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Pegah Rahmati Nezhad

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To My Pure Love,
My Dearest,
My Kindest,
My Most Caring and Compassionate,
The Most Radiant Light of My Life:
My Mom, Forough,
Who is The Unconditional Love Herself

UNIVERSITY OF TURKU
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ABSTRACT

The most prevalent metastatic skin cancer is keratinocyte-derived cutaneous squamous cell carcinoma (cSCC), the incidence of which is growing worldwide. Although the most common mutations in cSCC have been identified, its molecular pathogenesis is yet to be completely understood. At present, there are no biomarkers or therapeutic targets for high-risk cSCCs. Inflammation is part of the cSCC microenvironment. The complement system is the backbone of innate immune defense, and regulates immune and inflammatory responses.

In this study, the role of two complement components in cSCC progression was investigated. Additionally, the complement-targeted therapeutics under clinical and preclinical trials were comprehensively reviewed. Elevation of complement factor D (FD) expression was revealed in cSCC cells and cSCC tumors. The immunohistochemistry (IHC) analysis showed more intense FD labeling in invasive margins of human cSCC xenograft tumors, and in cytoplasm of tumor cells in recessive dystrophic epidermolysis bullosa-associated SCC, cSCC metastases, and metastatic and primary cSCCs vs. cSCC *in situ*, actinic keratosis and normal skin. Targeted inhibition of FD by small-molecule FD inhibitor danicopan (ACH-4471) suppressed ERK1/2 activation and proliferation of cultured cSCC cells. Furthermore, knockdown of complement factor I (FI) downregulated MMP-2 and -13 expressions by cSCC cells *in vitro* and *in vivo*, and inhibited invasion of cSCC cells in culture. FI overexpression upregulated the expression of MMP-2 and -13, increased ERK1/2 activation, and enhanced proliferation and invasion capacity of cSCC cells *in vitro*.

In conclusion, autocrine FD and FI promote the development and progression of cSCC, and can be identified as potential biomarkers and promising therapeutic targets in invasive cSCC. Besides, for the first time, the results propose small-molecule FD inhibitor danicopan as a highly specific and sensitive drug for precision cSCC therapy.

KEYWORDS: cutaneous squamous cell carcinoma, complement factor D, complement factor I, complement-targeted therapeutics, danicopan, ACH-4471, biomarker, cancer

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen laitos
Iho- ja sukupuolitautioppi
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TIIVISTELMÄ

Yleisin metastaattinen ihosyöpä on keratinosyyteistä peräisin oleva ihon levyepiteelisyöpä (cSCC), jonka ilmaantuvuus kasvaa maailmanlaajuisesti. Vaikka cSCC:n yleisimmät mutaatiot on tunnistettu, sen molekyylipatogeneesiä ei ole vielä täysin ymmärretty. Tällä hetkellä ei ole olemassa biomarkkereita tai terapeuttisia kohteita korkean riskin cSCC:lle. Tulehdus on osa cSCC-mikroympäristöä. Komplementtijärjestelmä on synnynnäisen immuunipuolustuksen selkäranka ja säätelee immuuni- ja tulehdusvasteita.

Tässä tutkimuksessa tutkittiin kahden komplementtikomponentin roolia cSCC:n etenemisessä. Lisäksi kliinisissä ja prekliinisissä tutkimuksissa komplementtikohdistetut lääkkeet arvioitiin kattavasti. Komplementtitekijä D:n (FD) ilmentymisen kohoaminen paljastettiin cSCC-soluissa ja cSCC-kasvaimissa. Immunohistokemiallinen (IHC) analyysi osoitti voimakkaampaa FD-leimausta ihmisen cSCC-ksenograft-kasvainten invasiivisilla marginaaleilla ja kasvainsolujen sytoplasmassa resessiivisessä dystrofisessa epidermolyysissä, bullosa-assosioituneessa SCC:ssä, cSCC-etäpesäkkeissä sekä metastaattisissa ja primaarisissa cSCC:issä vs. in situ cSCC:t, aktiiniset keratoosit keratoosit ja normaali iho. FD:n kohdennettu esto pienimolekyylisellä FD-estäjän danikopaanilla (ACH-4471) tukahdutti ERK1/2-aktivaation ja viljeltyjen cSCC-solujen lisääntymisen. Lisäksi komplementtitekijä I:n (FI) hiljentäminen vähensi cSCC-solujen MMP-2:n ja -13:n ilmentymistä in vitro ja in vivo, ja esti cSCC-solujen invaasiota viljelmässä. FI:n yliekspressio lisäsi MMP-2:n ja -13:n ilmentymistä, lisäsi ERK1/2-aktivaatiota ja tehosti cSCC-solujen proliferaatio- ja invaasiokapasiteettia in vitro.

Yhteenvetona voidaan todeta, että autokriininen FD ja FI edistävät cSCC:n kehittymistä ja etenemistä, ja ne voidaan tunnistaa mahdollisiksi biomarkkereiksi ja lupaaviksi terapeuttisiksi kohteiksi invasiivisessa cSCC:ssä. Lisäksi ensimmäistä kertaa tulokset ehdottavat pienimolekyylistä FD-estäjää danikopaania erittäin spesifiseksi ja herkäksi lääkkeeksi täsmälliseen cSCC-hoitoon.

AVAINSANAT: ihon okasolusyöpä, komplementtitekijä D, komplementtitekijä I, komplementtikohdistetut lääkkeet, danicopan, ACH-4471, biomarkkeri, syöpä

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Abbreviations

AK	Actinic keratosis
BCC	Basal cell carcinoma
CD35	Cluster of differentiation 35 (CR1)
CD46	Cluster of differentiation 46 (MCP)
CD55	Cluster of differentiation 55 (DAF)
CDC	Complement-dependent cytotoxicity
CFH	Complement factor H (Factor H; FH)
CFI	Complement factor I (Factor I)
CR1	Complement receptor type 1 (CD35)
cSCC	Cutaneous squamous cell carcinoma
cSCCIS	Cutaneous squamous cell carcinoma in situ
CT	Computed tomography
DAF	Decay accelerating factor (CD55)
DEB	Dystrophic epidermolysis bullosa
EB	Epidermolysis bullosa
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FB	Factor B (Complement factor B)
FCS	Fetal calf serum
FD	Factor D (Complement factor D)
FH	Factor H (Complement factor H)
FI	Factor I (Complement factor I)
HaCaT	Immortalized non-tumorigenic human keratinocyte-derived cell line
iC3b	Inactivated C3b
IHC	Immunohistochemistry
MAC	Membrane attack complex
MAPK	Mitogen activated protein kinase
MASP	Mannose-associated serine protease
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein (CD46)
MEK	Mitogen-activated protein kinase kinase

MMP	Matrix metallproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NHEK	Normal human epidermal keratinocyte
p53	Tumor protein 53
PET-CT	Positron emission tomography-computed tomography
PI3K	Phosphoinositide 3-kinase
RDEB	Recessive dystrophic epidermolysis bullosa
RDEBSCC	Recessive dystrophic epidermolysis bullosa-associated SCC
RNA-seq	RNA sequencing
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SCID	Severe combined immunodefficiency
siRNA	Small interfering RNA
TMA	Tissue microarray
UT-SCC	Human cutaneous squamous cell carcinoma cell line
UV	Ultraviolet

List of Original Publications

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

I Riihilä P, Nissinen L, Knuutila J*, Rahmati Nezhad P*, Viiklepp K*, Kähäri VM, 2019. Complement system in cutaneous squamous cell carcinoma. *Int J Mol Sci,* 20:3550.

II Rahmati Nezhad P, Riihilä P, Piipponen M, Kallajoki M, Meri S, Nissinen L, Kähäri VM, 2021. Complement factor I upregulates expression of matrix metalloproteinase-13 and -2 and promotes invasion of cutaneous squamous carcinoma cells. *Exp Dermatol,* 30:1631-1641.

III Rahmati Nezhad P, Riihilä P, Knuutila JS, Viiklepp K, Peltonen S, Kallajoki M, Meri S, Nissinen L, Kähäri V-M, 2022. Complement factor D is a novel biomarker and putative therapeutic target in cutaneous squamous cell carcinoma. *Cancers,* 14:305.

* Equal contribution

1 Introduction

The incidence of skin cancer is growing. This issue poses a substantial challenge to our health care system. Therefore, early detection of skin cancer and identification of high-risk premalignant lesions is of paramount importance. Also, more specific therapeutic approaches are required. The most common metastatic skin cancer is cutaneous squamous cell carcinoma (cSCC). Precursor lesions, actinic keratosis (AK) and cSCC in situ (cSCCIS) develop to primary invasive and metastatic cSCC. In addition to UV irradiation, other predisposing factors for cSCC are immunosuppression and chronic skin ulcers. Although surgical excision is a cure for most primary cSCCs, 3-7% metastasize and show poor prognosis. There are no biomarkers or therapeutic targets for high-risk cSCCs at the moment, and the molecular mechanisms of cSCC progression are not completely known. Several mutations in driver and suppressor genes have been identified in cSCC. However, a high amount of these mutations can also be detected in normal sun-exposed epidermal keratinocytes. Hence, other factors seem to be involved in cSCC progression. These important factors are proved to be changes in the microenvironment of premalignant lesions, inflammation, and exposure to microbial agents.

Previous work has demonstrated the essential role of autocrine complement synthesis in the progression of cSCC. A panel of inflammation-related genes including complement alternative components CFH, FI, FB and C3 has been identified in cSCC. It has been shown that CFH, FI and FB promote proliferation and migration of cSCC cells via activation of ERK1/2 signaling pathway, and FI, FB and C3 promote growth of cSCC xenografts *in vivo*. A new molecule of interest has been the rate-limiting enzyme of the alternative pathway, complement factor D (FD), which cleaves FB and plays a key role in both alternative pathway initiated C3 activation and subsequent amplification of the activation loop. In this thesis, the role of FI and FD in cSCC development and progression along with complement-targeted therapeutics under clinical and preclinical trials was studied.

2 Review of the Literature

2.1 Cutaneous squamous cell carcinoma (cSCC)

2.1.1 Overview

There are two main categories of skin cancers, namely melanoma and keratinocyte-derived non-melanoma skin cancer. Keratinocyte carcinoma is predominantly comprised of cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC) (Madan et al, 2010), and is the most prevalent cancer globally (Nehal et al, 2018; Nagarajan et al, 2019). cSCC constitutes 20% of keratinocyte carcinomas (Alam & Ratner, 2001; Green et al, 2017), and is considered to be the most common metastatic skin malignancy. Metastatic cSCC is associated with poor prognosis, causing approximately 20% of the skin cancer–associated mortalities. (Green et al, 2017; Rogers et al, 2015; Karia et al, 2013; Que et al, 2018; Schmults et al, 2013; Knuutila et al, 2020). The estimated rate for metastasis of primary invasive cSCC is 3-7% (Green et al, 2017; Rogers et al, 2015; Karia et al, 2013; Que et al, 2018; Schmults et al, 2013; Knuutila et al, 2020). Less than 35% of the patients with metastatic cSCC survive in a 5-year period (Tufaro et al, 2011), and the mortality rate of cSCC is reported to be 3% (Parikh et al, 2014; Czarnecki, 2017; Robsahm et al, 2015).

According to the Finnish Cancer Registry, in Finland, the incidence of cSCC ranks fifth and sixth among the most common cancers in males and females, respectively. However, the incidence of cSCC in the older population (age group 70 and over) increases, ranking fourth in both males and females amongst the most common cancer types in Finland. Overall, the incidence of cSCC in both genders has been increasing constantly since 2000s in Finland. cSCC has been reported to be the sixth most prevalent cancer in men and the seventh most prevalent cancer in women in Finland (Finnish Cancer Registry Report, 2020).

2.1.2 cSCC development

cSCC develops in a multistage process, meaning that the final clinically detectable lesion is preceded by multiple progressive changes on the molecular and cellular

level (Seebode et al, 2016). The first visible skin lesion in the process of cSCC development is called actinic keratosis (AK). AK is defined as the preneoplastic dysplasia of epidermal keratinocytes (Ackerman, 2003; Heaphy & Ackerman, 2000). The pink, red, or brownish scaly and rough patch of AK (also known as solar keratosis) is caused by years of sun exposure (Cockerell, 2000; Alam & Ratner, 2001). In a duration of more than a decade, only 0.025% - 20% of AKs will turn into cSCCs, and the more the number of lesions, the higher the risk of neoplastic change (Ratushny et al, 2012). In clinic, AK is categorized into three groups, i.e., mild, moderate, and severe based on the quantity and thickness of the lesions (Olsen et al, 1991).

A more progressive lesion than AK is cSCC *in situ* (cSCCIS) or Bowen's disease which is identified by the full-epidermis thickness keratinocyte atypia (or dysplasia), and is considered to be the very early stage of skin malignancy (Cockerell, 2000). The clinical appearance of cSCCIS is described as a large, slightly prominent, scaly and redish plaque which is more coarse and crusty in comparison to AK (Sterry & Stockfleth, 2009). Untreated cSCCIS has a great potential for further progression and can develop into invasive cSCC (Schmults et al, 2013; Ratushny et al, 2012) (**Figure 1**).

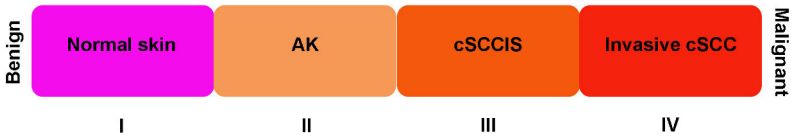

Figure 1. Schematic representation of progressive stages within the human epidermal carcinogenesis spectrum. The malignant cutaneous SCC develops from normal skin to premalignant lesion actinic keratosis (AK) to cSCC *in situ* (cSCCIS) and finally to invasive cSCC.

2.1.3 cSCC pathogenesis

The most important predisposing factors for cSCC include ultraviolet (UV) irradiation, chronic ulcerations, premalignant skin lesions (AK), cSCCIS, immunosuppressive diseases (e.g. AIDS), immunosuppressive therapies (e.g. organ transplantation medications), UVA and methoxsalen therapy, genodermatosis (e.g. dystrophic epidermolysis bullosa), human papilloma virus (HPV) infection, smoking (tobacco use), and carcinogenic chemicals (Kivisaari and Kähäri, 2013; Green & Olsen, 2017) (**Table 1**).

The UV wavelength determines the molecular mechanism of cSCC pathogenesis. UVB radiation (wavelength: 290-320 nm) directly damages both RNA and DNA materials of the cells and leads to the formation of cyclobutane pyrimidine dimers (as DNA damage byproducts) (Madan et al, 2010; Valejo

Coelho et al, 2016; Pfeifer et al, 2005). On the other hand UVA irradiation (wavelength: 320-400 nm) causes a photo-oxidative stress and damages the cellular DNA material indirectly (Madan et al, 2010; Ridley et al, 2009; Yaar & Gilchrest, 2007). Also, it is indicated that UV irradiation can suppress the migration of macrophages and induce immunosuppression (Kivisaari & Kähäri, 2013).

The two main molecular alterations in cSCC pathogenesis are inactivating and activating genetic mutations (Chang et al, 2021) (**Table 2**). Inactivating mutation in tumor suppressor gene *TP53* which encodes the p53 protein is caused by ultraviolet irradiation (Brash, 2006), and is predominantly found in metastatic cSCCs (~ 95%) compared to primary cSCCs (around 55%) (Pickering et al, 2014; Li et al, 2015; South et al, 2014; Yilmaz et al, 2017). *TP53* mutation has been propounded to be an initiatory mutation for cSCC pathogenesis as it is prevalently seen in premalignant lesion AK and cSCCIS (Campbell et al, 1993; Ziegler et al, 1994; Taguchi et al, 1994).

Also, the loss of p16 (INK4a) and p14 (ARF) functions as cell cycle regulators or tumor suppressor proteins due to inactivating mutations in the *CDKN2A* gene is commonly detected in primary cSCCs (Brown et al, 2004; Pickering et al, 2014; South et al, 2014; Li et al, 2015; Al-Rohil et al, 2016; Inman et al, 2018; Cho et al 2018; Mortier et al, 2002; Kanellou et al, 2008). The Notch signaling as the regulator of cell proliferation, differentiation, and apoptosis has been shown to partake in normal skin development, homeostasis and tumorigenesis (Nowell & Radtke, 2013). The inactivating mutations in both Notch genes (*NOTCH1* and *NOTCH2*) have been frequently detected in cSCC (Nowell & Radtke, 2013; Wang et al, 2011; Pickering et al, 2014; South et al, 2014; Li et al, 2015; Al- Rohil et al, 2016; Inman et al, 2018; Cho et al, 2018). Noteworthily, Notch1 is regulated by p53, and its down-regulation is suggested to be the downstream consequence of *TP53* mutation in human keratinocytes (Kraft & Granter, 2014; Ratushny et al, 2012; South et al, 2014; Wang et al, 2011).

In cSCC, the *HRAS* gene mutation is the most prevalent activating mutation followed by *NRAS* and *KRAS* mutations (Pickering et al, 2014; South et al, 2014; Bamford et al, 2004; Li et al, 2015; Al-Rohil et al, 2016; Inman et al, 2018; Cho et al, 2018). Triggered by UVB radiation, the *RAS* activating mutation causes downstream consecutive functional defects in Raf, MEK, and ERK1/2 signaling pathways (Ratushny et al, 2012).

Another important activating mutation in cutaneous SCC is directed at the epidermal growth factor gene (*EGFR*) (Li et al, 2015; Al-Rohil et al, 2016). As a result, EGFR overactivation prompts RAS signaling and then activates the MAPK and PI3K signaling pathways, which in turn cause turbulence in the normal

homeostasis of epidermis and lead to unconstrained cell survival, proliferation and growth (Doma et al, 2013).

Table 1. The most important predisposing factors for cSCC.

cSCC predisposing factors
Ultraviolet (UV) irradiation
Chronic ulcerations
Premalignant skin lesions (AK)
cSCCIS
Immunosuppressive diseases (e.g. AIDS)
Immunosuppressive therapies (e.g. organ transplantation medications)
UVA and methoxsalen therapy (e.g. for psoriasis and vitiligo treatment)
Genodermatosis (e.g. dystrophic epidermolysis bullosa)
Human papilloma virus (HPV) infection
Smoking (tobacco use)
Carcinogenic chemicals (e.g. arsenic, tar)

Table 2. The most frequent molecular alterations in cSCC.

Mutated gene	**Protein**	**Alteration in cSCC**
TP53 (inactivating mut.)	p53	Downregulated
CDKN2A (inactivating mut.)	p14 (ARF)	Downregulated
CDKN2A (inactivating mut.)	p16 (INK4a)	Downregulated
NOTCH (esp. *NOTCH1*) (inactivating mut.)	Notch (esp. Notch1)	Downregulated
RAS (esp. *HRAS*) (activating mut.)	Ras (esp. Hras)	Upregulated
EGFR (activating mut.)	EGFR	Upregulated

2.1.4 Diagnosis of cSCC

The clinical feature of cSCC is a persistent, protruding and fast-growing erythematous nodule in the skin. The lesion is firm or scaly and might spontaneously bleed or be ulcerated. There is also a need for physical examination of the regional lymph nodes as they might be metastatic in which case, they are usually attached to the local overlying skin and appear to be rigid and entangled. Different imaging modalities such as ultrasound, MRI, CT and PET-CT scans are

implemented to evaluate the extent of metastasis in advanced cSCC (Stratigos et al, 2020a; Madan et al, 2010; Sterry & Stockfleth, 2009). Furthermore, histopathological assessment of the lesion (excised or biopsied) as well as dissected lymph nodes is mandatory for the definitive diagnosis of cSCC.

Histopathologically, cSCC is identified by dysplastic proliferation of keratinocytes crossing the basal lamina and spreading to the dermal layers of the skin. Based on the histopathological analysis, cSCC is classified into three categories, namely well-differentiated, moderately-differentiated and poorly-differentiated cSCC (Sterry & Stockfleth, 2009).

2.1.5 Treatment of cSCC

Most of the times, primary cSCC is completely cured by surgical excision. The width of the tumor excision margins depends on the estimated risk of recur or spread based on the tumor staging system. If the tumor is considered as low-risk, the suggested advised excision margin of normal skin is 4-6 mm (5 mm according to the European consensus group) (Stratigos et al, 2020b), and if it is deemed to be high-risk, an excision margin of 6-10 mm is recommended. Low-risk cSCC tumors have a diameter of less than 2 cm while high-risk ones are more than 2 cm in diameter, and are usually located on the head area (ear, temple or lip). Also high-risk tumors have a thickness of more than 6 mm histologically or invade beyond the subcutaneous fat or perineurally. Poor differentiation or desmoplasia are amongst other histopathological features of high-risk cSCCs (Stratigos et al, 2020b). Aside from surgical excision, Mohs micrographic surgery is another treatment modality for primary high-risk cSCCs which is preferred over surgical excision if available (Stratigos et al, 2020b; Alam & Ratner, 2001; Bonerandi et al, 2011; Ferrandiz et al, 2012; Kivisaari & Kähäri, 2013; Madan et al, 2010). Nodal dissection is advised for metastatic cSCCs with regional lymph node involvement (Stratigos et al, 2020b). Irrespective of tumor stage (being primary or metastatic), irradiation is often used for non-surgical candidates or inoperable tumors/lymph nodes (Stratigos et al, 2020b).

There are limited options for systemic treatment of metastatic or locally advanced cSCCs that are unresponsive to curative surgery or irradiation. The first-line systemic therapeutics for these types of cSCC are anti-programmed cell death protein-1 (PD-1) antibodies, which are immune checkpoint inhibitors; To date cemiplimab is the only approved systemic drug for advanced cSCCs within this category. It was approved by the Food and Drug Administration (FDA) in 2018 and by the European Medicines Agency (EMA) in 2019 (Stratigos et al, 2020b; Migden et al, 2018). In a recent study the concomitant use of cemiplimab and radiation therapy was confirmed to be efficient in treatment of advanced cSCCs by

improving the local response to radiotherapy, and resulting in faster radiological response with no safety issues (Bailly-Caillé et al, 2023). The second-line systemic therapy for advanced cSCCs comprises the off-label use of EGFR inhibitors (cetuximab) in combination with radio- or chemotherapy. Systemic cetuximab treatment can lead to a profile of challenging adverse effects along with acquired drug resistance by tumor cells (Stratigos et al, 2020b; Migden et al, 2018). In general, multidisciplinary board meetings are essential for therapeutic decisions regarding advanced cSCC cases that are in need of more than operation (Stratigos et al, 2020b).

2.1.6 Prognosis of cSCC

Prognosis of primary cSCC tumors depends on several risk markers as follows (Stratigos et al, 2020a; Knuutila et al, 2020):

I) T stage in the TNM staging system, i.e., size (diameter and thickness) and depth of invasion: A diameter of greater than 2 cm doubles the recurrence rate and there is a higher recurrence potential in lesions more than 6 mm of thickness. Also, invasion of the subcutaneous fat leads to a poorer prognosis.

II) N stage in the TNM staging system, i.e., involvement of regional lymph nodes: In case of nodal metastasis, less than 20% of patients survive in a 10-year period.

III) M stage in the TNM staging system, i.e., the presence of distant metastasis: Once hematogenous metastasis occurs, the 10-year survival rate drops down to lower than 10%.

IV) Perineural invasion: The presence of perineural invasion is indicative of a poor prognosis.

V) Histopathological grade of differentiation: Having a postoperative recurrence rate of 13%, well-differentiated cSCC tumors show a better prognosis compared to poorly-differentiated ones with a recurrence rate of 25%.

VI) Desmoplasia

VII) Bone erosion

VIII) Immunosuppression: Similar to the previous two features (VI & VII), this feature is indicative of a poor prognosis for cSCC.

IX) Anatomical location: There is a higher risk of recurrence or metastasis in cSCC of ear or lip, i.e., 10 to 25% compared to other anatomical sites (Stratigos et al, 2020a; Alam & Ratner, 2001; Kivisaari & Kähäri, 2013; Madan et al, 2010; Sterry & Stockfleth, 2009).

X) Comorbidity: As much as 40% of cSCCs that are originated from and anchored in long-term ulcerations, metastasize (Königová & Rychterová, 2000; Novick et al, 1977).

The 5-year post-diagnosis metastasis and recurrence rates of invasive cutaneous SCC are estimated to be 5% and 5-16%, respectively. High-risk cSCCs have a greater chance of postoperative recurrence (16%) than low-risk ones (5 to 8%) (Alam & Ratner, 2001; Kivisaari & Kähäri, 2013; Madan et al, 2010; Sterry & Stockfleth, 2009; Knuutila et al, 2020).

2.2 Recessive dystrophic epidermolysis bullosa-associated cSCC

Epidermolysis bullosa (EB) is a rare genetic disorder that causes fragility of skin or other epithelia leading to blisters. The blisters take shape in the skin or mucosa because of the mildest injury such as scratching or even with no stimulus (Pearson, 1962). There are four distinct types of EB, and each has its own specific genetic, histopathological and clinical feature: 1) EB simplex (EBS), 2) Junctional EB (JEB), 3) Dystrophic EB (DEB) and 4) Kindler syndrome. EBS involves the epidermis, and JEB involves the epidermo-dermal junction. Dermolysis is the characteristics of DEB, and kindler syndrome is a compound histopathological condition.

As DEB blisters are deeply located (in the lamina densa of basement membrane along with the upper part of dermis), they mostly turn into massive scarring and finally long-term ulceration (Bruckner-Tuderman et al, 1989). Two main subtypes of DEB are autosomal dominant and autosomal recessive DEB abbreviated as DDEB and RDEB, respectively. The latter is worse in terms of severity (Fine et al, 2014). The *COL7A1* gene is mutated in all DEBs (Uitto & Christiano, 1994). In cases that *COL7A1* mutation results in very little to no production of collagen VII protein, the generalized severe subtype of RDEB (a.k.a RDEB-GS) is formed. RDEB-GS was previously known as Hallopeau-Siemens, and is considered the most critical subclass of DEB. There is a very high risk of aggressive cSCC development in RDEB-GS patients. The tumors appear within the chronic RDEB ulcers, and early mortality is the consequence (Fine et al, 2009; Reed et al, 1974; Condorelli et al, 2019; Montaudié et al, 2016).

2.3 Complement system

The complement system is a fundamental arm of host immunity and links the innate and adaptive immune responses. It clears the invading pathogens, immune complexes and cellular debris from circulation and tissue environment (Ricklin et

al, 2010; Serna et al, 2016; Rutkowski et al, 2010; Bohlson et al, 2019). The complement system components are protein molecules that are either soluble or membrane-bound. The soluble complement compounds account for almost 5% of the total plasma protein. Complement proteins are mainly synthesized in the liver. However, they can also originate from local tissues. Many complement components function as serine proteinases. These include C1r, C1s, MASP1, MASP2, MASP3, C3, complement factor B (FB), complement factor D (FD), C5, and complement factor I (FI) (Forneris et al, 2012; Merops database, 2019; Ricklin et al, 2010; Sim & Laich, 2000; Sim & Tsiftsoglou, 2004).

Activation of the complement system is through 3 different pathways, i.e., classical, lectin and alternative, each of which eventually activates the lytic a.k.a terminal cascade.

In general, the complement pathways start off by a "foreign agent- complement component" interplay, but it is the nature of the foreign agent or the stimulus that determines which complement pathway is going to get activated. The activation process in each complement pathway is sequential.

The final outcomes of the complement system activation are as follows:1) Production of activated complement cleavage fragments namely C3a and C5a which are anaphylatoxic, and recruit immune cells to the inflammation site. 2) Formation of the membrane attack complex (MAC) that is a pore like protein complex (C5b-9) assembled on the membrane of the target cell, leading to cell lysis. 3) Deposition of the C3b and iC3b complement fragments on the target cell membrane followed by phagocyte attachment and opsonization (a.k.a complement dependent cytotoxicity (CDC)) (Janssen & Gros, 2006; Sim & Tsiftsoglou, 2004; Mamidi S. et al, 2017) (**Figure 2**).

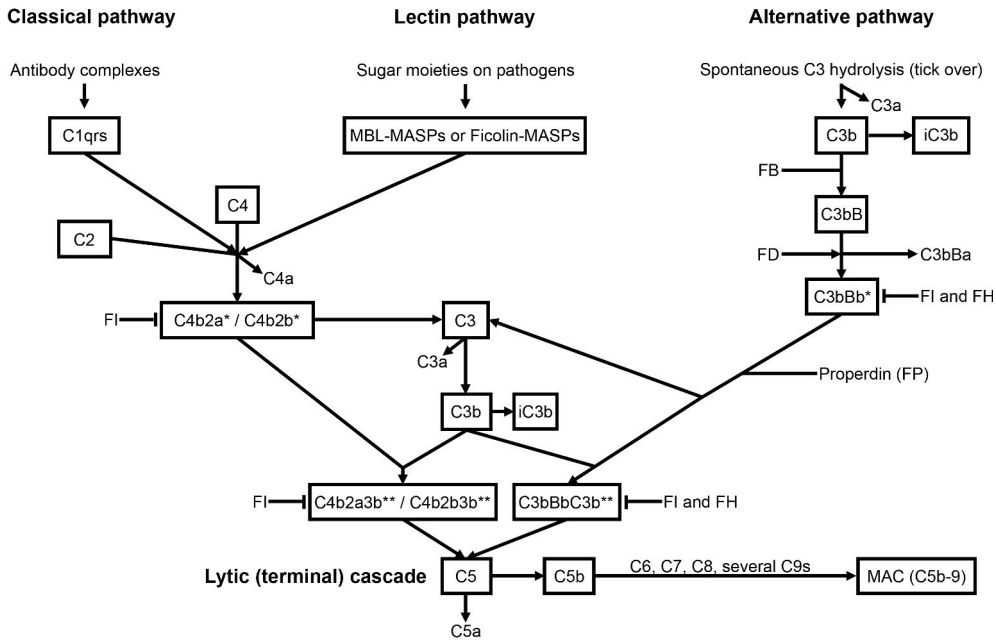

Figure 2. A schematic view of the complement system activation. Activation of the complement system is through three different pathways namely classical, lectin and alternative, each of which eventually activates the lytic (terminal) cascade. The activation trigger of the classical pathway is the interaction between antigen-antibody complexes and C1q component of the C1qrs complex. The activation stimulus of the lectin pathway is the interplay between ficolins or MBLs (mannose binding lectins) attached to specific sugar moieties on cell membranes of pathogens, and MASPs (mannose-associated serine proteases). The alternative pathway is persistently activated by spontaneous hydrolysis of circulated C3 component (a.k.a tick over). All three pathways converge at C3 and initiate the lytic cascade, which results in the formation of the membrane attack complex (MAC), i.e., C5b-9. The MAC pore causes cell lysis, and the anaphylatoxins C5a and C3a induce inflammation. The complement system is strictly regulated by membrane-bound complement regulatory proteins (mCRPs) and soluble regulators including FI and FH. FI and FH inhibit C3 and C5 convertases. C3 and C5 convertases are indicated with one (*) and two (**) asterisk(s), respectively.

2.3.1 Classical and lectin pathways

Classical pathway: The activation trigger of the classical pathway is the interaction between Fc region of IgG or IgM antibodies within antigen-antibody complexes and C1q component of the C1qrs complex. This causes a conformational change in the C1qrs complex which is followed by the activation of C1r and C1s, respectively. Activated C1s then splits both C4 and C2. The combination of resulted cleavage products, i.e., C4a, C4b, C2a, and C2b generates the C3 convertase of classical pathway, i.e., the C4b2a or C4b2b complexes (Gaboriaud et al., 2004). Cleavage of C3 by C3 convertase sets the lytic (terminal) cascade on motion. Other activation triggers of the classical pathway through C1q include

pentraxin (PTX3), C-reactive protein or CRP, serum amyloid A or SAA, and membrane blebs on apoptotic cells that resemble antibodies in structure (Bottazzi et al., 1997; Deban et al.,2008; Agrawal et al, 2001; Navratil et al, 2001; Ricklin et al, 2010).

Lectin pathway: The activation stimulus of the lectin pathway is the interplay between ficolins or mannose binding lectins a.k.a MBLs attached to specific sugar moieties on cell membranes of pathogens, and MASPs (1, 2 or 3) (Gros et al, 2008; Zipfel & Skerka, 2009, Lu et al, 1990). C2 and C4 are subsequently cleaved after MASP activation leading to the formation of C3 convertase, i.e., C4b2a or C4b2b and kindling of the lytic cascade (Lu et al, 1990) (**Figure 2**).

2.3.2 Alternative pathway

The alternative pathway is persistently activated by a specific phenomenon called "tick-over", which is defined as spontaneous and steady hydrolysis of circulated C3 component. The ensuing C3(H2O) complex adheres to FB which is in turn targeted by FD and fragmented into Bb and Ba, ending in the formation of fluid-phase C3 convertase C3(H2O)Bb. The fluid-phase C3 convertase keeps cleaving C3 and as a result the target cell surfaces get saturated with C3b. Membrane-bound C3bs attach to FB. Thereupon FD splits the FB and the genuine C3 convertase of the alternative pathway (C3bBb) is generated (Gros et al, 2008; Ricklin et al, 2010; Serna et al, 2016; Rutkowski et al, 2010; Bohlson et al, 2019). Since C3bBb is very short-lived by nature, factor P (FP) or properdin binds to it to create a combo with a much higher stability (Pangburn & Muller-Eberhard, 1986; Fearon & Austen, 1975; Kemper et al, 2010). Eventually, more C3b accumulates on the membranes of target cells causing cytotoxicity (i.e., CDC) (**Figure 2**).

2.3.2.1 Complement factor D (FD)

Complement factor D (FD) is the essential rate-limiting enzyme of the alternative pathway that is of crucial importance for switching it on (**Figure 2**). FD has the lowest concentration amongst all complement proteins (1-2 μM) in plasma (Tian et al, 2015). It is also known as adipsin, as the adipose tissue is recognized to be the chief source of its production (White et al, 1992). Other sources of FD genesis comprise liver, monocytes and macrophages (Maibaum et al, 2016). Factor D is eliminated from the body through kidneys (Tian et al, 2015).

FD is often synthesized as a single polypeptide chain with 253 amino acids (variant 1) comprising the signal or leader peptide, pro-peptide a.k.a activation peptide, and serine protease domain (Podos et al, 2018, RCSB Protein Data Bank (PDB) (www.rcsb.org)) (**Figure 3**). Variant 2 of factor D consists of 260 amino

acids. The mature form of FD that is found in circulation is a 228 amino acid monomer with a molecular weight of 24.4 kDa which is the cleavage product of the above-mentioned variant 1 or 2. The mature FD has the same structure whether produced from variant 1 or variant 2. It has 4 disulphide bonds internally that connect cysteine 51 to 67, 148 to 214, 179 to 195, and 204 to 229 (Podos et al, 2018). The proFD enzyme is dormant in circulation until it identifies and comes into contact with its key substrates, i.e., C3(H2O)B or C3bB. Thereafter, FD becomes active upon a conformational change and selectively targets and splits factor B to create the fluid-phase C3(H2O)Bb or true C3bBb C3 convertases of the alternative pathway (Ricklin et al, 2010; Serna et al, 2016; Rutkowski et al, 2010; Bohlson et al, 2019). MASP3 is another specific activating substrate for FD (Dobó et al, 2016)

Figure 3. Schematic structure of FD (variant 1). *Pro-pep: pro-peptide.

2.3.3 Lytic (terminal) cascade

The lytic cascade starts off when C3b bonds with the classical or alternative pathway C3 convertases, resulting in generation of C5 convertase. The classical pathway C5 covertase namely C4b2a3b or C4b2b3b or the alternative pathway C5 convertase, i.e., C3bBbC3b then convert C5 into C5b and C5a hence the name. C5b attaches to the target cell membrane and together with the attracted complement compounds C6, C7, C8, and several C9s creates a large pore called membrane attack complex (MAC). The MAC pore causes a significant leakage through the cellular membrane leading to cell lysis. In addition, the C5a fragment which is an anaphylatoxic agent induces inflammation (Müller-Eberhard, 1986; Tschopp et al, 1984; Papadimitriou et al, 1991) (**Figure 2**).

2.3.4 Complement system regulators

2.3.4.1 Overview

The main role of complement system regulators is to protect the host cells from getting opsonized or lysed inadvertently. Also, they hinder the exhaustion of complement compounds by regulating their consumption. These regulators are either bound to the cell membrane and called membrane complement regulatory

proteins (mCRPs) or circulate in plasma in a soluble form. Soluble regulators include C1 inhibitor (C1-inh), C4b-binding protein (C4BP), FI, FH, clusterin (CLU) and vitronectin (VTN), whereas mCRPs include CD35, CD46, CD55, and CD59. C1-inh impedes the split of C2 and C4 via connecting to the classical pathway C1r/C1s or lectin pathway MASP2 (Ziccardi, 1982). C4BP is a cofactor for FI and inhibits the C3 convertase of the classical pathway (Gigli et al, 1979). FI and FH block both C3 and C5 convertases of the alternative pathway through C3b cleavage. FI also inhibits the classical pathway C3 and C5 convertases by cleaving C4b (Mamidi et al, 2017; Ponce-Castro et al, 2008).

CD35 or complement receptor 1 (CR1) suppresses the production of both alternative and classical pathway C3 and C5 convertases on the cell membrane via binding to C3b or C4b, respectively (Fearon, 1980; Medicus et al., 1983; Medof et al., 1982; Nicholson-Weller and Wang, 1994). Similar to CD35, CD46 is a cofactor for FI and targets C3b or C4b to prevent the function of C3 or C5 convertases. Accordingly it is also named as membrane cofactor protein (MCP) (Kojima et al., 1993; Seya & Atkinson, 1989; Seya et al, 1986). CD55 or decay accelerating factor (DAF) dismantles the C3 convertases of alternative or classical pathway by cleaving Bb or C2a, respectively. Therefore, it is not considered as a cofactor for FI (Fujita et al, 1987). Finally, the membrane-bound CD59 (protectin) along with soluble complement regulators CLU and VTN hinders the creation of MAC and subsequent cell lysis (Meri et al., 1990; Rollins & Sims, 1990; Davies et al 1989; Jenne & Tschopp, 1989; Podack & Muller-Eberhard, 1979).

2.3.4.2 Complement factor I (CFI; FI)

Complement factor I (CFI or FI) is a soluble complement regulator functionally categorized as a serine protease. It can hamper all three pathways of the complement system via cleaving C3b or C4b in the C3 or C5 convertase structure (Mamidi et al, 2017; Ponce-Castro et al, 2008; Nagasawa & Stroud, 1977). The activity of FI is strictly regulated by the amount of its highly specific substrates (Nilsson et al, 2011). In order to play its role efficiently, FI requires the company of a few cofactors including C4BP, FH, CD35 (CR1) and CD46 (MCP) (Gigli et al, 1979; Schulze et al, 1993; Medicus et al, 1983; Medof et al, 1982; Ross et al, 1982; Seya & Atkinson, 1989; Seya et al, 1986).

FI is chiefly produced by hepatocytes. Other sources of FI production encompass myoblasts, endothelial cells, monocytes, fibroblasts of the skin, and keratinocytes (Schlaf et al, 2001; Julen et al, 1992; Whaley, 1980; Vyse et al, 1996; Timar et al, 2007). FI is a polypeptide dimer with a molecular weight of 88 kDa. The whole polypeptide chain of FI comprises 583 amino acids including the signal peptide (RCSB Protein Data Bank (PDB) (www.rcsb.org)). Furin targets the

disulfide link within the inactive FI dimer and splits the original chain into one heavy chain of 51 kDa and one light active chain of 37 kDa (Lachmann & Müller-Eberhard, 1968; Goldberger et al, 1984; Ruddy & Austen, 1969; Nilsson et al, 2009). The heavy chain is consisted of five domains including membrane attack complex domain of FI (FIMAC), scavenger receptor cysteine-rich protein domain (SRCR), and low-density lipoprotein receptors class A1 and A2 (LDLRA1 and 2). The light chain is composed of the serine protease domain (**Figure 4**).

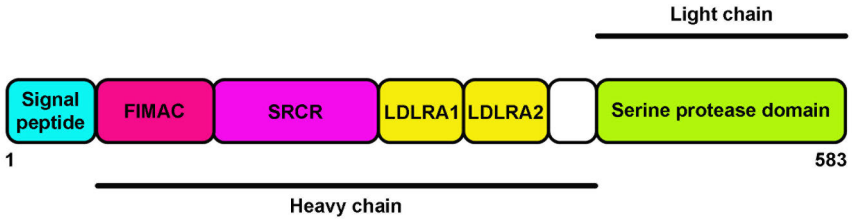

Figure 4. Schematic structure of FI. FIMAC: membrane attack complex domain of FI; SRCR: scavenger receptor cysteine-rich protein domain; LDLRA1 and LDLRA2: low-density lipoprotein receptor class A1 and A2.

2.3.5 The role of complement system in cancer

Complement is considered as a double-edged sword in the tumor field; although it is well known that complement activation induces an anti-cancer immune response, it can also promote carcinogenesis (Mamidi et al, 2017; Netti et al, 2021). The tumor microenvironment hugely impacts tumor growth and metastasis. Research over a decade has disclosed that malignant cells are able of generating a great spectrum of complement components on site (Roumenina et al, 2019; Reis et al, 2018). Complement components are present in the tumor microenvironment, but their role is complicated. They may enhance chronic inflammation or hinder the anti-tumor response of the T cells leading to cancer progression, or facilitate the phagocytosis of antibody-coated cancer cells. These antagonistic effects depend on the locations in which complement is activated, the sensitivity of cancer cell to complement attack, and constitution of tumor microenvironment (Balkwill and Mantovani, 2012; Grivennikov et al, 2010; Mantovani et al, 2008; Lujambio et al, 2013; Trinchieri, 2012; Rutkowski et al, 2010).

The expression of 50 complement genes has been analysed in 30 human cancers. Although there is only a little heterogeneity among tumor types, the heterogeneity in expression between the genes is strong (Roumenina et al, 2019). The C3 gene along with the genes encoding the components of the classical pathway (*C1QA*, *C1QB*, *C1QC*, *C1S*, *C1R*, *C4A* and *C2*) are intensely expressed in all types of malignancies. The genes encoding the alternative pathway complement

components (*FD* and *FB*) are highly expressed in cancer types with high *in situ C3* expression. Only prostate adenocarcinoma, chromophobe renal cell cancer, and uveal melanoma exceptionally show low expressions of *FD* and *FB.* On the contrary, genes of the lectin pathway are weakly expressed in most cancers (Roumenina et al, 2019). Moreover, tumor cells have developed mechanisms to survive the attack of the complement system by overexpression of complement inhibitors such as CD46, CD55, CD59 and FI or removal of the MAC from their surface membrane, or by promoting an immunosuppressive environment via recruitment of regulatory immune cells such as immune myeloid cells and Tregs (Reis et al, 2018; Revel et al, 2020; Mamidi et al, 2017; Fishelson et al, 2019). For example, increased levels of *FI* expression is detected in malignancies such as pancreatic and stomach adenocarcinoma, and glioblastoma (Roumenina et al, 2019).

Also, some components of the complement system have certain functional roles in addition to complement activation and inhibition which might play a part in cancer progression. For instance some complement components also function as serine proteinases. These include C1s, C1r, C2 and C4 in the classical pathway, FD and FB in the alternative pathway, MASPs 1-3 in the lectin pathway, C3, C5, and complement inhibitor FI (Sim and Laich, 2000; Forneris et al, 2012).

2.3.6 The role of complement system in cSCC

In recent years, it has been indicated that the complement system components originated from carcinogenic cells promote tumor progression. This interesting role of the autocrine complement components is shown to be separate from the systemic activation of complement system (Nissinen et al, 2016; Venables et al, 2018; Karia et al, 2013; Schmults et al, 2013; Nelson et al, 2017; Rowe et al, 1992). According to the preceding research, both cSCC tumors *in vivo* and cSCC cells in culture notably overexpress specific activators, i.e., C1r, C1s, FB and C3 and inhibitors, i.e., FH and FI of the complement system compared to normal skin and normal human epidermal keratinocytes (NHEKs), respectively (Riihilä et al, 2014; Riihilä et al, 2015; Riihilä et al, 2017; Riihilä et al 2020).

Complement activators C1r and C1s: The classical pathway C1r and C1s are demonstrated to have higher intensity of immunohistochemistry (IHC) staining in cSCC tumors vs. cSCCISs or premalignant AKs, and in cSCCISs compared with AKs, which is indicative of an association between their expression and the process of cSCC formation and progression. Also, the C1r and C1s IHC staining for aggressive RDEBSCC has been shown to be more intense when compared with non-RDEBSCC (Riihilä et al, 2020). The vascularization and growth of cSCC xenografts is hindered, and migration and proliferation of cultured cSCC cells is

blocked following C1r and C1s knockdown. In addition, C1r and C1s knockdown leads to the apoptosis of cSCC cells both *in vivo* and *in vitro* (Riihilä et al, 2020). It has been revealed that PI3K and ERK1/2 signaling pathways are significantly deactivated post-C1s knockdown. Furthermore, C1s has been found to be functionally active independent of C1q presence in cultured cSCC cells. These results elucidate that C1s promotes the progression of cutaneous SCC through a distinct mechanism from systematic activation of the classical complement pathway (Riihilä et al, 2020).

The expression of specific matrix metalloproteinases, i.e., MMP-1, MMP-10, MMP-12, and MMP-13 in cSCC cultures is downregulated after C1r CRISPR/Cas9 knockout. Besides, the *in vivo* cSCC cell production of MMP-13 and the xenograft tumor invasion are impeded following C1r knockout, which discloses the MMP-mediated mechanism of action of C1r in cSCC invasion (Viiklepp et al, 2021).

Complement activator FB: The alternative pathway FB is overexpressed in cultured cSCC cells compared to NHEKs (Riihilä et al, 2014; Riihilä et al, 2015; Riihilä et al, 2017). It has been shown that migration and proliferation of cSCC cells is inhibited after FB knockdown. Also, a potent inhibition of growth in cSCC xenografts is detected post-FB knockdown. In addition, the ERK1/2 signaling pathway is significantly inactivated following FB knockdown. Histopathologically, there is an overexpression of factor B component by cSCC cells *in vivo,* and an association between FB expression and the process of tumor formation and progression is noticed meaning that FB staining gets stronger in intensity from normal skin to AK, cSCCIS and cSCC, respectively. Also, the more aggressive the tumor is, the stronger the intensity of FB staining via IHC. In other words, aggressive RDEBSCC samples showcase the most intense FB staining in comparison with non-RDEBSCCs and cSCCISs (Riihilä et al, 2017).

Complement activator C3: C3 component is significantly overexpressed by both cSCC cells *in vitro* and *in vivo* vs. NHEKs and normal skin, respectively. Similar to FB, there is a correlation between C3 expression and the progression and aggressiveness of cSCC. cSCC tumors have a higher intensity of C3 IHC staining than cSCCISs or premalignant AKs, and the aggressive RDEBSCCs are stronger in C3 staining compared with cSCCs (Riihilä et al, 2014; Riihilä et al, 2015; Riihilä et al, 2017).

Furthermore, it has been revealed that proliferation and migration of cultured cSCC cells and growth of cSCC xenografts are hampered after C3 knockdown. The activation of ERK1/2 signaling pathway is notably blocked following C3 knockdown. Also, the level of C3 protein component in Ha-ras-transformed tumorigenic HaCaT cell lines disclose an association between the stage of epidermal carcinogenesis and autocrine C3 expression (Riihilä et al, 2017).

Complement inhibitors FH and FI: Both FH and FI are found to be significantly expressed by cultured cSCC cells as well as cSCC tumors *in viv*o, and the higher the stage of cSCC progression, the more is the expression level of these two chief inhibitors of the complement system. FH or FI knockdown results in remarkable deactivation of the ERK1/2 signaling pathway. Migration and viability of cSCC cells are shown to be potently inhibited after FI or FH knockdown. (Riihilä et al, 2014; Riihilä et al, 2015). There is also a noticeable decrease in the growth of human cSCC xenografts post-FI knockdown (Riihilä et al, 2015).

3 Aims

General Aim:

In this thesis the alternative pathway of complement system in cSCC was studied to find and characterize novel molecular biomarkers and therapeutic targets for the progression of cSCC.

Specific Aims:

I) To thoroughly study the complement-targeted therapeutic compounds

II) To elucidate the molecular mechanism of FI in cSCC progression

III) To explore the role of FD as a potential biomarker and putative therapeutic target of cSCC

4 Materials and Methods

4.1 Ethical Issues (II-III)

This research has the approval of the Ethics Committee of Turku University Hospital based on the Declaration of Helsinki (187/2006; 138/2007). The conduction of studies on skin samples and using the clinical data have been approved by the Clinical Research Center of Turku University Hospital (T80/2018) as well as Auria Biobank (AB15-9721). Written informed consent was obtained from all patients in the study before each operation. Permission for mice experiments was granted by the Project Authorisation Board of the Southern Finland (ESAVI15107/2020) under institutional guidelines.

4.2 Cell Cultures

4.2.1 Normal Human Epidermal Keratinocytes (NHEKs) (III)

NHEK cell cultures (n=10) were created using the skin samples collected from mammoplasty surgeries conducted in Turku University Hospital. The NHEK-PC cell line was provided by PromoCell (Heidelberg, Germany). The NHEK cells were cultured in keratinocyte growth medium-2 containing 0.5M calcium chloride and supplement mix (PromoCell), streptomycin-penicillin mixture as well as L-glutamine (Gibco, Paisley, Scotland, UK) (Riihilä et al, 2014).

4.2.2 HaCaT cell lines (III)

The spontaneously immortalized non-tumorigenic human keratinocyte-derived cell line (HaCaT) along with three Ha-Ras-transformed tumorigenic HaCaT-derived cell lines, i.e., A5, II-4 and RT3 were used to simulate the progressive stages of epidermal carcinogenesis *in vitro*. All these cell lines were provided by Dr. Norbert Fusenig from the German Cancer Research Center in Heidelberg, and were characterized previously (Boukamp et al, 1988; Boukamp et al, 1990). In nude mice, A5 cells generate benign tumors, whereas II-4 and RT3 cells form malignant low-grade and malignant high-grade tumors, respectively (Mueller et al, 2001).

The HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 100 μg/ml streptomycin, 100 IU penicillin G, 10% fetal calf serum (FCS), non-essential amino acids, and 2 mM L-glutamine. A5, II-4, and RT3 cell media contained G418 (200 μg/ml) too (Riihilä et al, 2014).

4.2.3 Human cSCC cell lines (II-III)

The establishment of human cSCC cell lines was performed by using surgically removed cSCCs (Farshchian et al, 2011). Five cSCC cell lines (UT-SCC-12A, -91, -105, -111, and -118) were established from primary cSCCs and three cSCC cell lines (UT-SCC-7, -59A, and -115) from metastatic cSCCs. Short tandem repeat (STR) DNA profiling was applied to authenticate the above cSCC cell lines (Farshchian et al, 2017). cSCC cells were cultured as HaCaT cells (Riihilä et al, 2014).

4.3 Tissue specimens of cSCC tumors and normal skin

4.3.1 Tissue RNA (III)

The tissue RNA was derived from normal skin and primary cSCC samples using RNeasy mini kit (Qiagen, Germantown, MD), and was analysed by RT-qPCR (Stokes et al, 2010). Normal skin samples (n=10) were obtained either from the upper arm area of volunteers in good health or via mammoplasty operations, and the samples of primary cSCC (n=6) were acquired from surgically removed malignancies in Turku University Hospital (Farshchian et al, 2011).

4.3.2 Tissue microarrays (III)

Overall 441 tissue specimens were gathered from the Auria Biobank and the Department of Pathology, both affiliated to the Turku University Hospital and University of Turku. The samples were embedded in paraffin and fixated in formalin, and the instruction for preparation of tissue microarrays (TMAs) from them was as previously published (Kononen et al, 1998). This collection of tissue samples consisted of a large panel of normal skin (n=80), premalignant AKs (n=65), cSCCISs (n=61), primary non-metastatic sporadic cSCCs (n=140), primary metastatic sporadic cSCCs (n=70), metastases of cSCC (n=9) and RDEBSCCs (n=16) (Knuutila et al, 2020; Kivisaari et al, 2008; Kivisaari et al, 2010).

4.4 Antibodies (II-III)

The antibodies used and mentioned in the original publications (II-III) are listed below (**Table 3**). (WB: Western blot; IHC: Immunohistochemistry)

Table 3. Antibodies used and mentioned in the original publications.

Antigen	Catalog No.	Supplier	Method	Publication
FI (Clone OX-21)	NBP1-02915	Novus Biologicals	WB	II
MMP-13	MAB3321, MAB13424	Merck Millipore	WB, IHC	II
MMP-2	HPA001939	Sigma	WB, IHC	II
phospho-p44/42 MAPK (Thr204/Tyr204, p-ERK)	9101S	Cell Signaling Technology	WB	II, III
P44/42 MAPK (ERK)	9102	Cell Signaling Technology	WB	II, III
TIMP-1	MAB3300	Millipore	WB	II, III
TIMP-2 (AB-1)	IM11L	CalbioChem	WB	II
β-Actin (AC-15)	A-1978	Sigma	WB	II, III
FD	SAB1301593	Sigma	WB, IHC	III
p-CREB	9191	Cell Signaling Technology	WB	III

4.5 Reverse transcription quantitative real-time PCR (RT-qPCR) (II-III)

Using RNeasy Mini Kit (Qiagen) total RNA was extracted from cultured cSCC cells, and synthesis of cDNA following the extraction of total RNA was accomplished according to the earlier instructions (Riihilä et al, 2014). Using the QuantStudio 12K Flex (Thermo Fisher Scientific) software, RT-qPCR analysis was then carried out. For this purpose, specific TaqMan probes and primers for MMP-2, MMP-13 and β-Actin were employed which are previously elaborated (Stokes et al, 2010). In addition, one specific TaqMan probe and two specific primers for FD were utilized:

Probe for FD: 5′-Fam-TCCCGAGCAATGAAGTCATCCAC-Tamra-3′

Forward primer for FD: 5′- GGGTCACCCAAGCAACAAAG -3′

Reverse primer for FD: 5′- CGTGGCCCATGCTGATCTC -3′

The analysis was performed on triplicate samples based upon a threshold cycle value (Ct) lower than 5% of the mean. The expression level of β-Actin was considered as control.

4.6 RNA-seq analysis (II)

Running the Illumina HiSeq3000 system, the isolated RNA from siRNA-transfected cSCC cells by miRNAeasy Mini kit (Qiagen) was sequenced at the Finnish Functional Genomics Centre, Turku, Finland. To elaborate, three cSCC cell lines (UT-SCC-7, -59A, and -118) were transfected with FI siRNA_1 and control siRNA (120 nM), and the RNA isolation process was carried out 3 days after transfection. The alignment of the reads to the Genome Research Consortium human build 38 (GRCh38 or hg38) was done post-sequencing. Normalization of data was accomplished using the trimmed mean of M (TMM) (Bioconductor edgeR 3.3, R version 3.3), and statistical data analysis was fulfilled with Limma package 3.10 (Farshchian et al, 2017). The mRNA-seq data is publicly available online on the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) through the Accession number GSE166421.

4.7 Western blot analysis (II-III)

Western blot analysis of the lysate and conditioned medium samples of cSCC cells was performed using antibodies listed previously (**Table 3**). The Western blotting for all media and lysates except complement components was performed under reducing conditions. The expression level of proteins was measured with LI-COR Odyssey® CLx fluorescent imaging system employing secondary antibodies labeled fluorescently (LI-COR Biosciences, Lincoln, USA). In general, the number of biological and technical replicates varied between 2 and 3.

4.8 Immunohistochemistry (IHC) (II-III)

The TMAs from normal skin, AK, cSCCIS, primary non-metastatic sporadic cSCC, primary metastatic sporadic cSCC, SCC metastase and RDEBSCC were stained with a rabbit polyclonal anti-FD antibody (**Table 3**) in the Histology Core facility of the Institute of Biomedicine affiliated to the University of Turku, Finland. The counterstaining of the slides was done with Hematoxylin. The slides were digitally visualized utilizing the Pannoramic 250 Slide Scanner or Pannoramic 1000 Slide Scanner (3DHistech). A semiquantitative analysis was carried out to assess the FD expression on the basis of cytoplasmic FD staining intensity: negative or (-) (= no FD expression), weak or (+) (= very low FD

expression), moderate or (++) (= moderate FD expression), and strong or (+++) (= very high FD expression). A separate analysis was performed on the distribution pattern of FD staining in TMAs. Furthermore, the tumor samples from human cSCC xenografts were processed and stained with rabbit polyclonal anti-FD, mouse monoclonal anti-MMP-13, and rabbit polyclonal anti-MMP-2 antibodies employing hematoxylin as counterstain.

A semiquantitative analysis of the staining intensities was accomplished following digital scanning of the slides by a Pannoramic 1000 Slide Scanner (3DHistech).

4.9 Generation of FI expression vector (II)

In order to synthesize the FI expression vector, the cDNA fragment of FI in pEX-A128 vector was acquired from Eurofins Genomics (Germany). Subsequently the FI fragment was inserted into pcDNA3.1 vector (Invitrogen), and the construct was sequenced to confirm the integrity and orientation of the clone.

4.10 Functional assays in culture

In general, the number of biological and technical replicates in all functional assays in culture were between 2 to 3.

4.10.1 Cell transfections

4.10.1.1 FI Gene knockdown by siRNAs (II)

To knock down the FI gene, cSCC cell cultures with a confluence of 50% were transfected with FI or AllStars negative control siRNAs (Qiagen) at a concentration of 120 nM applying siLentFect Lipid Reagent (Bio-Rad). For this purpose, two commercially accessible FI siRNAs were used each targeting a distinct area of FI gene:

1. FI siRNA_1 (Hs_FI_1):

   Sequence of target: 5′-AACTACCGTATCAGTGCCCAA-3′
2. FI siRNA_2 (Hs_IF_2):

   Sequence of target: 5′-TAAGACAATGTTCATATGCAA-3′

4.10.1.2 FI Gene overexpression using an FI expression construct (II)

To overexpress the FI gene, cultured cSCC cells were transfected with the FI expression construct (pcDNA3.1_FI) or an empty vector as control (pcDNA3.1). The transfection reagent used was Lipofectamine 3000 (Invitrogen).

4.10.2 Inhibitor assays

4.10.2.1 Specific inhibition of MAPKs (III)

In order to specifically inhibit MAPKs, cSCC cell cultures were treated with MEK1/2 inhibitor PD98059 (30 μM; Calbiochem), and p38 inhibitors SB203580 and BIRB796 (both 10 μM; Calbiochem) for 24 hours.

4.10.2.2 Targeted inhibition of FD (III)

To hinder the function of FD in cultured cSCC cells, small-molecule factor D inhibitor danicopan also known as ACH-4471 or ALXN2040 (MedChemExpress) was utilized. Plating of four cSCC cell lines (UT-SCC-12A, -59A, -91A, and -105) on 96-well plates was done using 10% fetal calf serum (4000 cells per well). Following an incubation period of 24 hours, the addition of various concentrations, i.e., 0.1 μM, 1 μM and 10 μM of small-molecule FD inhibitor danicopan to the wells was carried out in presence of serum-free medium. The vehicle control for control cell cultures was DMSO.

4.10.3 Cytokine assays (III)

To examine the effect of cytokines on cSCC cells, the cultures were treated with IFN-γ (100 U/mL) (Promega) or IL-1β (10 ng/mL) (Calbiochem) for 24 hours.

4.10.4 Cell proliferation assays (II-III)

cSCC cell cultures with a confluence of 50% were transfected with recombinant FI expression construct (pcDNA3.1_FI) or an empty pcDNA3.1 vector as control. Plating of the cultured cells on a 96-well plate was done after an overnight incubation period. Cell proliferation was tracked with IncuCyte S3 real-time cell imaging system (Essen Bioscience), and the data were analyzed running the IncuCyte S3 software (Essen Bioscience). The index used for cSCC cell proliferation was relative confluence.

4.10.5 Cell invasion assay (II)

cSCC cell cultures with a confluence of 50% were transfected with FI siRNA_1, FI siRNA_2 or negative control siRNA as well as recombinant FI expression construct (pcDNA3.1_FI) or negative expression vector, i.e., pcDNA3.1, and after an overnight incubation period, plating of the cells on an ImageLock 96-well plate coated with collagen type I (5 µg/cm^2, PureCol; Advanced BioMatrix) or Matrigel (100 µg/ml; Corning) was carried out. cSCC cells were left for 24 hours to attach to the collagen or Matrigel coat. The monolayer was then scratched by the IncuCyte® 96-well WoundMaker Tool (Essen Bioscience), and a second layer of collagen or Matrigel (4 mg/ml) was added to the cells and wounds to create a 3D matrix. The collagen type I solution (PH: 7.4) was a mixture of collagen type I (3.1 mg/ml; PureCol), 5× Dulbecco's Modified Eagle Medium (5×DMEM), HEPES buffer (0.2 M), and NaOH (1 M) at a proportion of 7:2:1 for the first three ingredients. The second coat of collagen I or Matrigel was allowed to get polymerized at +37 Celsius for two hours. Finally, the 0.5% fetal calf serum (FCS) medium was added on top. Applying the IncuCyte S3 real-time cell imaging system (Essen Bioscience), the wound closure was monitored. The acquired data were analyzed with IncuCyte S3 software (Essen Bioscience) using the relative wound density as an indicator of cell invasion.

4.11 Human cSCC xenografts (II-III)

UT-SCC-7 cells were transfected with control or FI siRNA_1. After 3 days of incubation, the cells were detached. Same process was done for UT-SCC-7 cells that were not transfected (FD experiment). In both FI and FD experiments, 5×10^6 cSCC cells in 100 µl of PBS were subcutaneously injected into the back of 6 to 8 weeks old female mice with severe combined immunodeficiency (SCID/SCID) (CB17/Icr-Prkdcscid/IcrIcoCrl) (Charles River Laboratories) (n = 8 for control and n = 6 for FI knockdown tumors; n=1 for FD experiment). The mice were sacrificed after 18 days and the tumors were excised. To prepare the tumors for immunostaining, they were fixed in fresh phosphate-buffered 4% paraformaldehyde at +4 Celsius overnight, and embedded in paraffin afterwards. Eventually, the processed tumors were dissected into 5-µm-thick slices and stained immunohistochemically.

4.12 Statistical analysis (II-III)

The statistical analysis between RT-qPCR sample groups in FI paper (II) was done with two-tailed Student’s t-test, and in FD paper (III) with Mann-Whitney U-test. Two-tailed Student’s t-test was also used for statistical analysis of the significance

of difference between sample groups in proliferation (II, III) and invasion (II) assays. To analyze the variations of FD immunostaining intensities, $\chi 2$ test was applied (III).

5 Results

5.1 Expression of FD in cSCC (III)

5.1.1 FD expression is upregulated in cSCC cultures and tumors (III)

The results of RT-qPCR analysis showed that the expression of FD mRNA is notably higher in cSCC cells and cSCC tumors in comparison with NHEKs and normal skin, respectively (III, Figure 1A and 1C). The Western blot analysis of the conditioned media of cultured cSCC cells revealed that the expression of FD protein is remarkably greater in specific cSCC cell lines (primary: UT-SCC-12A, -91A; metastatic: UT-SCC-7, -115) vs. NHEKs (III, Figure 1B). The IHC analysis of human cSCC xenograft tumor sample originated from the injection of metastatic UT-SCC-7 cell line into the back of SCID/SCID female mice verified that the cytoplasmic expression of FD is potently higher in the invasive margins of cSCC tumor (stronger intensity of FD staining) (III, Figure 1D).

Altogether, these findings disclosed that FD expression is significantly upregulated in cSCC cells and tumors in a specific manner.

5.1.2 FD expression by cSCC cells *in vivo* (III)

To evaluate the expression of FD by tumor cells in human cSCC *in vivo*, the immunostained TMA samples of progressive stages of skin carcinogenesis were semiquantitatively analyzed. The studied tissue samples consisted of normal skin (n=80), premalignant lesion AK (n=65), cSCCIS (n=61), non-metastatic primary cSCC (n=140), metastatic cSCC (n=70), metastases of cSCC (n=9), and RDEBSCC (n=16). Cytoplasmic expression of FD was particularly intense in the highly differentiated tumor cells and invasive margins of all forms of cSCC regardless of their metastatic condition (III, Figures 2A, 2B, 2C, 2D). The intensity of FD expression was notably fainter in normal skin, AK, and cSCCIS (III, Figures 2E, 2F, 2G), and there was a homogenous distribution of staining in the entire epidermis. FD labeling intensity was remarkably stronger in the invasive tumor islands of the metastatic cSCC compared to the neighboring epidermis (III, Figure

2H). The scoring system for this semiquantitative IHC analysis was based on the intensity of FD immunostaining in cytoplasm: negative = (-), weak = (+), moderate = (++) and strong = (+++). The results of the analysis demonstrated that most cSCC tumors, including non-metastatic and metastatic cSCCs as well as cSCC metastases and RDEBSCCs, scored (+++) irrespective of their aggressiveness status (III, Figure 2I). FD labeling intensity in non-metastatic primary cSCC was interestingly as strong as RDEBSCC, cSCC metastases and metastatic cSCC (III, Figure 2I). Besides, normal skin, AK, and cSCCIS samples mainly scored (-) (III, Figure 2I). These findings show that there is an upregulation of FD expression by cSCC cells *in vivo* during the process of epidermal carcinogenesis.

5.1.3 FD expression in HaCaT and tumorigenic Ha-Ras-Transformed HaCaT-derived cell lines (III)

The expression of FD in HaCaT and tumorigenic Ha-Ras-Transformed HaCaT-derived cells was studied to investigate the importance of FD in an *in vitro* model of various stages of epidermal carcinogenesis. HaCaT cells are non-tumorigenic immortalized cells established from human epidermal keratinocytes. The Ha-Ras–transformed HaCaT-derived cell lines used for this experiment comprised A5, II-4 and RT3 cells. To elaborate, A5 cells generate benign tumors, and II-4 and RT3 cells form primary invasive and metastatic malignancies *in vivo,* respectively (Boukamp et al, 1990; Mueller et al, 2001). Also, it has been shown that A5 cells have a significantly lower basal ERK1/2 activity compared with II-4 and RT3 cells (Toriseva et al, 2012). Results indicated that FD mRNA expression is considerably higher in benign A5 cells vs. non-tumorigenic HaCaT cells in which functional p53 gene is absent (III, Figure 4A). Also, the benign A5 cells interestingly expressed notably greater FD mRNA levels than primary invasive II-4 and metastatic RT3 cells (III, Figure 4A). In alignment with the RT-qPCR findings, the Western blot analysis revealed that A5 cells produce greater amount of FD protein in comparison with II-4 and RT3 cells (III, Figure 4B). In total, the outcomes provided proof that there is an upregulation of basal expression of FD in specifically early stages of skin carcinogenesis which is suppressed by constitutive ERK1/2 signaling activity.

5.2 Regulation of FD in cSCC (III)

5.2.1 FD expression in cSCC cultures is upregulated by IL-1β and IFN-γ (III)

To unveil how FD expression is regulated by inflammatory cytokines, cSCC cell lines with metastatic potential, i.e., UT-SCC-7 and -59A were treated with

interferon-γ (IFN-γ) and interleukin-1β (IL-1β) for 24 hours. The results displayed a noticeable upregulation of FD mRNA expression in IFN-γ and IL-1β treated cSCC cells (III, Figure 3A).

5.2.2 Expression of FD in cSCC cultures is regulated by p38 MAPK signaling pathway (III)

To further evaluate how FD expression is regulated in cutaneous SCC, treatment of the cSCC cell line 12A (UT-SCC-12A) with MEK1/2 inhibitor (PD98059) and p38 inhibitors (SB203580, BIRB796) was fulfilled for 24 hours. SB203580 is a selective inhibitor for p38α and p38β, and BIRB796 inhibits all isoforms of p38 including p38α, p38β, p38γ and p38δ. The Western blot analysis showed that there is a significant downregulation of basal FD expression in SB203580 and BIRB796 treated cSCC cells compared with the control cultures. However, the basal expression of FD protein in cSCC cells was not affected by PD98059 (III, Figure 3B). A potent reduction in the protein levels of a downstream effector of p38/MAPK signaling, namely p-CREB, was observed in SB203580 and BIRB796 treated cells which verifies the efficiency of these two p38 inhibitors. Also there was a remarkable inhibition of ERK1/2 signaling activity as measured by p-ERK1/2 protein levels that confirmed the proper function of PD98059 inhibitor (III, Figure 3B). The RT-qPCR analysis demonstrated that FD mRNA expression significantly decreases in SB203580 treated cSCC cells in comparison with control cultures (III, Figure 3C). As it has previously been indicated that cSCC cells do not express p38β and p38γ (Junttila et al, 2007), these results provide evidence that only p38α/MAPK takes part in the regulatory process of basal expression of FD in cSCC.

5.3 Molecular functions of FD and FI in cSCC (II-III)

5.3.1 Targeted FD inhibition suppresses cSCC cell proliferation via inhibition of ERK1/2 signaling activation (III)

cSCC cell line (UT-SCC-12A) was treated with the control vehicle DMSO or several dilutions of small-molecule factor D inhibitor danicopan (also known as ACH-4471) to investigate the effect of targeted FD inhibition on the proliferation of cSCC cells. The results of Western blot analysis demonstrated markedly decreased levels of a protein cleavage derivative of FB with a molecular weight of 60 kDa in danicopan-treated cSCC cultures after targeted FD inhibition (III, Figure

5A). There was a significant inhibition in proliferation of high FD expressing cSCC cell lines (UT-SCC-12A and 91A) treated with danicopan vs. the control group (III, Figure 5B). Interestingly, the cell response to danicopan treatment was revealed to be dose-dependent (III, Figure 5B). Also, it was elucidated that low FD expressing cSCC cells UT-SCC-59A and -105 were not affected by danicopan in terms of cell proliferation capacity (III, Figure 5B). In the UT-SCC-12A cell line the activity of the ERK1/2 signaling, measured by levels of p-ERK1/2 protein, was indicated to be hampered following targeted inhibition of FD (III, Figure 5C).

5.3.2 Gene expression profile alteration in cSCC cultures following FI knockdown (II)

To knock down FI expression by cSCC cells, cSCC cultures (UT-SCC-7, -59A, -118) were transfected with FI siRNA_1. Three days following transfection, mRNA sequencing was done (II, Figure 1). The heatmap created based on the mRNA-seq data analysis showed a significant reduction in the expression of CFH (a cofactor of FI) and other components of the complement system, i.e., C1QL1, C3 in addition to FI itself post-FI knockdown. On the contrary, the knockdown of FI, led to potent upregulation of FP or properdin and CD55 or DAF expression (II, Figure S1). The expression of other cofactors of FI (CD35, i.e., CR1 and CD46, i.e., MCP) was not affected significantly (II, Figure S1). The significantly regulated genes after FI knockdown were related to the Ingenuity Pathway Analysis (IPA) biofunctions *Growth of malignant tumor* and *Proliferation of cells*. Gene Ontology (GO) terms and Reactome data disclosed remarkably regulated genes in GO terms *Extracellular matrix component* and *Metallopeptidase activity* as well as Reactome *Degradation of extracellular matrix* following FI knockdown (II, Figure 1A). The heatmap of the top downregulated genes post-FI knockdown revealed *MMP10* and *MMP13* amongst the first 33 (II, Figure 1B). Besides, more in-depth analysis of the above three networks of GO terms and Reactome unveiled the significantly regulated genes following FI knockdown, and specific matrix metalloproteinase (MMP) coding genes associated with tumor invasion were found to be amongst the top downregulated (II, Figure 1C). The created heatmap on account of mRNA sequencing analysis demonstrated five MMP genes, i.e., *MMP2*, *MMP7*, *MMP10*, *MMP13* and *MMP14* that were significantly downregulated after FI knocdown (II, Figure 1D).

5.3.3 FI knockdown downregulates MMP-2 and -13 expression and attenuates cSCC cell invasion (II)

cSCC cell cultures were transfected with control or FI siRNAs (FI siRNA_1 or FI siRNA_2). The results of RT-qPCR analysis displayed a significant decrease in the

mRNA expression levels of MMP2 and MM13 in one primary (UT-SCC-105) and one metastatic (UT-SCC-7) cSCC cell line following FI knockdown (II, Figure 2A and Figure S2). Also, the protein expression of MMP-2 and MMP-13 was shown to be downregulated in metastatic cSCC cells UT-SCC-7 and -59A after knockdown of FI (II, Figure 2B). In addition, there was a significant reduction in the invasion capability of metastatic cSCC cells, i.e., UT-SCC-59A through collagen type I post-FI knockdown (II, Figure 2C). Similarly, the invasion of primary cSCC cell lines UT-SCC-105 was remarkably attenuated after FI knockdown (II, Figure S3).

5.3.4 FI knockdown inhibits MMP-2 and -13 expression in cSCC cells *in vivo* (II)

Generation of human cSCC xenograft tumors in SCID/SCID mice was performed following FI knockdown in cSCC cells UT-SCC-7 using FI siRNA_1 as mentioned in the materials and methods section. Also, contemporal incubation of the cSCC cells UT-SCC-7 following FI siRNA transfection proved that the efficiency of FI knockdown is persistent up to 15 days post-transfection.

cSCC xenograft samples were immunostained for MMP-2 and MMP-13. The results revealed stronger intensities of MMP-2 and MMP-13 staining in the control group compared to the FI siRNA_1-transfected tumors (II, Figure 3A). Besides, there was a higher quantity of cSCC cells positive for MMP-2 and MMP-13 in the control tumors in comparison with the FI knockdown group (II, Figure 3A). The semiquantitative IHC analysis demonstrated that MMP-13 labeling was moderate (++) in 37% and strong (+++) in 63% of the control xenograft tumors, while 17% of the FI siRNA_1-transfected tumors scored weak (+), 33% moderate (++), and 50% strong (+++) (II, Figure 3B). The expression of MMP-2 scored moderate (++) in 43% and strong (+++) in 57% of the control group, whereas the staining intensity was weak (+) in 40% and moderate (++) in 60% of the FI siRNA_1-transfected tumors. There was no strong (+++) scoring for MMP-2 labeling in the FI knocked down group (II, Figure 3C).

5.3.5 FI overexpression upregulates ERK1/2 signaling activation and promotes cSCC cell proliferation (II)

cSCC cells were transfected with control empty pcDNA3.1 vector or recombinant FI expression construct pcDNA3.1_FI to examine the role of FI in the proliferation of cSCC. Two metastatic cell lines (UT-SCC-7 and -59A) were studied in this functional assay using the IncuCyte S3 real-time cell imaging system, and relative confluence was used as an index for cell proliferation (II, Figure 4A). The results indicated a significant upregulation in proliferation of cSCC cells overexpressing

FI vs. the control negative vector-transfected group. The Western blot analysis showed an increase in ERK1/2 activity, measured by p-ERK1/2 protein levels, in cSCC cell line UT-SCC-59A following FI overexpression (II, Figure 4B).

5.3.6 FI overexpression upregulates MMP-2 and -13 expression and enhances cSCC cell invasion (II)

cSCC cell cultures were transfected with control vector pcDNA3.1 or FI expression construct pcDNA3.1_FI to illuminate the functional mechanism of FI in cSCC invasion. There was a potent overexpression of FI protein along with MMP-2 and MMP-13 proteins in the media of cSCC cell lines transfected with recombinant FI expression construct compared to the control group (II, Figure 4C). In addition, remarkable upregulation of cSCC cell invasion capacity through both collagen type I and Matrigel was observed after overexpression of FI (II, Figure 4D and Figure 4E).

5.4 Complement-targeted therapeutics (I)

Since complement system is a crucial pillar of the innate immunity and plays a significant part in numerous inflammatory diseases, it has been targeted as a promising therapeutic solution for these disorders (Bareke et al, 2018). Currently, there are many complement-targeted therapeutics in different phases of clinical studies or preclinical stage (I, Table 1 and 2) (Ricklin et al, 2018). It is expected that pinpointing distinct complement system components in various malignancies such as cutaneous SCC would provide an opportunity to aim at them using these drugs and test the potential of this novel strategy for cancer immunotherapy. At present, the receptor of the central component of complement system C5a is the only complement target in clinical trial for cancer immunotherapy. The drug tested in this phase I trial is IPH5401 which is a C5aR1 antibody whose effect on advanced solid tumors is being examined in combination therapy with a PD-L1 (programmed cell death ligand-1) inhibitor called durvalumab (I, Table 1). Avacopan/CCX168 is a small-molecule in phase II and III clinical trials that targets C5aR1, but its indication is in inflammatory conditions and not cancer (I, Table 1). Currently, there are five other C5aR1-targeting complement therapeutics in preclinical stage of which only two anti-C5aR1 peptides are being investigated for cancer therapy (I, Table 2); PMX-53 is in preclinical trial for SCC in combination therapy with paclitaxel (PTX), and AcF-(OPdChaWR) is in preclinical testing for cervical cancer (I, Table 2). Out of seven anti-C5a therapeutic compounds (two in clinical and five in preclinical phase), only one named AON-D21 or NOX-D21 has been studied for oncotherapy (I, Table 1 and 2); NOX-D21 is an L-RNA aptamer

(Spiegelmer) in preclinical stage that has shown promising results for lung malignancy in combination therapy with a PD-1 inhibitor (I, Table 2). Furthermore, there is only one complement therapeutic in preclinical trial that targets C3a receptor (C3aR); this small-molecule is called SB290157, and is being tested for melanoma and lung cancer indications (I, Table 2).

The main challenges in the field of complement-targeted therapeutics comprise 1) dosing of the drugs because of large-scale concentration and turnover of complement compounds in circulation 2) drug safety and 3) lack of transparency in molecular mechanism of targeted disorders (Harris et al, 2018). To tackle these problems, innovative strategies have been established. Novel recycling antibodies have been engineered in a way that their affinity to their antigen targets varies in reaction to pH-switch. These antibodies have a stronger affinity towards their targeted antigens in the neutral pH of plasma, i.e., pH 7.4, which smooths and accelerates the process of their intracellular transportation via endosomes. In reaction to the acidity of the endosomal environment, i.e., pH 6, the antibody is then resurfaced to the cell membrane after antigen release, hence the name recycling. The next generation anti-C5 antibodies Ravalizumab (ALXN1210) and Crovalimab (SKY59/RG6107/RO7112689) are two instances of this strategy (I, Table 1). Another approach is to develop antibodies that target the complement neoepitopes emerging on complement protein surfaces following their conformational transformation. The anti-C5a antibodies IFX-1 and ALXN1007 that specifically aim at C5a (not C5) are exemplars of this tactic (I, Table 1). As systemic suppression of the complement system makes the patient susceptible to infectious diseases, localized delivery of complement therapeutics can significantly alleviate the safety issue, enhance the drug efficacy, and assist drug dosing minimization. A decent example of this approach is the C3 convertase inhibitor Mirococept (or APT070) that has cytotopic localization, and specifically targets the cell membrane (I, Table 1). Computational structure-based drug design has revolutionized the world of drug development and in fact can be considered as a bypass in the tedious road to drug discovery. It has been used to design drug entities including proteins, peptides and small-molecules that aim at critical interaction sites on proteins; Small-molecule inhibitors of FD (danicopan or ACH-4471) and FB (LNP023) as well as next generation anti-C5 peptide RA101495 are instances of this approach (I, Table 1). Furthermore, the strategy of targeting responsible genes instead of proteins takes advantage of antisense oligonucleotide and siRNA drugs to knock down specific genes coding for complement proteins. The next generation anti-C5 oligonuleotides cemdisiran (ALN-CC5) and zimura (ARC1905) and also anti-FB oligonucleotide IONIS-FB-LRx are exemplars of therapeutics in this category (I, Table 1).

6 Discussion

6.1 The role of alternative complement factors in cSCC progression

6.1.1 Evaluation of the expression of FD in cancer and particularly in cSCC

Earlier research has shown that carcinoma cell lines including astroglioma U105-MG and gastric tumor-derived cells are able to synthesize FD (Kitano et al, 2002; Barnum et al, 1992). Also, FD produced by breast adipose cells has been demonstrated to boost the growth and proliferation of human mammary carcinoma and promote its stem cell features (Goto et al, 2019). According to the latest analysis of The Cancer Genome Atlas (TCGA), increased FD expression was detected in most carcinomas expressing high FB levels (Roumenina et al, 2019). Besides, elevated expression levels of FD were found to be related to poor prognosis of several malignancies such as glioblastoma, uveal melanoma, adrenocortical cancer, glioma (low-grade), and thyroid cancer (Roumenina et al, 2019).

Here, the function of FD in cSCC has been studied. Although earlier research has demonstrated the production of FD by hepatic cells, macrophages, adipocytes, and some carcinoma cells, its synthesis in normal skin keratinocytes or keratinocyte-derived carcinomas is unknown (White et al, 1992; Maibaum et al, 2016). Our findings display elevated expression levels of FD mRNA in cSCC tumors and cSCC cell lines vs. normal skin and NHEKs, respectively. Furthermore, increased protein expression of FD by specific cSCC cell lines was discovered via Western blot analysis; Primary cSCC cell lines UT-SCC-12A and -91A and metastatic cSCC cell lines UT-SCC-7 and -115 showed the greatest levels of FD expression. In human xenograft tumors originated from metastatic cSCC cells, i.e., UT-SCC-7, remarkable FD expression was noticed in the invasive edges of the cancer cells. Taken together, these observations indicate that there is an upregulation of the expression of FD in cSCC cells *in vivo* in a specific manner.

Immunohistochemical TMA analysis comprising a comprehensive panel of primary non-metastatic sporadic cSCC, primary metastatic sporadic cSCC,

metastases of cSCC, RDEBSCC, normal skin, AK, and cSCCIS disclosed specific FD labeling in the cytoplasm of cSCC cells *in vivo*. The intensity of FD labeling was markedly stronger in every cSCC tumor stated above, in comparison with cSCCIS, AK, and normal skin. The FD staining intensity was not reliant on the metastatic condition of the cSCC tumor. Intense expression of FD was specifically detected in highly differentiated cSCC cells and the invasive margins of tumor, while weak intensity of FD labeling with a homogenous distribution was noted in the entire epidermis in cSCCIS, AK and normal skin. In the TMA samples of primary metastatic sporadic cSCC with intense localized FD staining in tumor invasive islands, the intensity of labeling was clearly weaker in the neighboring epidermis. In total, these findings elucidate that FD is expressed in the invasive cSCC tumor cells, islands and edges in a specific and distinct manner, and propose an important function for FD in the process of cSCC progression.

Inactivation of the p53 due to mutation of the tumor suppressor gene *TP53* in skin keratinocytes is an early phenomenon in the process of cutaneous carcinogenesis, which leads to accrual of tumorigenic mutations necessary for cSCC development and progression (Piipponen et al, 2021). To evaluate the function of FD as a putative biomarker for skin carcinogenesis, a representative model of various stages of epidermal carcinogenesis *in vitro* was established and examined (Boukamp et al, 1988; Boukamp et al, 1990; Mueller et al, 2001). In HaCaT series all cell lines had mutated *TP53*, and Ras was activated in A5, II-4, and RT3 cells.

The basal FD protein and mRNA expression was markedly higher in benign Ha-Ras-transformed tumorigenic HaCaT-derived cell line A5 compared with the non-tumorigenic immortalized HaCaT cells. The FD expression was interestingly greater in the benign A5 cell line vs. the primary invasive oncogenic II-4 and metastatic RT3 cell lines, that both display increased basal ERK1/2 activity (Toriseva et al, 2012). These results unveil that mechanism of FD expression in cSCC is complicated and not solely reliant on Ha-Ras transformation or *TP53* inactivating mutation. In addition, the outcomes suggest FD as a potential molecular marker for the progression of cSCC during the primary stages.

6.1.2 Evaluation of the regulation of FD in cSCC

A representative histopathological feature of cSCC is the inrush of inflammatory blood cells in cancer microenvironment (Ratushny, 2012; Madan et al, 2010). The secretion of inflammatory cytokines by these blood cells triggers generation of invasion-related proteinases leading to the promotion of cSCC invasion and progression (Riihilä et al, 2021). Moreover, the cancer cell-secreted cytokines regulate the regional immune response to the tumor cell antigens (Lee et al, 1998;

Kim et al, 1995). In this research, it was discovered that the expression of FD by cSCC cell lines is enhanced by two cytokines, namely IL-1β and IFN-γ. In addition, it was shown that p38 MAPK pathway (specifically p38α MAPK) regulates the basal FD expression in cSCC cultures, which endorses the results of earlier studies indicating the importance of p38 MAPK signalling in progression of cSCC (Junttila et al, 2007; Johansson et al, 2000; Piipponen et al, 2016).

6.1.3 Evaluation of molecular functions of FD and FI in cSCC

To illuminate the molecular function of FD in the proliferation of cSCC, small-molecule FD inhibitor danicopan or ACH-4471 was applied (Maibaum et al, 2016; Yuan et al, 2017). Targeted FD inhibition in cSCC cultures expressing high levels of FD led to significant inhibition of the proliferation of cSCC cells (**Table 4**) and blockade of ERK1/2 signaling activity. Danicopan showed no suppressive influence on proliferation of low-level FD expressing cSCC cells. In addition, the cleavage of FB was noticed to be markedly decreased following FD targeted inhibition, which is in accordance with the molecular mechanism of FD in the alternative complement pathway, namely FD-mediated cleavage of FB. Taken together, these observations provide proof for the function of FD in the progression of cutaneous SCC via ERK1/2 regulation, and verify the specificity and sensitivity of small-molecule FD inhibitor danicopan as a putative targeted therapeutic for primary invasive cSCCs with high FD expression.

The results of the FI study clarified and elaborated the functional mechanism of FI in progression of cSCC. The gene expression profile alteration in cSCC cultures following FI knockdown was examined via mRNA sequencing, and the IPA biofunction *Proliferation of cells* was found to be significantly downregulated, which is compliant with our earlier findings demonstrating the effect of FI on the proliferation of cSCC cells (Riihilä et al, 2015) (**Table 4**). Furthermore, genes markedly regulated post-FI knockdown were associated with GO terms *Extracellular matrix component* and *Metallopeptidase activity* in addition to Reactome *Degradation of extracellular matrix*, proposing an important function for FI in cSCC progression and invasion. More in-depth analysis of the above three GO terms and Reactome networks, disclosed marked reduction in the expression level of several *MMP* genes coding for cancer invasion-related MMPs, i.e., *MMP2*, *MMP7*, *MMP10*, and *MMP13* after FI knockdown. We then concentrated on MMP2, i.e., gelatinase-A and MMP13, i.e., collagenase-3 as they both have been displayed to be expressed by cSCC cells and promote their invasion capacity (Kivisaari et al, 2008; Airola et al, 1997; Johansson et al, 1997; Ala-aho et al, 2004; Dumas et al, 1999; Lee et al, 2013;

Fundyler et al, 2004; Ayva et al, 2013). The RT-qPCR analysis validated the significant reduction in *MMP2* and *MMP13* mRNA expression levels following FI knockdown in both metastatic and primary cSCC cell lines. Besides, FI knockdown led to decreased protein expression of MMP-2 and -13 as well as marked suppression of cSCC cell invasion through collagen type I (**Table 4**). These results were verified applying the counter experiment of FI overexpression with an FI expression construct named pcDNA3.1_FI. FI overexpression led to ERK1/2 activation and significant elevation of cSCC cell proliferation which was in agreement with the previous studies (Riihilä et al, 2015) (**Table 4**). Also, increased expression level of MMP-2 and -13 proteins along with significant upregulation of cSCC cell invasion through Matrigel and collagen type I was detected after FI overexpression (**Table 4**). Altogether, these observations provide proof for the molecular function of autocrine FI as a potent promoter of invasion in cSCC (**Table 4**).

Table 4. Functional role of FD and FI in cSCC progression.

FD	FI
Cell proliferation ↑	Cell proliferation ↑
	Cell invasion ↑
	Cell migration ↑
	Tumor growth ↑

It has been reported that various MMPs are overexpressed by stromal and carcinoma cells in keratinocyte malignancies (Riihilä et al, 2021). MMP-13 has been demonstrated to be particularly produced by stromal fibroblasts and cancer cells in head and neck and cutaneous SCC with no expression in cSCCIS, AK, and normal epidermal keratinocytes (Kivisaari et al, 2008; Airola et al, 1997; Johansson et al, 1997). MMP-13 upregulates cSCC cell survival and invasion, and promotes the growth of human cSCC xenograft tumors *in vivo* (Ala-Aho et al, 2004). Increased expression of MMP-13 correlates with local invasion of the head and neck SCC and is associated with poor survival (Stokes et al, 2010; Luukkaa et al, 2006). Increased expression of MMP-2 has been spotted in the invasive edges of cSCC, while the expression was noticed to be weaker in cSCCIS and AK (Dumas et al, 1999; Lee et al, 2013; Fundyler et al, 2004). Also, MMP-2 is found to be expressed in the peritumoral epidermis in cutaneous SCC, proposing its connection to the UV-caused harm (Ayva et al, 2013). Dormant MMP-2 is specifically

targeted and activated by MMP-14, i.e., MT1-MMP, and it is revealed that the expression of both MMP-2 and -14 is associated with cSCC cell invasion (Kerkelä et al, 2001; Roh et al, 2015). Correspondently, elevated expression level of MMP-2 correlates with cSCC invasion (Chen et al, 2020). Also, dormant MMP-13 can be activated by MMP-2 and promote the invasion capacity of cSCC (Knäuper et al, 1996).

The serine protease FI inhibits all pathways of the complement system by blocking C3 and C5 convertases via splitting their C3b or C4b subunits. C3b cleavage in the alternative pathway results in the inhibition of the amplification loop and iC3b production at the same time. As C3b has an essential role in many biological functions, its synthesis and inactivation (iC3b production) are tightly regulated. FI activity is dependent on its cofactors namely CFH, CD35 (CR1), CD46 (MCP), and C4BP (Zipfel et al, 2009; Goldberger et al, 1984; Nilsson et al, 2011). As there are no endogenous FI inhibitors *in vivo*, its activity is regulated by the accessibility of its specific substrates (Nilsson et al, 2011). Accordingly, the results of this research elucidate that cSCC cultures highly express two FI cofactors, i.e., CD46 and CFH. This observation is in agreement with the former study revealing that the C3 generated by cSCC cultures is inactivated, demonstrating that the tumor cell-produced FI is functionally active (Riihilä et al, 2014). Additionally, an earlier study unveiled that FI knockdown leads to C3 activation and C3b accumulation on cancer cell membrane in human cSCC xenograft tumors *in vivo*, which is in accordance with the above-mentioned notions (Riihilä et al, 2015). It is quite probable that the promoting effect of autocrine FI on the invasion and proliferation of cSCC cells is linked with C3. Interestingly, it has been discovered that cSCC cells also express C3 which has been indicated to have an intracellular function, and be splitted into C3b and C3a fragments (Liszewski et al, 2013; Reichhardt et al, 2018). Nevertheless, the intracellular role of C3 in cutaneous SCC is yet to be fully investigated.

Previous research has shown that human normal skin keratinocytes express various components of the complement system including components of the lytic (terminal) cascade as well as FI (Dovezenski et al, 1992; Pasch et al, 2000; Timar et al, 2007). However, former and current studies display that there is no expression of the lytic cascade components, i.e., C6, 7, 8, and 9 in cSCC cultures. On this basis, it is possible that unlike normal skin keratinocytes, the tumor cells in cSCC are less prone to complement dependent cytotoxicity.

6.2 Complement components in precision medicine

6.2.1 Complement components as biomarkers of cancer

The local (autocrine) complement system components comprising complement factors and complement inhibitors have different roles beyond systemic complement activity. They can promote the growth, angiogenesis, proliferation, invasion and migration of tumor cells in cancers including cSCC, and assist the cancerous cells to evade from immune defense (Mamidi et al, 2017; Afshar-Kharghan et al, 2017; Reis et al, 2018; Roumenina, 2019; Bareke et al, 2018; Kolev et al, 2022; West & Kemper, 2023). Accordingly, there is a possibility to employ them as biomarkers of cancer to improve precision diagnosis, classification, prognostication and therapy of various malignancies.

6.2.2 Complement-targeted cancer therapy

More detailed understanding of the intricate interaction between the tumor cells and the immune response has unveiled the essential function of immune system in development and progression of malignancies (Bareke et al, 2018). As the backbone of the innate immune system, complement network plays a fundamental part in cancer progression in addition to inflammatory disorders (Mamidi et al, 2017; Afshar-Kharghan et al, 2017; Reis et al, 2018; Roumenina, 2019; Bareke et al, 2018; Kolev et al, 2022; West & Kemper, 2023). Currently, many complement-targeted therapeutics are under investigation in different phases of clinical studies and preclinical settings. However, these therapeutics are mainly focused on inflammatory conditions. For instance, clinical studies on 3 small-molecule factor D inhibitors for inflammatory disease indications are ongoing. Danicopan (ACH-4471) is being tested for targeted treatment of geographic atrophy due to age-related macular degeneration, immune complex membranoproliferative glomerulonephritis and C3 glomerulopathy (all in phase II), paroxysmal nocturnal hemoglobinuria (PNH; phase II and III), and COVID-19 (Big Effect Trial or ACTIV-5; phase II). BCX9930 and vemircopan, i.e., ACH-5228/ALXN2050 are other small-molecule inhibitors of FD that are under clinical trials for PNH (both in phase II). Latest research has disclosed the significance of autocrine expression of complement system components in comparison with systemic complement production originating from liver (Riihilä et al, 2014; Riihilä et al, 2015; Riihilä et al, 2017; Riihilä et al, 2020; Viiklepp et al, 2021). Nevertheless, merely anaphylatoxin C5a and its receptor C5aR1, anaphylatoxin receptor C3aR, and complement inhibitor CFH have been aimed at for oncotherapy. IPH-5401 is a

C5aR1 inhibiting antibody currently in clinical testing (phase I) for targeted treatment of advanced solid cancers in combination with durvalumab which is a PD-L1 blocking antibody (STELLAR-001). The idea is on account of previous preclinical studies that inhibition of C5aR leads to downregulation of PD-L1 expression in a rodent model of lung tumor (Corrales et al, 2012). By the same token, it has been recently elucidated that the combination of a PEGylated anti-C5a L-aptamer (Spiegelmer) named AON-D21 (also known as NOX-D21) and a PD-1 inhibitor impedes the growth and metastasis of lung cancer more effectively than single therapy with a PD-1 inhibitor under preclinical settings. AON-D21 is also under clinical trial (phase I) in healthy men (ClinicalTrials.gov Identifier: NCT05018403). Aside from AON-D21, other successful complement therapeutics in preclinical stages are as follows: SB290157 is an anti-C3aR small-molecule that is demonstrated to hamper the metastasis and growth of lung cancer and melanoma in mice. Cyclic anti-C5aR1 hexapeptides AcF-(OPdChaWR) and PMX-53 have been indicated to inhibit cervical and squamous cell carcinoma, respectively. The efficacy of the latter has been shown to be reliant on simultaneous paclitaxel (PTX) chemotherapy. Also, a recombinant anti-CFH antibody has been displayed to result in the CDC, i.e., complement dependent cytotoxicity of tumor cells *in vitro* along with inhibition of lung cancer growth in mice via causing a conformational transformation in CFH (Bushey et al, 2016).

The results of the current study introduce FD as a promising target for precision therapy of cSCC, and for the first time in science propose small-molecule FD inhibitor danicopan as a highly specific and sensitive complement-targeted therapeutic for treatment of primary invasive cutaneous SCCs expressing ample levels of FD.

7 Summary/Conclusions

In this study, the molecular function of components FI and FD in cSCC development and progression was investigated. Also, the role of complement components as putative novel targets for precision oncotherapy was studied. The mRNA-seq analysis revealed marked regulation of specific genes in IPA biofunctions *Growth of malignant tumor* and *Proliferation of cells*, GO terms *Extracellular matrix component* and *Metallopeptidase activity* along with Reactome *Degradation of extracellular matrix* following FI knockdown. Analysis of the last three pathways elucidated downregulation of several invasion-related MMP-coding genes post-FI knockdown. The downregulation of MMP-2 and -13 was verified at levels of mRNA (via RT-qPCR), protein (via Western blot), and tissue (via IHC). FI knockdown attenuated cSCC cell invasion through collagen type I. FI overexpression upregulated the expression of MMP-2 and -13, promoted invasion through Matrigel and collagen type I, and enhanced proliferation and ERK1/2 signaling activity in cSCC cultures. In regards to FD, RT-qPCR analysis disclosed significant elevation of FD mRNA levels in cSCC cells and cSCC tumors vs. NHEKs and normal skin, respectively. Western blotting indicated high FD expression by specific cSCC cell lines. The IHC analysis showed more intense FD labeling in the invasive margins of human cSCC xenograft tumors. Stronger FD staining was noted in the cytoplasm of tumor cells in RDEBSCC, cSCC metastases, and metastatic and primary cSCCs compared to cSCCIS, AK and normal skin. FD expression in cSCC cultures was regulated by P38 MAPK, and cytokines IFN-γ and IL-1β. Targeted inhibition of FD by danicopan (ACH-4471) suppressed ERK1/2 activation and proliferation of cultured cSCC cells. Also, a comprehensive review on the complement-targeted therapeutics under clinical and preclinical studies was accomplished.

Taken together, it is concluded that autocrine FI and FD promote the development and progression of cSCC, and can be identified as potential biomarkers and promising therapeutic targets in invasive cSCC. Furthermore, for the first time in science, small-molecule FD inhibitor danicopan is proposed as a highly specific and sensitive therapeutic for precision cSCC therapy.

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