Identification of dysregulated genes in cutaneous squamous cell carcinoma

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Abstract. Carcinogenesis is a multi-step process resulting from the accumulation of genetic mutations and subsequently leading to dysregulated genes, but the number and identity of differentially expressed genes in cutaneous squamous cell carcinoma (SCC) is unknown at present. In order to identify dysregulated genes, we examined the relative mRNA expression present in cutaneous SCC and its precursor lesion actinic keratosis (AK) by comparison to normal skin. Snap frozen biopsies from 20 specimens of normal skin, 10 AK, and 10 cutaneous SCC were examined. Total-RNA was extracted, reversely transcribed, and 14 genes were investigated using gene-specific intron-flanking primers and quantitative real-time reverse transcription PCR. Specificity was confirmed by sequencing of the PCR amplicons. Ten of 14 genes were significantly dysregulated in AK and/or cutaneous SCC by comparison to normal skin. The genes *CNN2*, *COX4I1*, *COX5B*, *COX7C*, *CRLF3*, *CTSC*, *NDRG1,* and *LMNA* showed increased expression in skin cancer (p<0.02), while *RPL15* and *LGTN* were down-regulated (p<0.03). The genes differentially expressed during skin carcinogenesis may prove useful in order to understand the origin and progression of cutaneous SCC and for diagnostic approaches.

Introduction

Non-melanoma skin cancer (NMSC) is the most common tumor among populations of European origin. Actinic keratosis (AK) is an early stage of cutaneous squamous cell carcinoma (SCC), and approximately 10% of cases progress to SCC (1-3). Several studies suggest cumulative lifetime exposure to ultraviolet (UV-)radiation of the sun as the primary risk factor for NMSC (4,5). The mechanism of UV-induced carcinogenesis includes the mutation of the *p53* tumor suppressor gene (6), but the detailed molecular pathways remain to be determined.

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The step-wise accumulation of mutations and hence dysregulation of genes are key factors for neoplastic development (7). The identification of distinct genetic aberrations, which may provide useful information on tumor classification, prognosis of etiopathology and response to therapy is, therefore, of prime importance in cancer research (8). Approximately 700 genes (1-1.5% of 30,000-50,000 different expressed genes per cell) showed altered expression in breast cancer, and comparable amounts of genes were affected in ovarian and colon tumors (9,10). However, the number and identity of genes that are differentially expressed in NMSC are largely unknown at present.

In NMSC cell lines, genes from at least three functional categories are dysregulated by comparison to cells from normal skin (11), including apoptosis genes, DNA repair genes and extracellular matrix proteases (12-14). Genes that are involved in extracellular matrix production and apoptosis are already altered in pre-neoplastic cells, while those involved in DNA repair or epidermal growth are dysregulated in later stages of tumorigenesis (15).

Despite the limited explanatory power of cancerous cell-line investigation (16,17), studies on dysregulated genes investigating NMSC biopsies are scare. Dooley and colleagues (18) were the first to describe dysregulated genes for cutaneous SCC in both *in vitro* and *in vivo*. In a cDNA microarray, 5 differentially expressed genes were frequently found in cell lines and human tissues, namely *FN1*, *ANAX5*, *G3P2*, *ZNF254,* and *HAPIP*.

In a previous approach, we have examined the mRNA expression of genes from skin cancer biopsies (comprising normal skin, AK, and SCC) by cDNA microarray analyses and revealed up- or down-regulated genes, respectively (unpublished data). In this study, we investigated the expression of 14 selected genes involved in different functional categories (e.g., adhesion, communication, differentiation, metabolism, proliferation, respiration) present in a larger cohort using quantitative real-time reverse transcription PCR (qRT-PCR). Ten of these genes were significantly dysregulated in normal skin by comparison to both AK and cutaneous SCC, and *LMNA* and *NDGR1*, as well as genes of the respiratory chain are known to play essential parts in various types of cancer.

Materials and methods

Patients. Forty punch biopsies (diameter 4 mm) were collected at the Charité, University Hospital (Berlin, Germany) from

Table I. Primer sequences and quantitative real-time RT-PCR conditions of 14 genes.

Gene	T_{ann} (˚C)	MgCl$_2$ (mM)	Forward (5'-3')	Reverse (5'-3')	Amplicon (bp)
CNN2	55	3	AAC CGC TCC ATG CAG AAC	CCC ACT CTC AAA CAG GTC GT	120
COX4I1	60	3	CTA GTT GGC AAG CGA GCA AT	TCA CGC CGA TCC ATA TAA GC	104
COX5B	55	3	ACT GGG TTG GAG AGG GAG AT	TCT TCC CTG GTG CCT GAA	95
COX7CP1	60	3	GCA TTT GCT ACA CCC TTC CT	CAC TTC CAG AGG CTG CAC	117
CTSC	60	5	TTA CTG CAA CGA GAC AAT GAC TG	AGG TGT GCT GTG TTG ACA TAC	120
CRLF3	60	4	AAA CCT GGA GGC ATC ATT GT	GCG CAG ACT CTG AAC TGG TA	182
DNCLI2	60	3	CCA GTG TGC CTA GCT CCT C	CTG ACT CTT TGG CTG TGC TCT	177
EMP2	55	3	ACC AAC AAC ACG AAT TGC AC	GAA GAT GAA GAA GGC GAT GC	120
JTB	60	3	GTG GTC CCA CAG GAT ATG TAG A	CGT TGT TCC ATC AAA GCT GA	91
LGTN	60	4	CTT GTG GCG GTC TGG AAA CA	AGG GTC ATG TGC CTC ATG TCT	135
LMNA	55	3	CCG ATA AGG AAG GTC AGC AT	TGC CCG AAA TAC TCT CTC AAA	236
NDRG1	60	3	GCT GAA ATG CTT CCT GGA GT	CTC CAC CAT CTC AGG GTT GT	120
RPL15	60	3	GTT CTG GCC AAA CAA CCC TA	CAA AGT GGG TGC ACA AGT GA	71
TARDBP	60	4	TGC TTC GGT GTC CCT GTC	GGG CTC ATC GTT CTC ATC TT	100

T_{ann}, annealing temperature; bp, base pairs.

three diagnostic stages of tumorigenesis comprising 20 specimens of normal skin (49-85 years, median 70 years), 10 AK (55-77 years, median 61 years), and 10 SCC (46-94 years, median 63 years). Half of the tissue was immersed in liquid nitrogen within 2 min of resection and stored at -70˚C. The other half of each biopsy (excluding normal skin specimens) was fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin for histology. The study was approved by the local ethics committee at the Charité, University Hospital, Berlin, Germany (no. Si. 248).

RNA extraction. Total-RNA was isolated using a modified RNeasy Micro Kit protocol (Qiagen, Hilden, Germany). The modification included the homogenisation of the frozen tissue in 300 µl of buffer RLT (Qiagen) with 20 ng glycogen (Roche, Mannheim, Germany) using a rotor-stator homogeniser Ultra Turrax T25 (Janke & Kunkel, Staufen, Germany). The homogenised tissue was digested using 0.1 mg proteinase K (Roth, Karlsruhe, Germany) at 55˚C for 15 min. Subsequently, the sample was digested with DNase I (Invitrogen, Karlsruhe, Germany). Quantification of total-RNA was performed by RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions.

Quantification using real-time RT-PCR. Reverse transcription was performed with the Superscript First-Strand Synthesis-System for qRT-PCR (Invitrogen) using oligo-dT as described by the manufacturer. The concentration of cDNA was quantified with OliGreen ssDNA Quantification Kit (Molecular Probes). Gene-specific intron-flanking primers were designed for each of the 14 genes under investigation in order to avoid genomic DNA contamination (Table I) using the software

Primer 3 (19). The primers were synthesised by Metabion (Planegg-Martinsried, Germany).

qRT-PCR was conducted using LightCycler technology with calibrated standard curves (Roche) and was performed in duplicate for each sample. For specific primers, annealing temperature and concentration of MgCl$_2$ were optimised (Table I). The amplification mix (20 µl) contained 20 ng of cDNA, 500 nM of each primer, 2 µl LightCycler FastStart Reaction Mix SYBR Green I (Roche), 3-5 mM MgCl$_2$, and double-distilled water. The qRT-PCR protocol included 10 min initial denaturation at 95˚C and 40 cycles: 10 sec at 95˚C, 5 sec at 60˚C, 10 sec at 72˚C. Specificity of PCR products (comprising the genes *CNN2*, *COX4I1*, *COX5B*, *COX7CP1*, *CRLF3*, *CTSC*, *DNCLI2*, *EMP2*, *JTB*, *LGTN*, *LMNA*, *NDRG1*, *RPL15*, and *TARDBP*) was verified by melting curve analysis. Furthermore, PCR products were sequenced on the ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) using gene specific primers (Table I) and DNA sequencing kits (Applied Biosystems). Sequence analysis confirmed the specificity of all 14 genes under investigation.

The relative expression levels of 14 genes were quantified in 3 diagnostic groups of cancerous tissues, including 20 normal skins, 10 AK and 10 SCC. Expression levels were determined as the ratio between the gene under investigation (as the mean of the results of 2 independent qRT-PCR experiments) and the reference gene *RPS9* in order to correct for variation in the mRNA levels. Ratios were then normalised such that the mean ratio of all 20 normal skin samples equaled 1.00.

Statistical analysis. For analysis of the relative expression rates of normal skin versus AK and normal skin versus
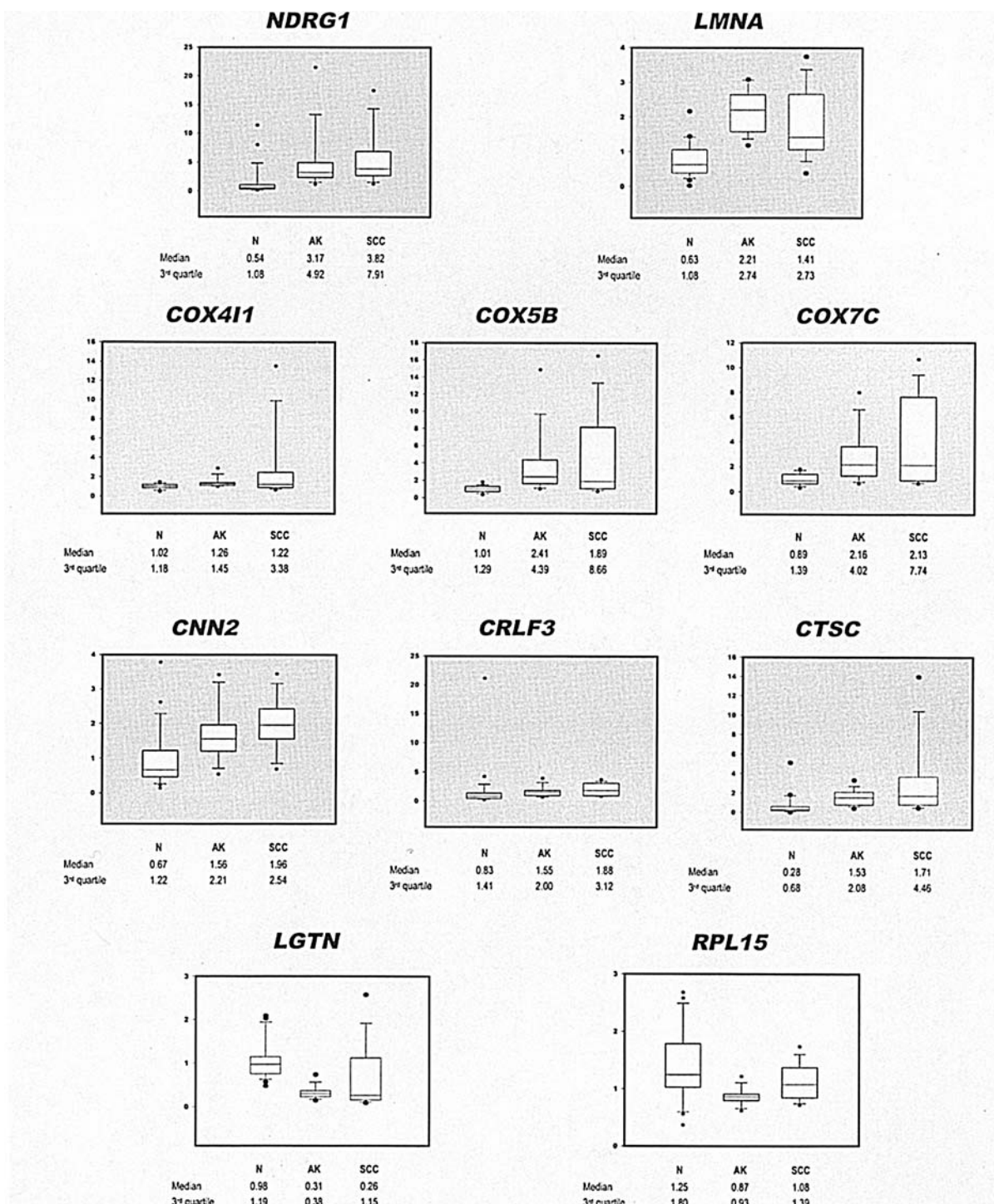
Figure 1. Ten genes with significant dysregulated RNA expression in AK and SCC. Quantitative real-time RT-PCR was used to investigate the expression of 10 genes in 20 normal skin (N) versus 10 actinic keratoses (AK) versus 10 squamous cell carcinoma (SCC), respectively. Gene expression was equalised to *SPR9*, and the ratios were subsequently normalised to the mean value of the total normal skin samples, as described in Materials and methods. The p-values are summarised in Table II. The relative expression rates of each gene are shown at the vertical axis. Shaded diagrams indicate upregulated genes, while down-regulated genes have a white background. Horizontal line in box, median; box, lower and upper quantiles; horizontal line at end of whisker, 2.5 and 97.5% values; individual plots, values outside the range of the whiskers.

SCC (Table II, Fig. 1) the U-test by Wilcoxon, Mann, and Whitney was applied. A p-value of <0.05 was considered significant.

Results

The mRNA expression levels of 14 genes have been quanti-fied in 3 diagnostic groups of cancerous tissues, including 20 specimens from normal skin, 10 AK and 10 SCC (Table II). Significant differential expression was observed for 10 of 14 genes. Seven genes (*CNN2*, *COX5B*, *COX7C*, *CRLF3*, *CTSC*, *LMNA,* and *NDRG1*) were significantly upregulated in both stages AK (p=0.019 through p<0.001) and cutaneous SCC (p=0.019 through p<0.001) by comparison to normal skin (Table II, Fig. 1). An increase of gene expression concurring with the severity of the lesion was observed for 4 upregulated

Table II. List of 14 selected genes used for expression analysis in normal skin, AK and SCC.

Gene	Description	Function	Chromosome localisation	GeneBank accession no.	Change fold (median AK or SCC divided by median normal skin) (p-value of N versus AK or SCC)	
					AK	SCC
Adhesion						
CNN2	Calponin 2	Organisation of actin cyto-skeleton, widely distributed	21q11.1	NM_004368	2.33 (p=0.011)	2.93 (p=0.003)
LGTN	Ligatin	Receptor participating in intercellular adhesion and specific metabolic processes in neurons	1q31-q32	NM_006893	0.32 (p<0.001)	0.27 (p=0.031)
LMNA	Lamin A/C	Structural organization of nucleus and chromatin, ubiquitous distributed in differentiated cells	1q21.2-q21.3	NM_005572	3.51 (p<0.001)	2.24 (p=0.003)
Communication						
CRLF3	Cytokine receptor-like factor 3	ATP/GTP binding, precise distribution unknown	17q11.2	NM_015986	1.87 (p=0.019)	2.27 (p=0.008)
NDRG1	N-myc downstream regulated gene 1	Stress and hormone response, cell growth and differentiation in epithelial cells	8q24	NM_006096	5.87 (p<0.001)	7.07 (p<0.001)
Differentiation						
EMP2	Epithelial membrane protein 2	Endometrial protein necessary for blastocyst implantation, high expression in eye, lung, heart, thyroid, uterus	16p13.2	NM_001424	1.10 (ns)	0.74 (ns)
Metabolism						
CTSC	Cathepsin C	Protein degrading, pro-enzyme activating, highly expressed in lung, kidney, placenta, immune cells	11q14.1-q14.3	NM_001814	5.46 (p=0.001)	6.11 (p<0.001)
DNCLI2	Dynein, cytoplasmic, light intermediate polypeptide 2	Involved in retrograde organelle transport, ATP/nucleotide binding	16q22.1	NM_006141	0.86 (ns)	0.96 (ns)
Proliferation						
JTB	Jumping translocation break-point	Fusing with telomeric repeats of acceptor telomeres at jumping translocation, ubiquitous distribution	1q21	NM_006694	0.89 (ns)	1.17 (ns)
RPL15	Ribosomal protein L15	Structural constituent of ribosomes, ubiquitous distribution	3p24.2	NM_002948	0.70 (p=0.005)	0.86 (ns)
TARDBP	TAR DNA binding protein	Binding to bulge regions of TAR RNA, activating HIV-1 long terminal repeat (LTR), ubiquitous distribution	1p36.2	NM_007375	0.90 (ns)	1.50 (ns)

Table II. Continued.

Gene	Description	Function	Chromosome localisation	GeneBank accession no.	Change fold (median AK or SCC divided by median normal skin) (p-value of N versus AK or SCC)	
					AK	SCC
Respiration						
COX4I1	Cytochrome-c oxidase subunit IV isoform 1	Catalysing electron transfer from reduced cytochrome c to oxygen, ubiquitous distribution	16q22-qter	NM_001861	1.24 (p=0.011)	1.20 (ns)
COX5B	Cytochrome-c oxidase subunit Vb	Catalysing electron transfer from reduced cytochrome c to oxygen, ubiquitous distribution	2cen-q13	NM_001862	2.39 (p<0.001)	1.87 (p=0.010)
COX7C	Cytochrome-c oxidase subunit VIIc	Catalysing electron transfer from reduced cytochrome c to oxygen, ubiquitous distribution	5q14	NM_001867	2.43 (p=0.006)	2.39 (p=0.019)

Significantly dysregulated genes that have been identified using quantitative real-time RT-PCR are indicated in bold. AK, actinic keratosis; SCC, squamous cell carcinoma; N, normal skin; ns, not significant.

genes, with the exception of *LMNA* and the 3 *COX* genes exhibiting higher expression in AK than in SCC. Two genes (*LGTN* and *RPL15*) showed a lower expression level in each sample of both AK and SCC compared to normal skin. Significant down-regulation from normal skin to skin cancer was observed for LGTN in AK (p<0.001) and SCC (p=0.031) and for *RPL15* exclusively in AK (p=0.005), respectively. *RPL15* and *COX4I1* were the only genes showing significant dysregulation exclusively in AK, whereas the other 8 genes were significantly dysregulated both in AK and in SCC by comparison to normal skin.

The increase of significant gene expression ranged from 1.24 (AK of *COX4I1*) through 7.07 (SCC of *NDRG1*) and the decrease from 0.70 (AK of *RPL15*) through 0.27 (SCC of *LGTN*), respectively (Table II). The highest differences of expression in normal skin versus AK and SCC were observed for the up-regulated genes *NDRG1* (5.87 and 7.07) and *CTSC* (5.46 and 6.11) and the lowest for the down-regulated gene *RPL15* (0.70). Using qRT-PCR, 4 of 14 genes (*DNCLI2*, *EMP2*, *JTB,* and *TARDBP*) were not significantly dys-regulated in AK and/or SCC by comparison to normal skin.

Discussion

We investigated the mRNA expression of 14 selected genes in specimens from normal skin, AK, and cutaneous SCC. Ten of these genes (*CNN2*, *COX4I1*, *COX5B*, *COX7C*, *CRLF3*, *CTSC*, *LGTN*, *LMNA*, *NDRG1*, and *RPL15*) showed significantly differential expression in NMSC by comparison to normal skin. Predominantly, they are known to exhibit primary functions within the cytoplasm or are associated with

the nucleus, a noteworthy concordance to insights in the cellular biology of autoimmune diseases (20,21). Aberrant expression levels were frequent already in the early cancerous stage AK suggesting that the dysregulation of these genes are crucial early steps in the pathogenesis of skin cancer. The genes of the respiratory chain, lamins (*LMNA*), and *NDRG1* were already in previous studies considered to play important roles in various types of cancer including skin carcinoma. However, this is the first report of *CRLF3* and *RPL15* to be involved in carcinogenesis of cutaneous SCC and that *CNN2*, *CTCS,* and *LGTN* are integral parts of the malignant disease.

Altered communication of cells in their micro- and macro-environment is essential for tumor development. *NDRG1* encodes a 394-amino acid protein with a molecular mass of 43 kDa and is upregulated in response to cellular stress such as hypoxia and DNA damage (22,23) contributing to cellular differentiation (24-26). The gene has been frequently associated with neoplastic developments, but its role in tumori-genesis is controversial. *NDRG1* (or its protein) is expressed at lower levels in colon, breast and prostate cancers than in normal tissue of human biopsies using Northern blots (27) or based on both Western blot analysis and immunohisto-chemistry (28). To the contrary, *NDRG1* (or its protein) was upregulated in mouse skin carcinoma as inferred from Northern blots (24), human oral SCC using both qRT-PCR and Western blots (26), and additionally in a variety of human cancer biopsies, including brain, liver, lung, and renal tumors based on immunohistochemistry (23). In our study, *NDRG1* showed the highest increase of expression in the genes under investigation in cancer specimens by comparison to those of normal skin. *NDRG1* may thus prove useful as biomarker for

early stages of cutaneous SCC, and the overexpression in dysplastic cells may reflect stress response to dysfunction of entire cells.

Cell adhesion proteins play important roles in cell migration and invasion, and knowledge upon their function and regulation is therefore crucial in cancer research. *LMNA* encodes A-type lamins by alternate splicing such as lamin A (664 amino acids, 74 kDa) and lamin C (572 amino acids, 65 kDa), which exhibit important functions during various steps of post-mitotic nuclear reassembly including cross-linking of chromatides and nuclear lamina assembly (29). Mutations occurring in this gene lead to diseases such as muscular dystrophy, conduction-system disease, cardiomyopathy and partial lipodystrophy (30). A-type lamins have been extensively studied and are considered to interfere intensely in cell functions during tumorigenesis. They were reduced or absent in proliferating cells of human biopsies on the mRNA or protein level, including various types of cancer such as leukaemia and lymphomas (31), ovarian cancer (32) and gastrointestinal neoplasms (33). Conflicting results on lamin expression were reported in NMSC by immunohistochemistry showing either a lower (34,35) or a higher expression in dysplastic cells by comparison to normal skin (36). We observed increased mRNA expression of *LMNA* in cutaneous SCC, and the highest mRNA levels were detected in AK. This suggests an involvement of *LMNA* in early stages of NMSC supporting the results of Tilli and colleagues (36). The importance of increased *LMNA* expression in skin cancer remains to be elucidated by further studies, but may indicate that such neoplasms arise by amplified cell survival and resistance to apoptosis rather than by hyperproliferation.

Mitochondria play essential roles in cellular energy metabolism, free radical generation, and apoptosis, and mtDNA is particularly susceptible to damage and oxidative stress. Thus, alterations in respiratory activity and genetic aberrations of mitochondria are considered as inherent to a wide range of cancers (37). Durham and colleagues (38) investigated the genetic deletion spectrum of mtDNA in NMSC by comparison to normal skin, but it remains unclear at present if mtDNA damage has a direct impact on skin cancer. *COX7C* is a 63-amino acid respiratory protein with a molecular mass of 7 kDa and was significantly upregulated in NMSC by comparison to normal skin in our study. Whether its increase in cancerous tissue simply responds to degradation of entire cells has to be elucidated in future studies.

CNN2 encodes a 309-amino acid protein with a molecular mass of 36 kDa and may be involved in the structural organisation of actin cytoskeleton (39,40). Initially, *CNN2* was detected in various cell types of the human heart (39), but was additionally found in osteoblasts (41) and at the cytoplasmic region of cell-to-cell junctions in cultured keratinocytes and human skin tissue (42). Furthermore, it may play an important role in migration of endothelial cells, and the expression is critical for proper vascular development (43). In our study, *CNN2* was upregulated both in AK and in SCC, but its precise function during tumorigenesis is elusive at present.

The translation of *LGTN* yields a 584-amino acid membrane protein with a molecular mass of 65 kDa occuring during embryonic development and in early differentiated stages. It is a trafficking receptor for the attachment of phosphoglycoproteins within endosomes and at the cell periphery, where it participates in metabolism and intercellular adhesion (44). We observed a lower mRNA expression of *LGTN* both in AK and in cutaneous SCC. Histologically, *LGTN* is largely restricted to the cell body region of rat hippocampal neurons with little or no dendritic and axonal expression (45), but the correlation to human carcinogenesis of the skin remains to be determined.

The translation of *CTSC* results in a lysosomal 463-amino acid protease with a molecular mass of 200 kDa, and its main functions are considered to be protein degradation and pro-enzyme activation (46). Defects cause the Papillon-Lefevre syndrome, a rare autosomal recessive disorder that is characterised by hyperkeratotic skin lesions and early-onset periodontitis (47,48). In our study, *CTSC* mRNA levels were significantly increased in NMSC by comparison to normal skin. The constitution of the immune system has a strong influence on the development of NMSC (49), and this may be reflected by the significant upregulation of *CTSC* in AK and cutaneous SCC.

The present study identified 10 genes that were significantly differentially expressed during various stages of cutaneous SCC using qRT-PCR. Respiratory chain genes, lamins and *NDGR1* are well-known with respect to the origin and progress of various kinds of cancer, but this is the first report of *CRLF3* and *RPL15* to be involved in cutaneous SCC and of *CNN2*, *CTCS*, and *LGTN* to play integral roles in tumorigenesis. The dysregulation of genes in both AK and SCC compared to normal skin suggest involvement in the development of skin cancer. These genes may prove useful for diagnostic approaches, and studies analysing their precise function are warranted.

Acknowledgements

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