Complement Factor H: A Biomarker for Progression of Cutaneous Squamous Cell Carcinoma

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The incidence of cutaneous squamous cell carcinoma (cSCC) is increasing globally. We have studied the expression of complement system components in cSCC. Expression profiling of cSCC cell lines ($n=8$) and normal human epidermal keratinocytes ($n=5$) with Affymetrix and quantitative real-time PCR (qPCR) revealed upregulation of complement factor H (CFH) and factor H-like protein-1 (FHL-1) in cSCC cell lines. The expression of CFH and FHL-1 mRNAs was also significantly higher in cSCC tumors ($n=6$) than in normal skin ($n=11$). Analysis of CFH and FHL-1 expression *in vivo* in invasive cSCCs ($n=65$), *in situ* cSCCs ($n=38$), and premalignant lesions (actinic keratoses, $n=37$) by immunohistochemistry showed that they were specifically expressed by tumor cells in cSCCs and the staining intensity was stronger in cSCCs than in *in situ* cSCCs and actinic keratoses. The expression of CFH by cSCC cells was upregulated by IFN-γ and the basal CFH and FHL-1 expression was dependent on extracellular signal–regulated kinase (ERK)1/2 and p38 signaling. Knockdown of CFH and FHL-1 expression inhibited proliferation and migration of cSCC cells and inhibited basal ERK1/2 activation. These results provide evidence for a role of CFH and FHL-1 in cSCC progression and identify them as progression markers and potential therapeutic targets in SCCs of skin.

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INTRODUCTION

The incidence of nonmelanoma skin cancer is increasing globally and cutaneous squamous cell carcinoma (cSCC), which constitutes approximately 20% of nonmelanoma skin cancer cases, is the second most common cutaneous malignancy in white population worldwide (Rogers *et al.*, 2010). Solar UV radiation is the major risk factor for cSCC and other important risk factors include chronic ulceration of skin and immunosuppression (Madan *et al.*, 2010). Inflammation is detected in a subset of cSCC and its precursors, actinic keratosis (AK) and cSCC *in situ* (cSCCIS, Bowen's disease), as in many other types of cancer (Coussens and Werb, 2002; Ratushny *et al.*, 2012).

Complement system is an important part of host defense in the innate immune system and its function is to destroy microbes and apoptotic cells, and initiate the inflammatory response (Ricklin *et al.*, 2010, Rutkowski *et al.*, 2010). Complement system consists of three distinct pathways, i.e., classical, alternative, and lectin pathway, that converge and activate complement C3 resulting in the formation of membrane attack complex (MAC) and lysis of the target cell (Supplementary Figure S1 online) (Ricklin *et al.*, 2010). The activity of the complement system is strictly regulated by cell surface–bound and soluble inhibitors, which protect cells against complement-mediated lysis. Complement factor H (CFH) is an important soluble inhibitor of continuously activated alternative pathway (de Cordoba and de Jorge, 2008; Zipfel and Skerka, 2009). It inhibits binding of complement factor B to the C3 activation product C3b, and displaces complement factor B cleavage product Bb from the C3bBb complex (Supplementary Figure S1 online). CFH also functions as a cofactor for complement factor I in the inactivation of C3b to iC3b (Schulze *et al.*, 1993). CFH consists of 20 short consensus repeats (SCRs) (de Cordoba and de Jorge, 2008). As a result of alternative splicing of the primary transcript, CFH is present in two isoforms with similar functions: CFH (150 kDa) and factor H-like protein-1 (FHL-1, 45 kDa) (Zipfel and Skerka, 1999). Previous studies have shown that normal epidermal keratinocytes express complement components and inhibitors, including CFH (Dovezenski *et al.*, 1992; Timar *et al.*, 2007).

Here, we have examined the role and regulation of CFH and FHL-1 in cSCC. The results demonstrate overexpression of CFH and FHL-1 by cSCC cells in culture and *in vivo*, and that the proportion of tumors with high expression increases with

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Abbreviations: CFH, complement factor H; cSCC, cutaneous squamous cell carcinoma; cSCCIS, cSCC in situ; FHL-1, factor H-like protein-1; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; qPCR, quantitative real-time PCR; TGF, transforming growth factor; TMA, tissue microarray; TNF, tumor necrosis factor

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the progression of cSCC from early intraepithelial form, AK to invasive cSCC. In addition, the results show that CFH promotes proliferation and migration of cSCC cells. The results identify CFH as tumor cell–associated biomarker for progression of SCCs of the skin.

RESULTS

Overexpression of CFH and FHL-1 in cSCC cells

The expression of complement components in cSCC cells was determined by oligonucleotide array–based (Affymetrix, Santa Clara, CA) expression profiling of eight cSCC cell lines (five primary and three metastatic), and primary normal human epidermal keratinocytes (NHEKs) established from normal skin of five individuals. In general, the expression of mRNAs for most complement system components, except for C1qBP and C3, was low in NHEKs (Figure 1a). Interestingly, the mRNA levels of certain complement components (C1r, C1s, C3, and complement factor B) were elevated in cSCC cells, as compared with NHEKs (Figure 1a). Among the complement inhibitors, the expression of CFH and FHL-1, as well as complement factor I, were markedly increased in cSCC cells (Figure 1a).

Verification of CFH and FHL-1 mRNA expression by quantitative real-time PCR (qPCR) revealed that the mean levels of CFH and FHL-1 mRNA were significantly higher in cSCC cell lines than in NHEKs (Figure 1b). Specific bands corresponding to CFH (155 kDa) and FHL-1 (45 kDa) were noted in conditioned media of cSCC cell lines, but not NHEKs by western blot analysis (Figure 1c).

The expression of CFH and FHL-1 *in vivo* was analyzed with qPCR of RNA samples obtained from cSCC tumors ($n=6$) and normal skin ($n=11$). The expression of CFH and FHL-1 mRNAs in normal skin was low, whereas the mean expression level of mRNAs for both inhibitors was significantly higher in cSCCs (Figure 1d).

Overexpression of CFH and FHL-1 by tumor cells in cSCCs *in vivo*

The expression of CFH and FHL-1 during progression of cSCCs *in vivo* was examined by immunohistochemistry (IHC) using tissue microarrays consisting of a large panel of tissue samples representing different stages of epidermal carcinogenesis, i.e., invasive cSCCs ($n=65$), cSCCIS (Bowen's disease; $n=38$), and premalignant lesions (AKs; $n=37$), as well as normal skin ($n=12$). The CFH antibody used for IHC recognizes both CFH and FHL-1. The results showed prominent tumor cell–associated staining for CFH/FHL-1 in cSCCs (Figure 2a–d). No clear difference in the staining intensity was detected between inflamed and non-inflamed cSCCs. Analysis of CFH and FHL-1 expression in *in situ* cSCCs and AKs revealed in general weaker immunostaining intensity in most AKs (Figure 2e and f) and cSCCIS sections (Figure 2h). The epidermal layer in normal skin samples was in general negative for CFH/FHL-1 (Figure 2g). Semiquantitative analysis of the immunostainings revealed that the proportion of tumor sections showing strong staining was significantly higher in cSCC group than in cSCCIS and AK groups (Figure 2i).

CFH and FHL-1 expression is upregulated in Ha-*ras*-transformed HaCaT cells

To gain further insight into the role of CFH in epidermal carcinogenesis, we determined the expression of CFH and FHL-1 mRNAs in immortalized nontumorigenic keratinocyte–derived cell line (HaCaT) lacking functional p53, and in a series of Ha-*ras*-transformed tumorigenic HaCaT cell lines (A5, II-4, and RT3; Mueller *et al.*, 2001). CFH and FHL-1 mRNA levels were low in parental HaCaT cells, whereas abundant expression of both CFH and FHL-1 mRNAs was noted in Ha-*ras*-transformed HaCaT cell lines (Figure 3a). Accordingly, the production of CFH and FHL-1 was clearly upregulated in Ha-*ras*-transformed HaCaT cells, as well as in metastatic cSCC cell line (UT-SCC-7), as determined by western blotting (Figure 3b).

Regulation of CFH expression by cSCC cells by cytokines and growth factors

To investigate the regulation of CFH production, UT-SCC-7 cells in culture were treated for 24 hours with inflammatory cytokines and growth factors present in the microenvironment of cSCCs. Analysis of the cell lysates by western blotting indicated that the production of CFH was upregulated by interferon-γ (IFN-γ), interleukin-1β (IL-1β), and transforming growth factor-α (TGF-α), and less potently by transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) (Figure 4a).

CFH and FHL-1 inhibit the C3 activation cascade in the alternative pathway by promoting the proteolytic cleavage of C3b to iC3b (Supplementary Figure S1 online). C3 consists of α- and β-chains linked by a disulfide bond and the N- and C-terminal regions of the α-chain are also connected by a disulfide bond. Stepwise proteolytic inactivation of C3b by complement factor I results in the generation of smaller cleavage fragments. The C3b degradation promoting activity of CFH/FHL-1 was evaluated by analyzing C3 cleavage products in conditioned media of cSCC cell cultures by western blot analysis. The presence of C3α′43, as a marker of CFH and FHL-1 activity, was detected in media of primary (UT-SCC-59A and 91) and metastatic (UT-SCC-7) cSCC cells (Figure 4b). C3β′ + α′67 fragment of iC3b generated by proteolytic cleavage and disruption of the disulfide bridge between the C- and N-termini of the α-chain was also detected in the media (Figure 4b).

CFH and FHL-1 expression by cSCC cells is regulated by extracellular signal–regulated kinase (ERK)1/2 and p38

To further investigate the regulation of CFH and FHL-1 expression in cSCC cells, primary cSCC (UT-SCC-118) cultures were treated in parallel for 24 hours with the inhibitor of MEK1/2 (MAPK/ERK kinase 1/2; PD98059), or the inhibitor of p38α and p38β mitogen-activated protein kinases (MAPKs; SB203580), or for 48h with BIRB796, an inhibitor of all four p38 isoforms. Basal CHF and FHL-1 mRNA levels were potently downregulated by PD98059 as compared with the untreated control cells (Figure 4c). Interestingly, CFH and FHL-1 expression was also potently

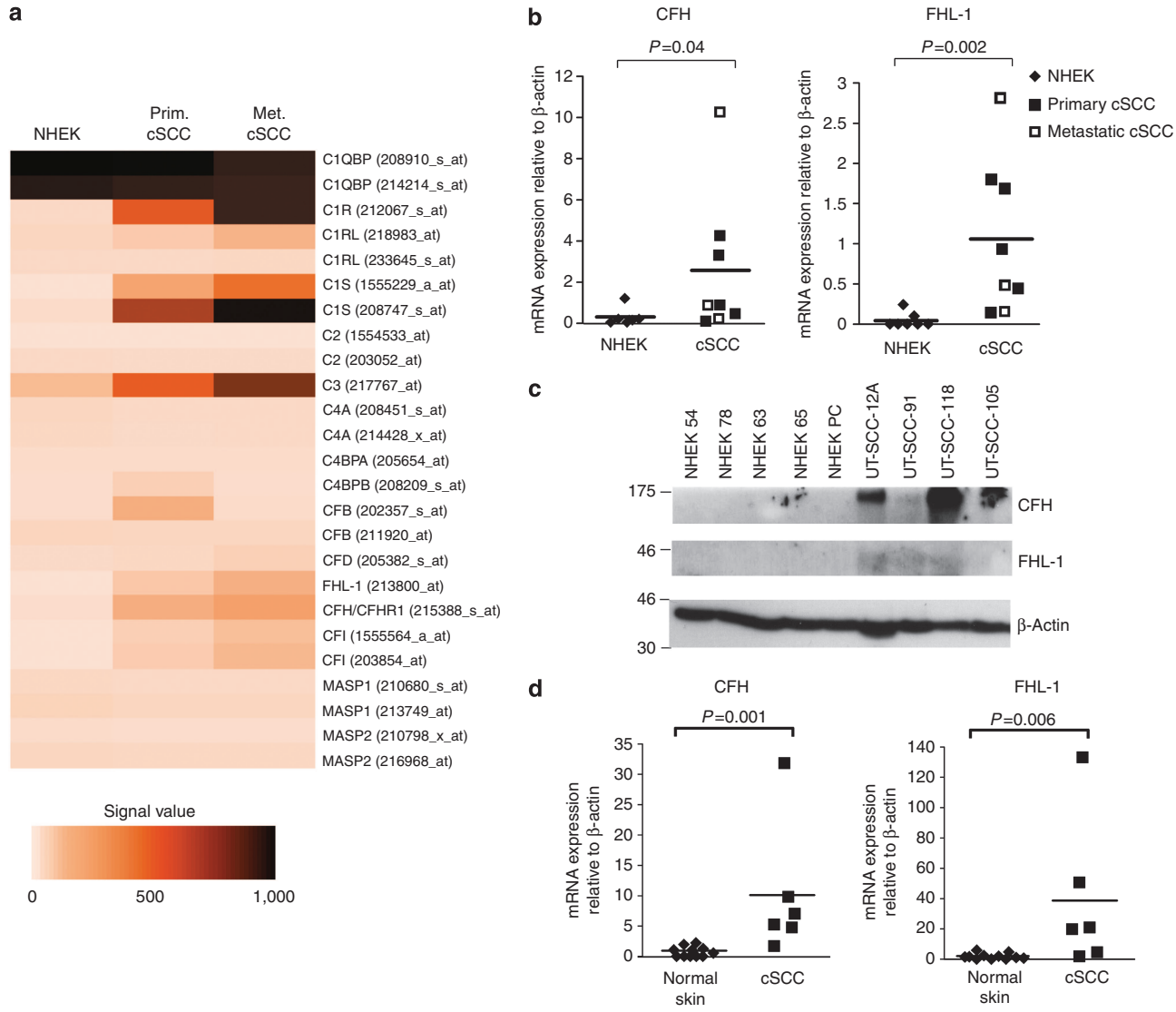

Figure 1. Expression of complement system components in cutaneous squamous cell carcinoma (cSCC) cells. (**a**) Oligonucleotide array (Affymetrix)–based gene expression profiling of normal human epidermal keratinocyte (NHEK) cultures ($n=5$), primary (Prim. cSCC; $n=5$) and metastatic (Met. cSCC; $n=3$) cSCC cell lines. The mean signal values for NHEKs, primary cSCC cell lines, and metastatic cSCC cell lines are shown as a heat map. (**b**) The expression of complement factor H (CFH) and factor H-like protein-1 (FHL-1) mRNAs in NHEKs ($n=7$) and cSCC cell lines ($n=8$) were analyzed by quantitative real-time PCR (qPCR) and corrected for the levels of β-actin mRNA in the same samples. The mean values for each group are indicated by horizontal bars. (**c**) The conditioned media of NHEK and cSCC cell lines were analyzed by western blotting for the presence of CFH (155 kDa) and FHL-1 (45 kDa). β-Actin was used as a loading control. Migration positions of molecular weight markers (in kDa) are shown on the left. (**d**) CFH and FHL-1 mRNA levels in RNA samples from normal skin ($n=11$) and primary cSCCs ($n=6$) were analyzed by qPCR, as in (**b**). The mean values for each group are indicated by horizontal bars. Statistical analysis (**b**, **d**) was performed with the Mann–Whitney two-way *U*-test.

inhibited by p38 inhibitors SB203580 and BIRB796 (Figure 4c). These findings are interesting in the light of previous reports showing the role of ERK1/2 and p38 MAPKs in growth and invasion of cutaneous SCC cells (Johansson *et al.*, 2000; Junttila *et al.*, 2007).

CFH and FHL-1 regulate cSCC cell proliferation and migration

To examine the functional role of CFH and FHL-1 in cSCC cells, the expression of CFH and FHL-1 mRNAs was knocked down using specific small interfering RNA (siRNA; Figure 5a). A significant reduction in the number of viable cells (UT-SCC-118) was detected at 48 and 72 hours following the inhibition of CFH/FHL-1 expression as compared with control siRNA-transfected cultures (Figure 5b). Inhibition of cell proliferation was associated with potent inhibition in the levels of activated, phosphorylated ERK1/2 at 72 hours after CHF/FHL-1 siRNA transfection (Figure 5c). In addition, migration of UT-SCC-118 cells was significantly reduced 72 hours after knock down of CFH/FHL-1 expression (Figure 5d). Similar results on cell proliferation and migration were obtained with another cSCC cell line (UT-SCC-91) (data not shown).

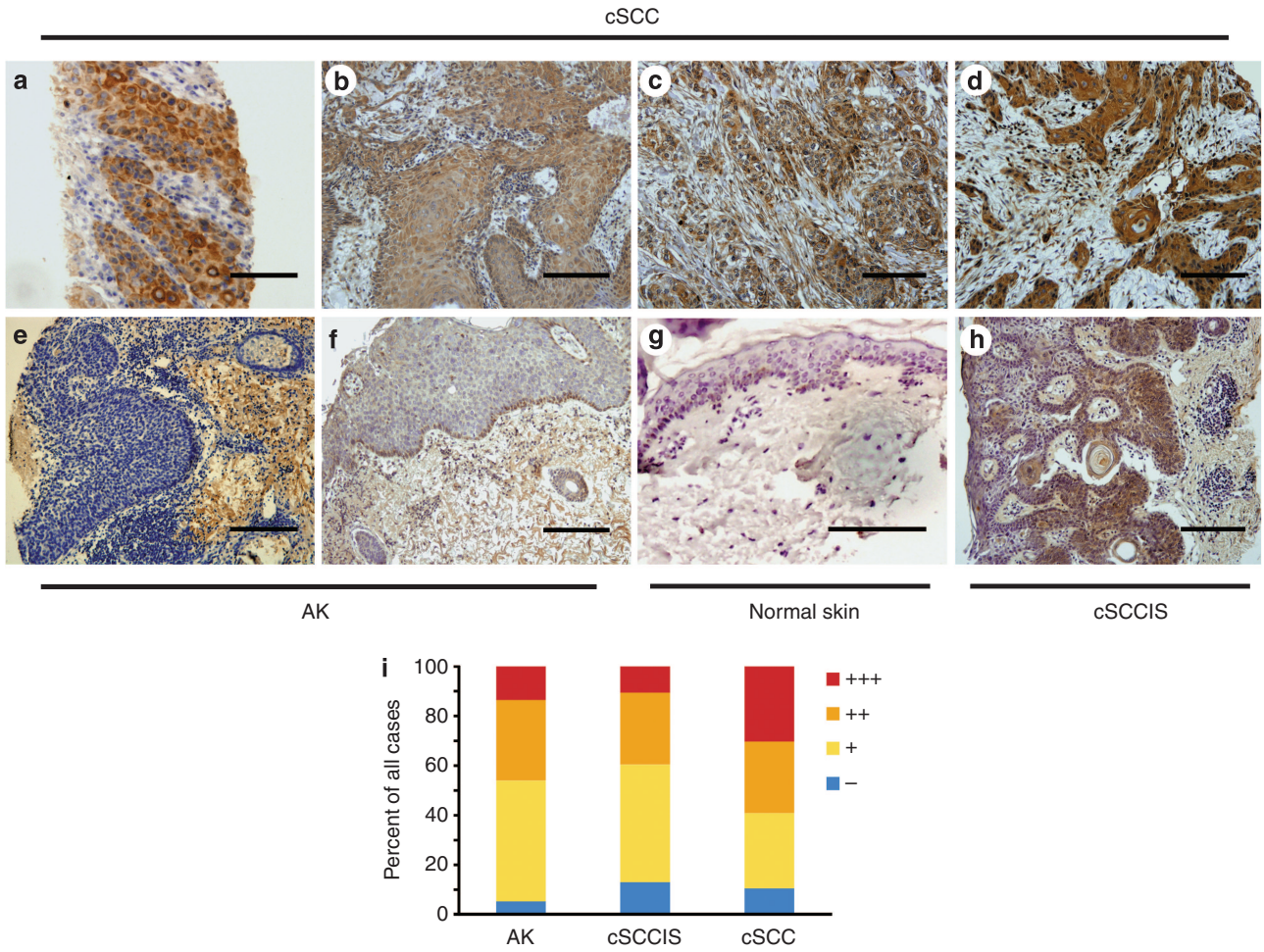

Figure 2. Expression of CFH/FHL-1 by tumor cells in cutaneous squamous cell carcinoma (cSCC). (**a**–**h**) Sections of tissue microarray blocks containing cSCC ($n=65$), cSCCIS (cSCC *in situ*, Bowen's disease; $n=38$), premalignant lesions (actinic keratosis (AK); $n=37$), and normal skin ($n=12$) were stained with complement factor H/factor H-like protein-1 (CFH/FHL-1) antibody. Strong cytoplasmic staining is seen in tumor cells in moderately differentiated (G2) cSCCs (**a**, **b**) and in poorly differentiated (G3) cSCCs (**c**, **d**). Staining for CFH/FHL-1 is absent (**e**) or weak (**f**) in most AKs. Epidermal layer in normal skin is negative for CH/FHL-1 (**g**). In cSCCIS tissue sections, moderate CFH/FHL-1 staining is detected in tumor cells (**h**). Bar = 200 μm. (**i**) Semiquantitative analysis of CFH/FHL-1 stainings of AK, cSCCIS, and cSCC tissue sections. Intensity of tumor cell–specific immunostaining was scored negative (−), weak (+), moderate (++), and strong (+++). The percentage of tumors with strong tumor cell–specific staining is significantly higher in cSCCs than in cSCCIS and AK sections ($P=0.035$; χ^2-test).

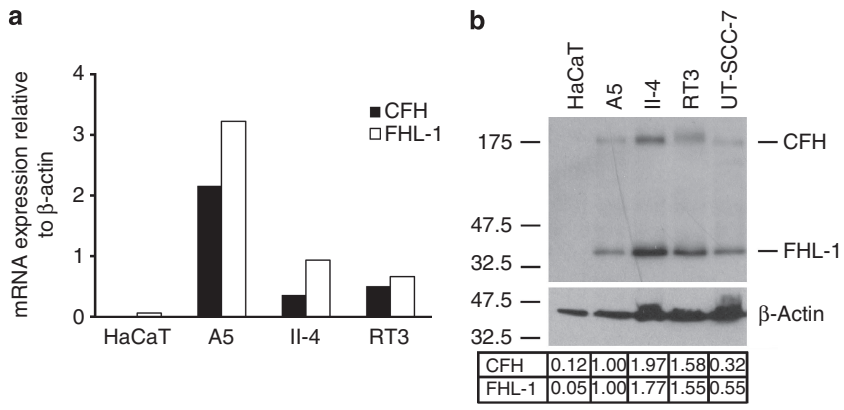

Figure 3. Upregulation of complement factor H (CFH) and factor H-like protein-1 (FHL-1) expression in Ha-*ras*-transformed HaCaT cells. (**a**) Expression levels of CFH and FHL-1 mRNAs in HaCaT cells and in tumorigenic Ha-*ras*-transformed HaCaT cell lines (A5, II-4, and RT3) were analyzed by quantitative real-time PCR (qPCR) and corrected for the levels of β-actin mRNA in the same samples. (**b**) The cell lysates were analyzed by western blotting for the presence of CFH (155 kDa) and FHL-1 (45 kDa). Migration positions of molecular weight markers (in kDa) are shown on the left. CFH and FHL-1 levels quantitated densitometrically and corrected for β-actin levels in the same samples are shown below the western blots relative to levels in A5 cells (1.00).

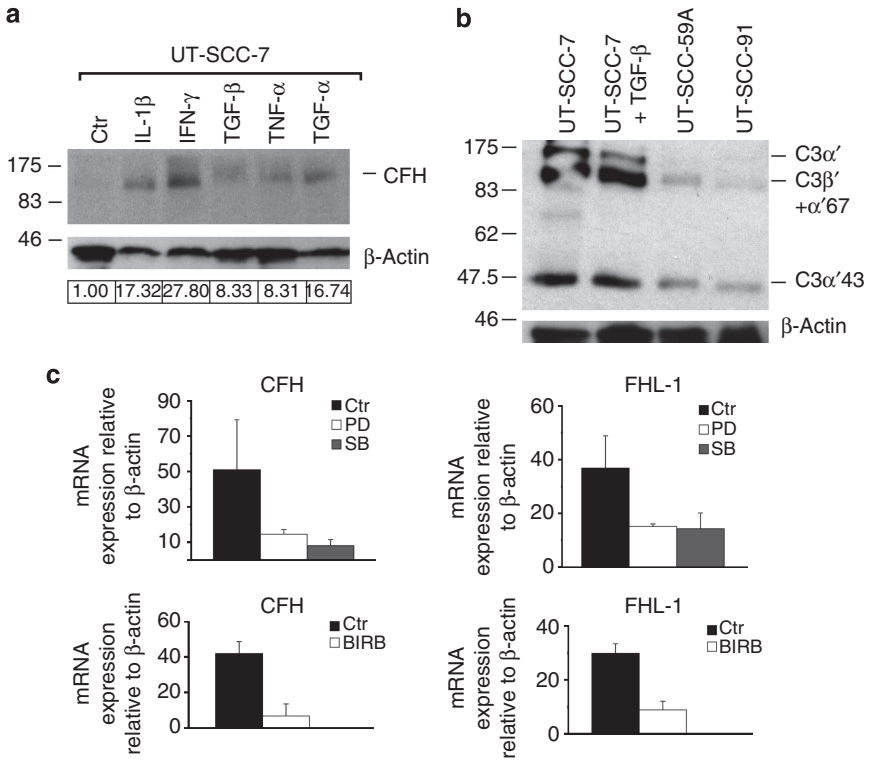

Figure 4. Regulation of complement factor H (CFH) expression in cutaneous squamous cell carcinoma (cSCC) cells. (**a**) Metastatic cSCC cell line (UT-SCC-7) cultures were treated with interleukin-1β (IL-1β (10 ng/ml), interferon-γ (IFN-γ (100 U/ml), transforming growth factor-β (TGF-β; 5 ng/ml), tumor necrosis factor-α (TNF-α; 20 ng/ml), and transforming growth factor-α (TGF-α; 50 ng/ml) for 24 h. Cell lysates were analyzed by western blotting for CFH (155 kDa). CFH levels quantitated densitometrically and corrected for β-actin levels in the same samples are shown below the western blots relative to levels in untreated control (Ctr) cells (1.00). (**b**) Primary (UT-SCC-59A and UT-SCC-91) and metastatic (UT-SCC-7) cSCC cells were cultured for 24 hours. UT-SCC-7 cell line was treated in parallel with TGF-β (5 ng/ml). The conditioned media were analyzed for C3 inactivation fragments C3α′43 and C3β′ + α′67 as indicators for CFH/FHL-1 activity with western blotting using C3 antibody. β-Actin levels were determined in the corresponding cell lysates as the loading control. Migration positions of molecular weight markers (in kDa) are shown on the left. (**c**) cSCC cells (UT-SCC-118) in culture were treated for 24 hours with MEK1/2 inhibitor PD98059 (PD; 30 μM) and with p38 inhibitor SB203580 (SB; 10 μM), for 48h with p38 inhibitor and BIRB796 (10 μM) as indicated. Levels of CFH and FHL-1 mRNAs were analyzed by qPCR and corrected for the levels of β-actin mRNA in the same samples.

DISCUSSION

The complement system has an important role in host defense as a part of innate immunity. Complement can be activated via three distinct pathways, i.e., classical, lectin, and alternative pathways (Supplementary Figure S1 online). The alternative pathway is continuously activated *in vivo*. Therefore, specific inhibitors are required to protect the host tissues from damage exerted by complement activation (Zipfel and Skerka, 2009). CFH and its splicing variant FHL-1 are key inhibitors in the alternative pathway. They inhibit activation of the alternative pathway by promoting proteolytic inactivation of C3b and by inhibiting the activity of C3 convertase C3bBb (Supplementary Figure S1 online). Escape from immune attack has an important role in cancer progression, and expression of complement inhibitors, including CFH, has been documented in malignant tumors such as bladder cancer (Cheng *et al.*, 2005) and ovarian cancer (Junikkala *et al.*, 2002).

The results of this study show that tumor cells of cSCCs produce functionally active complement inhibitors CFH and FHL-1 in culture and also express these inhibitors *in vivo*. The expression of the mRNAs for CFH and FHL-1 was elevated in most cSCC cell lines studied and in all cSCC tumors compared with NHEKs or normal skin. Production of CFH and FHL-1 was also detected by cSCC cell lines, but not by NHEKs. For further investigation of CFH and FHL-1 expression *in vivo* we performed IHC analysis of tissue microarrays consisting of a large panel of cSCCs, cSCCIS, and AKs. The antibody used does not distinguish between CFH and FHL-1, but the results of qPCR of tumor tissue RNA verified that both CFH and FHL-1 mRNAs were expressed in cSCCs *in vivo*. Results of IHC analysis revealed that CFH and FHL-1 were specifically expressed by tumor cells, whereas epidermal layer in normal skin was negative. Although the most prominent staining for CFH/FHL-1 was detected in cSCCs, moderate and even strong staining was also noted in a subset of AK and cSCCIS sections indicating that the expression is induced early in cSCC progression. Analysis of Ha-*ras*-transformed HaCaT cells representing different stages of epidermal carcinogenesis (Mueller *et al.*, 2001) revealed that CFH and FHL-1 expression was low in parental HaCaT cells and markedly higher in *ras*-transformed HaCaT cells. Thus, inactivation of p53, an early event in epidermal carcinogenesis, is not sufficient for induction of CFH and FHL-1 expression, but activation of *ras* signaling is also required. Constitutive

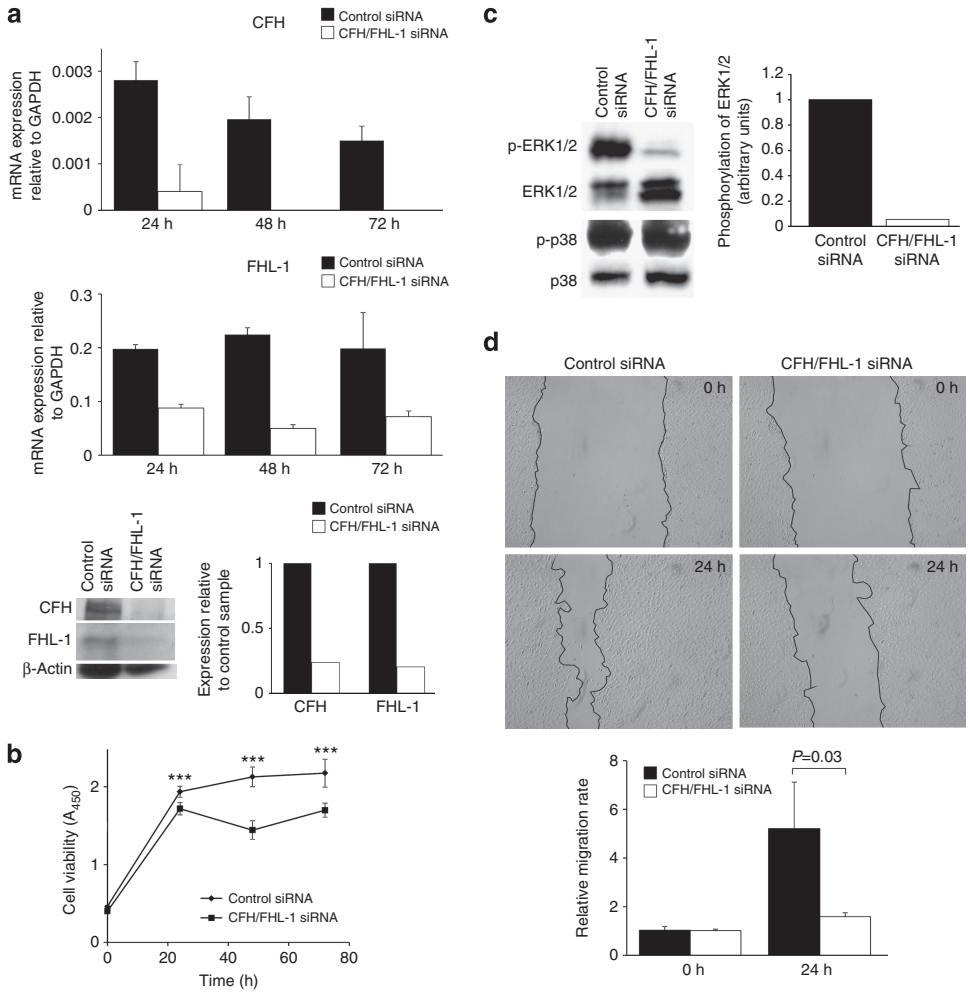

Figure 5. Complement factor H (CFH) and factor H-like protein-1 (FHL-1) regulate proliferation and migration of cutaneous squamous cell carcinoma (cSCC) cells. (**a**) Parallel cultures of primary cSCC cells (UT-SCC-118) were transfected with CFH/FHL-1 small interfering RNA (siRNA) and control siRNA (75 nM). The CFH and FHL-1 mRNA levels were subsequently measured with quantitative real-time PCR (qPCR) at 24, 48, and 72 hours and corrected for the levels of β-actin mRNA in the same samples (upper panels). The cell lysates of control and CFH/FHL-1 siRNA–transfected UT-SCC-118 cells at 72 hours were analyzed by western blotting for the levels of CFH and FHL-1. CFH and FHL-1 levels were quantitated by densitometry and corrected for the levels of β-actin in the same samples (lower panels). (**b**) Number of viable cells was determined after siRNA transfection using WST-1 assay ($n=3$; ***$P<0.0001$, *t*-test). (**c**) Cell lysates were analyzed for the levels of phosphorylated extracellular signal–regulated kinase (ERK)1/2 (p-ERK1/2), total ERK1/2, phosphorylated p38 (p-p38), and total p38 by western blotting 72 hours after siRNA transfection (left panel). p-ERK1/2 blots were quantitated by densitometry and corrected for the levels of total ERK1/2 (right panel). (**d**) Control and CFH/FHL-1 siRNA–transfected cells were incubated for 48 hours. To inhibit cell division, cells were then treated with hydroxyurea (1 mM) for 6 hours. A scratch was created with a pipet tip in the cell monolayer and incubation was continued in 1% fetal calf serum in DMEM and 0.5 mM hydroxyurea for 24 h. A representative image of the experiment is shown (upper panel). Quantitation of the relative migration rate of cultures is shown (lower panel; $n=3$, $P=0.03$, *t*-test).

activation of *ras* signaling results in permanent activation of ERK1/2 signaling in cSCC cells (Toriseva *et al.*, 2012). Accordingly, our results showed that CFH and FHL-1 expression in cSCC cells was potently downregulated by inhibition of ERK1/2 signaling. Interestingly, p38 MAPK signaling was also found to have an important role in regulating basal CFH and FHL-1 expression. Together these results identify CFH and FHL-1 as targets for ERK1/2 and p38 MAPK signaling cascades in cSCC cells.

It has been proposed that tumor cells utilize complement inhibitors for immune evasion (Gorter and Meri, 1999). CFH and FHL-1 have also been suggested to have a role in the escape of malignant cells from complement-mediated cytotoxicity in colon (Wilczek *et al.*, 2008), lung (Ajona *et al.*, 2004; Ajona *et al.*, 2007), ovarian (Junnikkala *et al.*, 2002), and bladder cancers (Cheng *et al.*, 2005) and in glioblastoma (Junnikkala *et al.*, 2000). CFH has also been shown to bind osteopontin and bone sialoprotein, which in this way may promote CFH-mediated inhibition of cell lysis by sequestering CFH to cell surface and blocking the activity of the alternative pathway (Fedarko *et al.*, 2000). Our results show that certain complement components are also overexpressed in cSCC cells as compared with NHEKs (Figure 1a). C3 is a key component of complement cascade

as activation of all three different pathways leads to activation of C3. Our results show that although NHEKs express C3, the expression is elevated in cSCC cells. These findings provide a possibility for complement activation by cSCC cells, which in this way may promote inflammation and attract phagocytic cells to the site of activation. Our results also provide evidence that cSCC cells produce functionally active CFH, as shown by the presence of iC3 in culture media, which reflects CFH activity and is a marker for recent complement activation and inhibition (Schulze *et al.*, 1993). Thus, cSCCs appear to create a microenvironment that protects the cells from complement-mediated cell killing.

CFH has additional documented functions that may have a role in cancer progression. CFH has been shown to regulate activation of matrix metalloproteinase-2 and 3 by an interaction with three small integrin-binding ligand N-linked glycoproteins (SIBLINGs) osteopontin, bone sialoprotein, and dentin matrix protein-1 (Fedarko *et al.*, 2004). Furthermore, FHL-1 has been shown to promote cell adhesion (Hellwage *et al.*, 1997; Zipfel and Skerka, 1999). Our results provide evidence for two other previously unreported cellular functions of CFH and FHL-1 relevant in cancer progression, i.e., regulation of cell proliferation and migration. In this respect, it is interesting, that knockdown of CFH and FHL-1 expression resulted in potent inhibition of ERK1/2 activation in cSCC cells. CFH and FHL-1 have been shown to bind complement receptor 3 (CD11b/CD18; $\alpha_M\beta_2$ integrin) on neutrophils (Losse *et al.*, 2010), but it is not known whether this receptor has any role in mediating effects of CFH on cSCC cells. Inflammation is noted in a subset of cSCCs. Interestingly, inflammatory cytokines IFN-γ, IL-1β, and TNF-α upregulated the expression of CFH by cSCC cells. These results are in accordance with previous studies showing upregulation of CFH expression by IFN-γ in epidermal keratinocytes (Timar *et al.*, 2006).

Taken together, the results of the present study show that cSCCs express CFH and FHL-1, the main inhibitors of the alternative pathway of the complement system. Apparently, these complement regulators have an important role in the progression of cSCCs by allowing tumor cells to escape complement attack. In conclusion, our results show that CFH and FHL-1 expression is specifically induced during cutaneous carcinogenesis, suggesting these complement inhibitors as putative diagnostic biomarkers and as potential targets for anticancer therapy in skin SCCs.

MATERIALS AND METHODS

Ethical issues

The study was conducted according to the Declaration of Helsinki. The use of archival tissue specimens, and collection of normal skin and cSCC tissues was approved by the Ethics Committee of the Hospital District of Southwest Finland. The study was carried out with the permission of Turku University Hospital and with written informed consent of all patients before surgery.

Cell cultures

NHEKs were established from skin samples obtained from mammoplasty ($n=6$). Primary human epidermal keratinocytes (NHEK-PC) were obtained from PromoCell (Heidelberg, Germany). NHEKs were cultured in keratinocyte growth medium-2 with supplement mix and calcium chloride (PromoCell), L-glutamine and penicillin–streptomycin mixture (Gibco, Paisley, Scotland, UK) (Junttila *et al.*, 2007). The spontaneously immortalized nontumorigenic human keratinocyte–derived cell line (HaCaT; Boukamp *et al.*, 1988) and three Ha-*ras*-transformed tumorigenic HaCaT cell lines (A5, II-4, and RT3; Boukamp *et al.*, 1990) were kindly provided by Dr Norbert Fusenig (Deutsche Krebsforschungszentrum, Heidelberg, Germany). A5 cells form benign, II-4 cells low-grade malignant, and RT3 cells high-grade malignant tumors *in vivo* in nude mice (Mueller *et al.*, 2001). cSCC cell lines ($n=8$) were established from the cSCCs at the time of operation in Turku University Hospital (Farshchian *et al.*, 2011). Of these, five were derived from primary SCCs (UT-SCC-12A, 91, -105, -111, and -118) and three from metastases (UT-SCC-7, -59A, and -115). SCC and HaCaT cells were cultured in DMEM (Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum (FCS), 100 IU penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. G418 (200 μg/ml) was included in A5, II-4, and RT3 cell medium.

Microarray-based expression profiling

The gene expression profiling on NHEKs ($n=5$) and cSCC cell lines ($n=8$) was assessed using Affymetrix Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) and the data analyses were performed at the Microarray Centre of Turku Centre for Biotechnology. Normalization of the arrays was performed using RMA assay, Chipster software (CSC – IT Center for Science, Espoo, Finland). Mean signal level of NHEKs was used as the control for signal level of each cell line. Sequence specificity of Affymetrix probes was verified by BLAST search.

Tissue RNA

Primary cSCC ($n=6$) samples were obtained from surgically removed tumors in Turku University Hospital (Farshchian *et al.*, 2011). Normal skin samples ($n=11$) were obtained during mammoplasty operation in Turku University Hospital and from the upper arm of healthy volunteers. Total RNA was isolated from the tissue samples and analyzed by qPCR (Stokes *et al.*, 2010).

qPCR

cDNA was synthesized from total RNA using 1 μg of RQ1 DNase–treated (Promega M610A; Promega, Madison, WI) RNA, Random Primers (Promega C118A), M-MLV Reverse Transcriptase (Promega M531A) and RNase H minus polymerase (Promega M368B). qPCR analysis of cDNA samples for CFH and FHL-1 mRNA levels was performed in duplicate with specific primers and fluorescent probes (Supplementary Table S1 online; Stokes *et al.*, 2010). In each measurement, range of the threshold cycle values were $<5\%$ of the mean. The results were corrected for the levels of β-actin or glyceraldehyde-3-phosphate dehydrogenase mRNA in the same samples.

Tissue samples and IHC

Altogether 152 formalin-fixed paraffin-embedded tissue samples from sporadic, UV-induced cSCC ($n=65$; mean age 77 years, range 45–96 years), cSCCIS (Bowen's disease; $n=38$; mean age 82 years, range 70–95 years), AK ($n=37$; mean age 78 years, range 58–95 years), and

normal skin ($n=12$) were obtained from the archives of the Department of Pathology, Turku University Hospital. Tissue microarray blocks were generated as described (Kononen *et al.*, 1998). The sections were stained with mouse monoclonal anti-CFH antibody, which also detects FHL-1 (clone OX-24, AbD Serotec, Oxford, UK), diluted (1:850) in PBS containing 1.5% horse serum. Immunostainings were performed with avidin–biotin–peroxidase complex technique (VectaStain ABC Kit; Vector Laboratories, Burlingame, CA) in combination with diaminobentzidine and Mayer's hematoxylin (Sigma-Aldrich Chemie, Steinheim, Germany) as a counterstain (Kivisaari *et al.*, 2008). For negative control stainings, the primary antibody was replaced with PBS. The staining intensity was scored by two independent observers (PR and MK) as negative (−), weak (+), moderate (++), or strong (+++) based on the intensity of cytoplasmic staining.

Western blot analysis

The production of CFH by NHEKs and cSCC cell lines was determined by western blotting of cell lysates and media using specific polyclonal goat anti-CFH antibody (1:1000; Calbiochem, La Jolla, CA). The levels of C3 cleavage products in conditioned media were determined using polyclonal goat anti-C3 antibody (1:1000; Calbiochem). UT-SCC-7 cells were maintained in serum-free DMEM for 24 hours, and then treated with IL-1β (10 ng/ml; Calbiochem), IFN-γ (100 U/ml; Promega), TGF-β1 (5 ng/ml; Sigma Aldrich, St Louis, MO), TNF-α (20 ng/ml; Sigma Aldrich), or TGF-α (50 ng/ml; Peprotech, Stockholm, Sweden) for 24 hours. To study the role of MAPK signaling on expression of CFH, cSCC cell cultures were serum starved for 24 hours and then treated with MEK1/2 inhibitor PD98059 (30 μM), or with p38α/p38β inhibitor SB203580 (10 μM), for 24h or with p38α/p38β/p38γ/p38δ inhibitor BIRB796 (10 μM) (all from Calbiochem) for 48 hours.

siRNA knockdown of CFH/FHL-1 expression

cSCC cells were grown to 50% confluency and transfected with negative control siRNA or siRNA targeting both CFH and FHL-1 (75 nM; Qiagen, Hilden, Germany) using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA) (Kivisaari *et al.*, 2010). Cells were harvested 24, 48, and 72 hours after transfection, the levels of CFH and FHL-1 mRNAs were determined by qPCR, and CFH and FHL-1 protein levels by western blotting.

Cell proliferation

cSCC cells were transfected with negative control siRNA or CFH/FHL-1 siRNA (75 nM). After 24 hours transfected cells (10^4 cells/well) were seeded on 96-well plates. The number of viable cells at 0, 24, 48, and 72 hours was determined by WST-1 cell proliferation reagent (Roche Diagnostics, Mannheim, Germany). The experiment was performed with 5–6 parallel wells in each time point with two cSCC cell lines (UT-SCC-91 and 118). The activation of ERK1/2 and p38 was verified by western blotting of the cell lysates with antibodies specific for phospho-ERK1/2 and phospho-p38 (Cell Signaling Technology, Berverly, MA), respectively.

Cell migration

Cells were transfected with negative control siRNA or CFH/FHL-1 siRNA (75 nM) and grown to confluency in complete growth medium for 48 hours. To inhibit cell division, cells were treated with 1 mM hydroxyurea (Sigma Aldrich) in DMEM with 1% FCS for 6 hours. A scratch in the cell monolayer was created with pipet tip and incubation was continued in DMEM with 1% FCS and 0.5 mM hydroxyurea for 24 hours. Microscopy was performed with Olympus IX70 (Olympus Optical, Tokyo, Japan) inverted microscope. Cell migration was studied in three independent experiments with two cSCC cell lines (UT-SCC-91 and 118). In each experiment there were three parallel wells and the cell-free area was measured from four areas in one well with ImageJ software (Schneider *et al.*, 2012).

Statistical analysis

Mann–Whitney *U*-test was used for determining the significance of differences between two non-normally distributed independent sample groups for qPCR. Two independent samples *t*-tests was used for proliferation and migration assays. χ^2-Test was used to compare IHC staining intensity.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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