targets for the recurrent and metastatic cSCC.

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