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Expression of claudin-11 by tumor cells in cutaneous squamous cell carcinoma is dependent on the activity of p38δ

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Abstract

The incidence of cutaneous squamous cell carcinoma (cSCC) is rapidly increasing, and the prognosis of patients with metastatic disease is poor. There is an emerging need to identify molecular markers for predicting aggressive behaviour of cSCC. Here, we have examined the role of tight junction (TJ) components in the progression of cSCC. The expression pattern of mRNAs for TJ components was determined with RNA sequencing and oligonucleotide array-based expression analysis from cSCC cell lines (n=8) and normal human epidermal keratinocytes (NHEK, n=5). The expression of *CLDN11* was specifically elevated in primary cSCC cell lines (n=5), but low or absent in metastatic cSCC cell lines (n=3) and NHEKs. Claudin-11 was detected in cell-cell contacts of primary cSCC cells in culture by indirect immunofluorescence analysis. Analysis of a large panel of tissue samples from sporadic UV-induced cSCC (n=65), cSCC in situ (n=56), actinic keratoses (n=31), seborrhoeic keratoses (n=7) and normal skin (n=16) by immunohistochemistry showed specific staining for claudin-11 in intercellular junctions of keratinizing tumor cells in well and moderately differentiated cSCCs, whereas no staining for claudin-11 was detected in poorly differentiated tumors. The expression of claudin-11 in cSCC cells was dependent on the activity of p38δ MAPK and knock-down of claudin-11 enhanced cSCC cell invasion. These findings provide evidence for the role of claudin-11 in regulation of cSCC invasion and suggest loss of claudin-11 expression in tumor cells as a biomarker for advanced stage of cSCC.

KEYWORDS
claudin, invasion, p38, skin cancer, tight junction

1 | INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) constitutes about 20% of all non-melanoma skin cancer cases, making it the second most common cutaneous malignancy in white population.[1] The major risk factors for cSCC are solar UV radiation, chronic ulcers and immunosuppression.[2] Although early excision of cSCC is associated with favourable outcome, the prognosis of patients with advanced and metastasized disease is poor.[3] Inactivation of tumor protein 53 gene (*TP53*, p53) by UV radiation is one of the early steps in initiation of cSCC.[4] Another early event in keratinocyte carcinogenesis is loss-of-function mutation of *NOTCH1*.[5,6] In cSCC, mutations in *EGFR*, *HRAS* and *KRAS* have also been detected but the molecular basis of cSCC

Abbreviations: AK, actinic keratosis; cSCC, cutaneous squamous cell carcinoma; cSCC in situ, IHC, immunohistochemistry; MAPK, mitogen activated protein kinase; qRT-PCR, quantitative real-time PCR; SK, seborrhoeic keratosis; TJ, tight junction.

progression is still incompletely understood.[4] Thus, markers for progression and metastatic capacity of cSCC are in need.[7] There is increasing evidence that the role of tumor microenvironment presents a significant role in initiation and progression of cSCC. The composition of epidermal basement membrane and dermal extracellular matrix, influx of inflammatory cells and presence of microbial structures have been revealed to affect cSCC progression.[8–10]

Epidermal keratinocytes are connected by four types of cell junctions: desmosomes, adherence junctions, gap junctions and tight junctions (TJ). TJs are localized in granular cell layer, whereas other cell junctions can be found between keratinocytes in all viable layers of the epidermis.[11,12] TJs regulate movement of macromolecules, ions and inflammatory cells in simple epithelia.[13] TJs consist of transmembrane proteins occludin and variable combinations of claudins, and peripheral plaque proteins, such as TJ proteins (TJP) 1-3 (zona occludens, ZO), which connect TJs to actin cytoskeleton. Phosphorylation of occluding, claudins and ZO-1 regulates the permeability of TJs.[14] The expression and localization of TJ proteins has been shown to be altered in various types of cancers in a stage and tumor-specific manner.[15] In addition, TJ molecules have been shown to be involved in cell-cell adhesion, apoptosis and tumor invasion.[16]

Claudin family consists of more than 23 members expressed in tissue-specific manner in various normal and malignant tissues.[17] Normal human epidermis contains claudin-1, claudin-4 and claudin-7.[16] Claudin-11, also known as oligodendrocyte-specific protein (OSP), is concentrated in central nervous system myelin,[18] and it is expressed in Sertoli cells in testes at all stages of the seminiferous epithelial cycle,[19] while it has not been detected in human epidermal TJs.[16] Increased expression of claudin-3 and claudin-4 has been revealed in malignant tumors including breast, colorectal, prostate and ovarian cancers.[15] During cSCC progression, decreased claudin-1 expression and increased claudin-2 expression have been noted in actinic keratosis (AK) and cSCC.[20] Furthermore, the expression of claudin-4 has been shown to be associated with keratinization in cSCC and cSCC in situ (cSCCIS).[21]

In this study, we have examined the role of TJ components in the progression of cSCC. The results show that the expression of claudin-11 is specifically upregulated in primary cSCC cell lines, whereas the expression is low or absent in NHEKs and metastatic cSCC cell lines. Claudin-11 is detected in cell-cell contacts of keratinizing tumor cells of well and moderately differentiated cSCC tumors, but not in the poorly differentiated cSCCs in vivo. The expression of claudin-11 in cSCC cells is dependent on the activity of p38δ mitogen activated protein kinase (MAPK), and knock-down of claudin-11 increases the invasion potential of cSCC cells. These results provide evidence for the role of claudin-11 in regulation of cSCC invasion and suggest loss of claudin-11 expression in tumor cells as a biomarker for advanced stage of cSCC.

2 | MATERIALS AND METHODS

2.1 | Ethical issues

Approval for use of archival tissue specimens and the collection of normal skin and cSCC tissues was obtained from the Ethics Committee of the Hospital District of Southwest Finland, Turku, Finland (187/2006; 138/2007). The study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Each patient gave their informed consent.

2.2 | Cell cultures

Human cSCC cell lines were established from surgically removed primary (n=5) and metastatic (n=3) cSCCs and cultured in DMEM supplemented with 6 nmol/L glutamine, non-essential amino acids and 10% FCS as described previously.[22] The authentication of cell lines was performed by STR DNA profiling.[23] Normal human epidermal keratinocytes (NHEK, n=4) originated from normal skin obtained from breast reduction operations at the Department of Surgery, Turku University Hospital, Turku, Finland. Additional NHEKs (NHEK-PC) were purchased from PromoCell (Heidelberg, Germany). NHEKs were cultured in Keratinocyte Basal Medium 2 (PromoCell GmbH), as previously described.[24] For p38 MAPK inhibitor treatment, the cells were first serum-starved for 24 hours, followed by treatment with p38 MAPK inhibitors SB203580 (10 μmol/L; Calbiochem, Darmstadt, Germany) or BIRB796 (10 μmol/L; Axion Medchem, Groningen, the Netherlands) in 10% DMEM for 24 hours.

2.3 | Oligonucleotide array-based expression and RNA sequencing analysis

RNA extraction was performed as described previously.[24] For RNA sequencing (RNA-seq), total RNA was isolated using miRNAeasy Mini kit (Qiagen, Chatworth, CA, USA) according to the manufacturer's instructions. Gene expression profiling was performed with Affymetrix human U133 Plus 2.0 gene chips at Finnish Microarray and Sequencing Center, Turku Center for Biotechnology, Turku, Finland.[25] RMA (Chipster; CSC, Helsinki, Finland) was used for normalization of the data. Sequence specificity of probes was verified by BLAST search.

Whole transcriptome libraries were constructed using the SOLiD™ Whole Transcriptome Analysis Kit (Applied Biosystems, Foster City, CA) at Finnish Microarray and Sequencing Center, Turku Center for Biotechnology, Turku, Finland.[25] The samples were processed with the SOLiD 3Plus instrument with 35 bp read length. The colourspace reads were aligned against the human reference genome (GRCh37 assembly) using the standard whole transcriptome pipeline and the colourspace alignment tool (Applied Biosystems). The data were normalized as described previously (25). The microarray data (accession number GSE66368) and RNA-seq data (accession number GSE66412) are available at the Gene Expression Omnibus (GEO, NCBI; http://www.ncbi.nlm.nih.gov/geo/).

2.4 | Quantitative real-time PCR

Preparation of cDNA was performed as described previously.[25] Quantitative real-time PCR (qRT-PCR) analysis of cDNA samples was performed with specific primers and fluorescent probes for *CLDN11*: forward primer 5′-CGTGGGTGGCTGTGTCATC-3′,

reverse primer 5′- GAGCCCGCAGTGTAGTAGAAAC-3′ and probe 5′-CTGCTGCGCTGGAGATGCCC -3′. Primers and probes were purchased from Oligomer (Helsinki, Finland). β-actin was used as a control.[22,26] Applied Biosystems 7900HT Fast Real-Time PCR System was used to perform qRT-PCR.

2.5 | Immunofluorescence stainings

Cultured cells were fixed with ice-cold methanol for 5 minutes, blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 30 minutes at room temperature and treated with primary antibody (rabbit polyclonal claudin-11; Abcam, Cambridge, UK) diluted in BSA/PBS. Highly precross-absorbed goat anti-rabbit antibody (conjugated to Alexa dyes 488) (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody.[27] Hoechst (Invitrogen) was used to visualize nuclei. The cells were mounted in Mowiol-DABCO (Sigma, St Louis, MO, USA) and examined with Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). AxioVision Release 4.9.1 software (Carl Zeiss, Jena, Germany) was used for imaging the samples.

2.6 | Tissue samples and immunohistochemistry

Formalin-fixed paraffin-embedded tissue samples consisting of normal sun-protected skin (n=16), premalignant lesions, that is actinic keratoses (AK, n=31), benign epidermal papillomas, that is seborrhoeic keratosis (SK, n=7), cSCC in situ (cSCCIS, n=56), and sporadic UV-induced invasive cSCCs (n=65) were accessed from the archives of the Department of Pathology, Turku University Hospital. To perform immunohistochemistry (IHC) of human tissue microarrays, automated immunostaining device (Ventana Medical System SA, Illkrich, France) was used.[24] Rabbit polyclonal claudin-11 (HPA013166; Sigma) antibody was used. As a positive control for claudin-11 staining, mouse brain tissue samples were used. Claudin-11 staining was analysed by three observers (L.N., P.R. and M.K.).

2.7 | Western blot analysis

For Western blot analysis, cell lysates were fractionated in 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA), as previously described.[25] The following antibodies were used in Western blotting: rabbit polyclonal antibodies against claudin-1, claudin-11 (both from Invitrogen), phospho-CREB (Cell Signaling Technology, Beverly, MA, USA), β-tubulin and p38δ/SAPK4 (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal antibodies against ZO-1 (Invitrogen); β-catenin (Dako, Glostrup, Denmark); p38α (SAPK2a, Upstate) and β-actin (Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (sheep anti-mouse IgG HRP-linked whole antibody; Amersham Biosciences, and swine anti-rabbit immunoglobulins; both from DakoCytomation, Glostrup, Denmark) were used and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

2.8 | Adenoviral gene delivery

Cutaneous SCC cells were infected with adenoviral vectors at MOI 600, for 6 hours after which the medium was changed, as previously described.[28] Recombinant adenoviruses for dominant negative mutants of p38δ (RAdp38δAF) and p38α (RAdp38αAF)[29] were kindly provided by Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA). Recombinant adenovirus RAdLacZ, which contains the *Escherichia coli* β-galactosidase gene under the control of CMV IE promoter,[30] was kindly provided by Dr. Gavin W. G. Wilkinson (University of Cardiff, UK).

2.9 | siRNA knock-down experiments

The following siRNAs (all from Qiagen) were used: for claudin-11 Hs_CLDN11_5 (sense 5′-GCAAGUGAGUAUAACUCUATT-3′, antisense 5′-UAGAGUUAUACUCACUUGCAC-3′), for claudin-11 Hs_CLDN11_7 (sense 5′-GGUAUAUCAGUAUCUGAGATT-3′, antisense 5′-UCUCAGAUACUGAUAUACCAT-3′), for p38α Hs_MAPK 14_6 (sense 5′-GAGAACUGCGGUUACUUAATT-3′, antisense 5′-UUAA GUAACCGCAGUUCUCTG-3′), for p38δ Hs_MAPK13_5 (sense 5′-GGAGUGGCAUGAAGCUGUATT-3′, antisense 5′-UACAGCUUC AUGCCACUCCGG-3′). Non-specific siRNA (Qiagen) was used as a negative control. cSCC cell lines were transfected with siRNA using siLentFect™ Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA), as previously described.[31] For Western blot analysis, cells were incubated 72 hours after siRNA transfections and total cell lysates were collected.

2.10 | Cell proliferation assays

cSCC cells were transfected with negative control siRNA (control siRNA) and claudin-11 siRNA_5 or claudin-11 siRNA_7 (75 nmol/L) and seeded (1.0×10^4 cells/well) on 96-well plates. The number of viable cells was determined with WST-1 cell proliferation assay (Roche Diagnostics, Mannheim, Germany) at 0, 24, 48 and 72 hours. The experiments were carried out with six parallel wells in every time point with two cSCC cell lines (UT-SCC-12A and -105).

2.11 | Invasion assays

To study the effect of claudin-11 in cSCC cell invasion, cells were transfected with negative control siRNA (control siRNA) and claudin-11 siRNA_5 or claudin-11 siRNA_7 (75 nmol/L). 48 hours after transfection, the cells were trypsinized, suspended in DMEM containing 0.1% BSA and seeded (5.0×10^5 cells/insert) to Matrigel coated invasion chambers (BD Bioscience, Franklin Lakes, NJ, USA). Chemoattractant (10% FBS in DMEM) was added into the lower chamber. After 24-hour incubation, cells on the upper surface of the insert were removed and the invaded cells on the lower surface were fixed with methanol. Hoechst 33342 (Invitrogen, Paisley, UK) was used to visualize nuclei, and the nuclei were then counted under fluorescent microscope. The experiment was carried out with two cSCC cell lines (UT-SCC-12A and UT-SCC118).

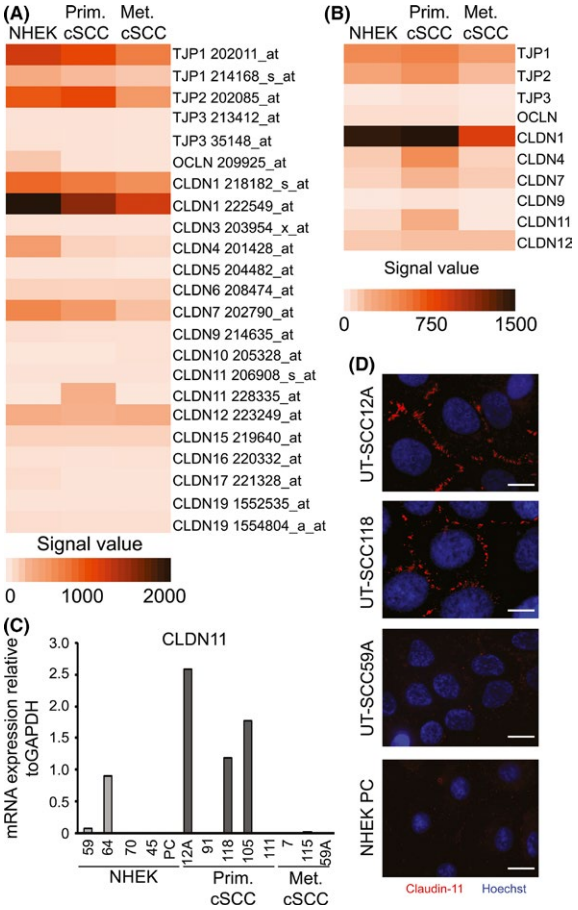

FIGURE 1 The expression of claudin-11 is upregulated in primary cSCC cell lines. The mRNA expression profile of tight junction molecules in normal human keratinocytes (NHEK), primary cSCC (n=5, Prim. cSCC) and metastatic cSCC (n=3, Met. cSCC) cell lines was analysed by (A) Affymetrix gene chip assay (NHEK, n=5) and (B) RNA sequencing analysis (NHEK, n=4). (C) Claudin-11 (CLDN11) expression was determined with quantitative real-time PCR in NHEKs, and primary and metastatic cSCC cells. (D) Cultured primary cSCC cells (UT-SCC12A and UT-SCC118), metastatic UT-SCC59A cells and NHEK-PC were labelled for claudin-11 with indirect immunofluorescence. Scale bar=10 μm

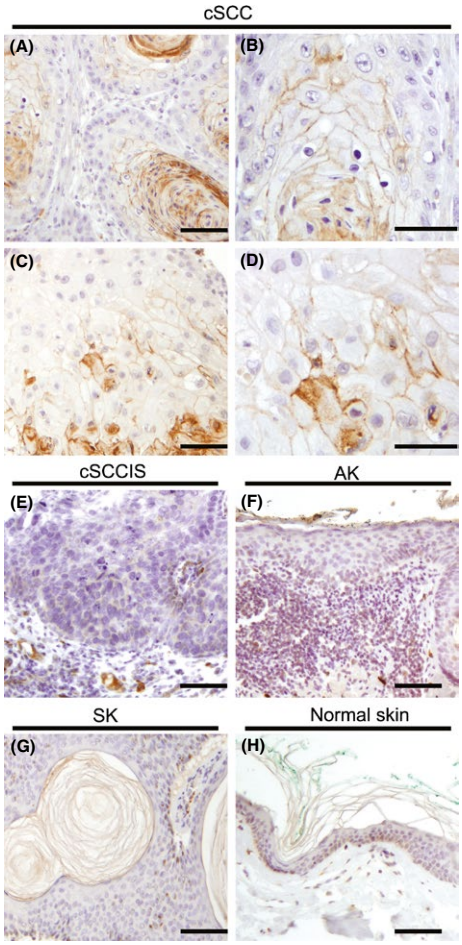

FIGURE 2 Expression of claudin-11 by tumor cells in cutaneous squamous cell carcinoma (cSCC). (A-H) Sections of cSCCs (n=65), cSCC in situ (cSCCIS, n=56), premalignant lesions (actinic keratosis, AK, n=31), benign epidermal papillomas (seborrhoeic keratosis, SK, n=7) and normal skin (n=16) were stained for claudin-11 by immunohistochemistry. Claudin-11 was detected in cell-cell contacts of well-differentiated areas of cSCC tumors (A-D). Normal skin (H), AK (F), cSCCIS (E) and SK (G) sections were negative for claudin-11. (A,C,E-H) Scale bars=100 μm. (B,D) Scale bars=50 μm

3 | RESULTS

3.1 | The expression of claudin-11 is upregulated in primary cSCC cell lines

The expression of TJ component mRNAs in primary (n=5) and metastatic (n=3) cSCC cell lines and NHEKs (n=5) was determined by oligonucleotide array-based expression profiling. The expression of mRNAs for *CLDN1*, *CLDN4*, *CLDN7*, *CLDN12*, *TJP1* (ZO-1) and *TJP2* (ZO-2) was detected both in cSCC cells and NHEKs (Figure 1A). The expression of *CLDN11* mRNA was upregulated in primary cSCC cell lines but not detectable in metastatic cSCC cells and NHEKs (Figure 1A). Additional analysis of the expression profile of mRNAs for TJ proteins in cSCC cell lines and NHEKs (n=4) with RNA-seq revealed elevated *CLDN11* mRNA levels in primary cSCC cells and very low levels in NHEKs and metastatic cSCC cell lines (Figure 1B). *CLDN11* mRNA levels in NHEKs, and primary and metastatic cSCC cells were also determined by qRT-PCR. The results showed that the mean level of *CLDN11* mRNA was higher in primary cSCC cell lines than in metastatic cSCC cell lines or NHEKs (Figure 1C). Indirect immunofluorescence labelling of cSCC cell cultures demonstrated the presence of claudin-11 in cell-cell contacts of primary cSCC cells, but not in metastatic cSCC cells or NHEKs (Figure 1D). Claudin-11 was selected for further characterization, because it was upregulated in primary cSCC cells and little-to-no expression was detected in NHEKs (Figure 1A-D).

3.2 | Tumor cell-specific expression of claudin-11 in cSCC in vivo

For analysis of claudin-11 in cSCCs in vivo, TMAs containing a large panel of tissue samples representing different stages of cSCC

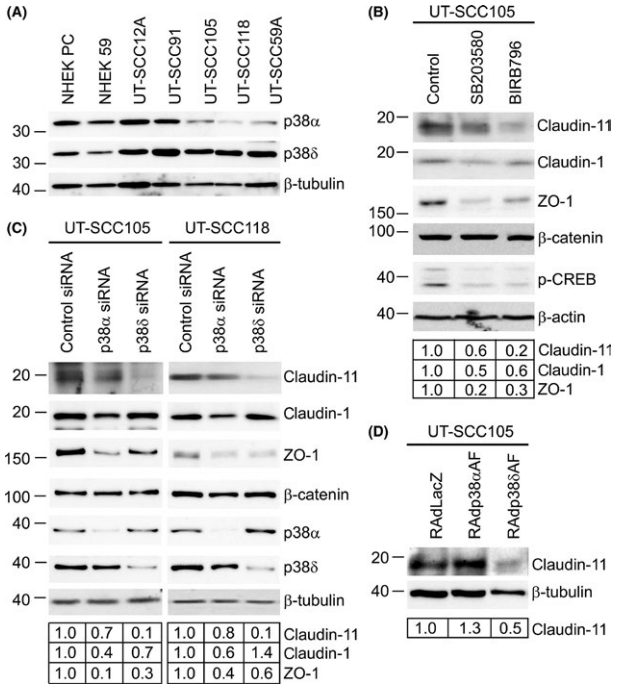

FIGURE 3 The production of claudin-11 in cSCC cells is regulated by p38δ. (A) The expression of p38α and p38δ subunits in normal human keratinocytes (NHEK) and cutaneous SCC cell lines (UT-SCCs) was analysed by Western blotting of total cell lysates. β-tubulin was determined as loading control. (B) UT-SCC105 cells were untreated (control) or treated with SB203580 (10 μmol/L) or BIRB796 (10 μmol/L) and incubated for 24 hours. Total cell lysates were analysed by Western blotting, and β-actin was used as loading control. Levels of phosphorylated CREB (p-CREB), a downstream mediator of the p38 MAPK pathway were determined by Western blot analysis to verify the effect of p38 inhibitors. Claudin-11, claudin-1 and ZO-1 levels quantitated densitometrically and corrected for β-actin level in the same sample are shown below the Western blots relative to levels in control cultures (1.0). (C) Cells were transfected with specific siRNAs (75 nmol/L) against p38α (p38α siRNA), p38δ (sip38δ siRNA) or control siRNA, and 72 hours after transfection, the levels of different cell junction proteins, p38δ, and p38α were determined by Western blotting of total cell lysates. β-tubulin was determined as loading control. Claudin-11, claudin-1 and ZO-1 levels quantitated densitometrically and corrected for β-tubulin level in the same samples are shown below the Western blots relative to levels in control cultures (1.0). (D) UT-SCC105 cells were infected with control adenovirus (RAdLacZ), adenoviruses harbouring dominant negative p38α (p38αAF) or dominant negative p38δ (p38δAF) and subsequently cultured for 72 hours. Total cell lysates were analysed by Western blotting. β-Tubulin was determined as loading control. Claudin-11 levels quantitated densitometrically and corrected for β-tubulin level in the same samples are shown below the Western blots relative to levels in control cultures (1.0)

progression were stained for claudin-11 by IHC. Samples consisted sporadic UV-induced invasive cSCCs (n=65), cSCCIS (n=56) and premalignant lesions (AK, n=31). Benign epidermal papillomas (SK, n=7) and normal skin samples (n=16) were examined as controls. Specific staining for claudin-11 was detected in the cell-cell contacts of keratinizing tumor cells in well and moderately differentiated cSCCs (20/65, 30% of all cSCC samples) (Figure 2A-D). No staining for claudin-11 was detected in unkeratinized tumor cells in less-differentiated areas of cSCCs. In addition, no staining for claudin-11 could be detected in poorly differentiated cSCC tumors (n=11). The expression of claudin-11 did not correlate with the level of inflammatory cells. All tissue samples from cSCCIS (Figure 2E), AK (Figure 2F), SK (Figure 2G) and normal skin (Figure 2H) were negative for claudin-11.

3.3 | Expression of claudin-11 in cSCC cells is regulated by p38δ

Our previous studies have demonstrated basal activation of p38 MAPKs in cSCC cells in culture and in vivo.[22] We have also shown that p38δ MAPK regulates the expression of ZO-1 in both NHEKs and cSCC cells.[27] In this respect, we investigated the role of p38 MAPK signalling in regulation of claudin-11 expression in cSCC cells. All cSCC cell lines and NHEKs studied expressed both p38α and p38δ isoforms (Figure 3A).[22] The role of p38 MAPK pathway in the regulation of the expression of junction proteins was first investigated by treating the cells with BIRB796 (10 μmol/L), a specific inhibitor of all four p38 isoforms (α, β, γ, δ) and with SB203580 (10 μmol/L), an inhibitor of p38α and p38β. Treatment with BIRB796 potently inhibited the expression of claudin-11, whereas SB203580 had no marked effect revealing the role of p38δ in the regulation of claudin-11 expression (Figure 3B). As shown previously, BIRB796 and SB203580 also inhibited the expression of ZO-1 in cSCC cells (Figure 3B).[27] Treatment with SB203580 or BIRB796 had no effect on claudin-1 or β-catenin levels in cSCC cells (Figure 3B). The role of p38δ in the regulation of claudin-11 was further examined using specific siRNAs for silencing of p38α and p38δ (Figure 3C). Knock-down of both p38α and p38δ inhibited the expression of ZO-1, but only silencing of p38δ inhibited the expression of claudin-11 in cSCC cells (Figure 3C). In contrast, knock-down of p38α or p38δ had no effect on the expression of claudin-1 or β-catenin in cSCC cell lines (Figure 3C). To confirm these results, the function of p38α and p38δ was inhibited by adenoviral expression of dominant negative mutants of p38α (p38αAF) and p38δ (p38δAF). Inhibition of p38δ downregulated the expression of claudin-11 in cSCC cell lines, whereas inhibition of p38α had no effect (Figure 3D).

3.4 | Claudin-11 regulates the invasion of cSCC cells

To elucidate the functional role of claudin-11 in cSCC cells, specific small interfering RNAs (claudin-11 siRNA_5 and claudin-11 siRNA_7, 75 nmol/L) were used to knock down the expression of claudin-11 (Figure 4A, Fig. S1A). Cutaneous SCC cultures transfected with control siRNA, or claudin-11 siRNAs (75 nmol/L) were incubated for 48 hours and the invasion of UT-SCC12A and UT-SCC118 cells through matrigel was subsequently determined. Knock-down of claudin-11 significantly increased the invasion of cSCC cells through matrigel (Figure 4B, Fig. S1B), but in the same time points, knock-down of claudin-11 had no effect on the number of viable cSCC cells (Fig. S2).

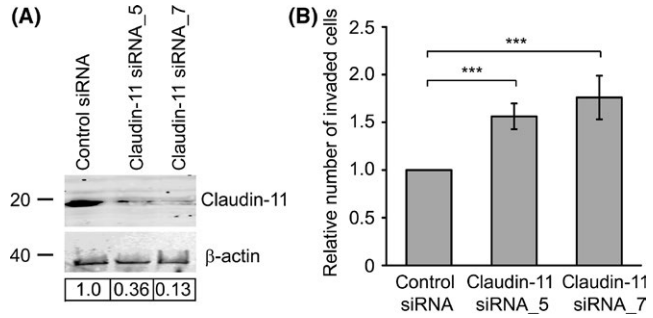

FIGURE 4 Claudin-11 knock-down increases invasion of cSCC cells. (A) UT-SCC12A cells were transfected with specific siRNAs against claudin-11 (claudin-11 siRNA_5 and claudin-11 siRNA _7) or control siRNA (75 nmol/L), and the cell lysates were analysed by Western blotting 72 hours after transfection. (B) UT-SCC12A cells were transfected with claudin-11 siRNA_5, claudin-11 siRNA_7 or control siRNA, and 48 hours after transfection, the cells were seeded to the upper chamber of the tissue culture inserts with 8.0 μm pore size coated with Matrigel. The cells were fixed, and the nuclei of the invaded cells to 10% FCS were visualized by Hoechst staining and counted after 24 hours. Data are shown as mean ± SEM (n=3). Statistical significance was determined by Student's *t* test

4 | DISCUSSION

In this study, we have examined the expression of TJ molecules in cSCC, the most common metastatic skin cancer.[4] The results of the oligonucleotide array, RNA-seq expression profiling and qRT-PCR of *CLDN11* mRNA revealed potent upregulation of claudin-11 mRNA expression in primary cSCC cells. Immunofluorescent staining of cSCC cells in culture detected claudin-11 in cell-cell contacts of cSCC cells. In contrast, little–to-no expression of claudin-11 was detected in NHEKs, in accordance with previous findings.[32] On the basis of these findings, claudin-11 was selected for further characterization to specify its role in the progression of cSCC.

Immunohistochemistry analysis of a large panel of tissue samples from cSCCs revealed specific staining for claudin-11 in cell-cell contacts of keratinizing tumor cells in well and moderately differentiated cSCCs in vivo, whereas no staining was detected in poorly differentiated cSCCs. The localization pattern of claudin-11 in cSCC was similar to that previously reported for claudin-4, and ZO-1 in cSCC[20] and resembles that of normal epidermis where TJs are present in well-differentiated cells in the granular layer. Altered expression of TJ molecules, for example claudins and ZO-1, has been described in various cancer types.[15] In addition, reduced expression of TJs and alterations in their function has been demonstrated during carcinogenesis associated with poor differentiation of tumors.[33] In this study, all tissue samples from preinvasive cSCCs (cSCCIS) and premalignant lesions (AK), as well as from benign skin tumors (SK) were negative for claudin-11 staining indicating that the induction of *CLDN11* is associated with progression of cSCC to the invasive stage.

Cutaneous SCC cells have been shown to express p38α and p38δ MAPK isoforms, and basal activation of p38 MAPK pathway promotes cSCC cell invasion.[22,34] The role for p38δ in cutaneous carcinogenesis has also been demonstrated in knockout mouse model of p38δ.[34,35] Furthermore, we have previously noted that the expression of ZO-1 is regulated by p38δ in NHEKs and cSCC cells.[27] The results of the present study demonstrate that claudin-11 expression in cSCC cells is specifically dependent on the activity of p38δ. It is therefore possible that activation of p38δ in the early stage of cSCC growth mediates induction of claudin-11 expression in cSCC cells in vivo.

In the present study, claudin-11 was absent in metastatic cSCC cell lines and in less-differentiated cSCC tumors in vivo. In addition, our results show that invasion of cSCC cells through matrigel is enhanced, when claudin-11 expression is silenced. These findings are also supported by previous studies in which the expression of claudin-11 was shown to decrease the invasiveness of bladder and gastric cancer cells in vitro.[36,37] Loss of claudin-11 could be due to epigenetic regulation observed for claudin family in human cancers. Methylation *of CLDN1* and *CLDN3* has been detected in colon and breast cancer and oesophageal and hepatocellular carcinoma, respectively.[38,39] Moreover, methylation of *CLDN4* and *CLDN5* genes has been demonstrated in bladder and pancreatic cancer.[40,41] Methylation of *CLDN11* has been detected in malignant melanoma, and methylation of this gene is significantly more frequent in skin metastases than in brain metastases.[42]

Expression of claudins is altered in various epithelial cancers in a stage- and tumor-specific manner making claudin family members potential biomarkers for these cancers.[15] During progression of cSCC, decrease in the expression of claudin-1 and increase in the expression of claudin-2 have been noted.[20] In addition, downregulation or loss of claudin-1 has been shown to promote brain metastasis of melanoma, and it has been suggested that expression of claudin-1 could serve as a prognostic marker for melanoma patients with a high risk of brain metastasis.[43] Our results reveal specific regulation of claudin-11 expression during cSCC progression and warrant further evaluation of the loss of claudin-11 as a biomarker for aggressive behaviour of cSCC.

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AUTHOR CONTRIBUTIONS

LN, ES, SP, JP and VMK contributed to the conception and design of the study. LN, ES, PR, MP and LR performed the experiments. LN, ES, PR, SP and VMK analysed and interpreted the data. MF, MK, PR and AK produced the TMAs. JP and SP established NHEKs. The manuscript was written and reviewed by LN, SP and VMK. All authors read the manuscript and approved the submission.

CONFLICT OF INTERESTS

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

FIGURE S1 Claudin-11 knockdown increases invasion of cSCC cells

FIGURE S2 Knockdown of claudin-11 has no effect on the number of viable cSCC cells

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