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Aberrant expression and high-frequency mutations of *SHARPIN* in nonmelanoma skin cancer

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Abstract. Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) have exhibited a marked increase in incidence in previous decades and are the most common malignancies in Caucasian populations. Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein (SHARPIN) has been identified as a commonly overexpressed proto-oncogene in several types of visceral cancer. However, to the best of our knowledge, the functions of SHARPIN in nonmelanoma skin cancer (NMSC) have not been described. The present study aimed to investigate the expression of SHARPIN protein and *SHARPIN* mutations in NMSC. A total of 85 BCC, 77 SCC and 21 keratoacanthoma (KA) formalin-fixed paraffin-embedded (FFPE) samples were collected. SHARPIN expression was detected using immunohistochemistry. DNA was extracted from the FFPE samples, and the sequences of *SHARPIN* were analyzed using polymerase chain reaction. In addition, high and moderate expression levels of SHARPIN were observed in normal skin tissues and KA samples. However, the expression of SHARPIN was absent in cancer nests and was significantly low in precancerous NMSC lesions. The total mutation frequency of SHARPIN was 21.8% in BCC and 17.0% in SCC. These data indicate that SHARPIN may serve a tumor-suppressing role and be a promising diagnostic, prognostic and therapeutic biomarker in NMSC.

Introduction

The incidence of nonmelanoma skin cancer (NMSC), which includes squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), has exhibited a marked increase in the previous decade and at present is the most common malignancy in Caucasian populations (1). NMSC is associated with a low rate of mortality but a high rate of disfigurement in cases where skin lesions are located on the head and neck. In addition, SCC occurs less frequently compared with BCC but is generally more aggressive. Sunlight (2), viral infection (3), diet (4), immunosuppression in organ transplant recipients (5) and induction of spontaneous genetic mutations (6) have been regarded as causes for NMSC. Tumors are markedly associated with chronic ultraviolet (UV) radiation exposure and occur primarily on sun-exposed areas of the body (7). Early detection and surgical removal may prevent the majority of complications. However, skin cancer has a high rate of recurrence and occasionally tumors progress to advanced stages that are difficult to treat with present therapeutic modalities; additionally, advanced-stage tumors become associated with high morbidity and decreased survival rates (8). At present, treatment options have remained limited for locally advanced or metastatic NMSC. Therefore, an in-depth understanding of the molecular basis of skin tumorigenesis is necessary in order to develop novel and specific diagnostic biomarkers and efficient therapies.

Src homology 3 and multiple ankyrin repeat domains protein (SHANK)-associated RH domain-interacting protein (SHARPIN) is a 387-amino acid protein that was originally identified as a SHANK-binding protein, which is enriched in the postsynaptic density of excitatory neurotransmitters (9). In addition, SHARPIN has been detected in cancer in the brain, spleen, lungs and other organs. Seymour *et al* (10) have identified *SHARPIN* as a gene mutated in chronic proliferative dermatitis (*cpdm*) mice (*Sharpin*$^{cpdm/cpdm}$) which spontaneously causes chronic inflammation, primarily in the skin, but also in other tissues including the gut, lung, liver and esophagus. SHARPIN has been previously identified as a common component of the linear ubiquitin chain assembly complex (LUBAC) which also contains E3 ubiquitin protein ligase ring finger protein 31 and RanBP-type and C3HC4-type zinc finger-containing protein 1 (HOIL-1L) (11). The C-terminal portion of SHARPIN consists of a ubiquitin-like (UBL) domain followed by an Npl4-zinc finger (NZF) domain and is important for the formation of a complex with the LUBAC component, haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1 interacting protein and ubiquitin (9).

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LUBAC is an important component of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which is a critical regulator of inflammation, immune response and lymphoid tissue development (12). NF-κB signaling is generally classified into canonical and non-canonical pathways. The canonical pathway, primarily triggered by tumor necrosis factor (TNF), lipopolysaccharides, and T and B cell receptors, occurs in the majority of cells as the principal NF-κB pathway. Upon stimulation, the downstream kinase inhibitor of κB (IκB) kinase (IKK) complex, composed of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit [NF-κB essential modulator (NEMO)], is activated, allowing the phosphorylation of the IκBα inhibitory protein. A linear form of polyubiquitin chains was previously identified in the NF-κB signaling pathway following TNF stimulation (13). The generation of linear ubiquitin polymers is catalyzed by LUBAC. Previous evidence indicates that LUBAC is recruited to TNF receptor complexes upon TNF induction, and then conjugates linear ubiquitin chains to the regulatory subunit NEMO of the IKK complex (14). This activates the kinase activity of IKK and ubiquitin-dependent degradation of phosphorylated IκBα, therefore enabling the nuclear translocation of NF-κB dimers and downstream gene expression (15). SHARPIN contains a PH (pleckstrin homology) domain at the N-terminus, which serves as a dimerization domain and may serve a role in other physiological functions of SHARPIN, including its tumor-associated role and its ability to inhibit β1-integrin activation (16). Furthermore, SHARPIN has been identified as a commonly overexpressed proto-oncogene and functionally serves tumor-associated roles during cancer progression according to previous studies (17-23). However, data regarding the function of SHARPIN in the pathogenesis and development of NMSC is lacking. These background data prompted the present study to investigate the expression and mutations of SHARPIN in skin tumors and identify a promising prognostic biomarker and therapeutic target for NMSC. Immunohistochemistry was utilized in the current study to assess SHARPIN expression in NMSCs and polymerase chain reaction (PCR) was used to detect mutations of *SHARPIN* in NMSCs. It was revealed that the expression of SHARPIN was absent in cancer nests and was significantly low in precancerous NMSC lesions. The total mutation frequency of SHARPIN was 21.8% in BCC and 17.0% in SCC.

Materials and methods

Literature retrieval. To acquire all literature regarding SHARPIN and NMSCs, PubMed (https://www.ncbi.nlm.nih.gov/pubmed) was searched using the following search string to identify relevant papers: (NMSC) OR non-melanoma skin cancer AND SHARPIN. No restrictions on publication date or language were imposed during the search strategy. No articles were identified.

Specimen selection. Anonymized control DNA samples from blood specimens of 100 normal individuals and skin tissues from 12 healthy volunteers who received cosmetic surgeries were obtained according to a protocol approved by the Southern Medical University Shenzhen Hospital Subject Review Board. All 100 normal individuals and 12 healthy volunteers did not have skin diseases. Formalin-fixed paraffin-embedded (FFPE) samples were retrieved from the Department of Dermatology of Shenzhen Hospital in Southern Medical University (Shenzhen, China). All samples from January 2012 to June 2017 were biopsied. All samples were fixed for 24 h in 10% formalin solution at room temperature. The thickness of the sections was 4 μm. A total of 85 BCC, 77 SCC and 21 keratoacanthoma (KA) FFPE samples were collected. The diagnoses of the samples were confirmed by pathologists from the Department of Dermatology of Shenzhen Hospital in Southern Medical University. Informed consent was obtained from all patients.

DNA extraction and mutation sequencing. DNA was extracted from the blood using the phenol-chloroform method (24). The FFPE genomic DNA was extracted using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany). To detect hotspot mutations, 8 exons and exon-intron adjacent sequences of the *SHARPIN* gene were amplified using PCR. In the DNA from the tumor samples, each amplification reaction was performed under standard conditions in a 20 μl PCR mixture containing 70-150 ng template DNA, 10 pmol primers, and 10 μl 2X Taq Master Mix (Dye Plus) (Vazyme, Piscataway, NJ, USA). The GC percentage of Exon 1 was relatively high; therefore, the 2X Taq Master Mix (Dye Plus) was replaced by 2X Phanta Max Master Mix (Vazyme) in the amplification of Exon 1. The 8 primer pairs that were used are listed in Table I. Exon 3 was amplified by PCR. The thermocycler conditions for the standard and nested PCR protocols are listed in Table II. PCR products were purified using QIAquick reagent (Qiagen GmbH) and directly sequenced based on the Big Dye Terminator sequencing chemistry (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA USA) in an ABI3130 automated sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). All mutations were confirmed through repeated bidirectional sequencing on the ABI sequencer. Gene sequences were blasted using DNASTAR Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA).

Immunohistochemistry. FFPE sections were deparaffinized in xylene at room temperature and rehydrated in 100, 95, 90, 80 and 70% alcohol solutions prepared with absolute ethyl alcohol and distilled water. For antigen retrieval, sections were heated in citrate buffer (pH 6.0) for 15 min at 100˚C in a microwave oven and naturally cooled to room temperature. Subsequently, the samples were blocked with a mixture of methanol and 0.75% hydrogen peroxide for 20 min at room temperature. Following washing with PBS, samples were incubated with SHARPIN antibody (cat. no., sc-98127; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution, 1:100) at 4˚C overnight. Subsequent to incubation, slides were washed three times with PBS. The slides were then processed using a 2-step Plus® Poly-horseradish peroxidase Anti-Mouse/Rabbit IgG Detection System (cat. no., PV-9000; ZSGB-BIO; OriGene Technologies, Inc., Beijing, China) and were developed with a DAB Detection kit (Enhanced Polymer; cat. no., PV-9000-D; ZSGB-BIO; OriGene Technologies, Inc.) for 3 min at room temperature. SHARPIN immunohistochemical staining was expected to be localized to the cytoplasm.

Table I. Primers used in the screening of Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein gene mutations.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')
1	CAGGTTCGCGGCCCGTGTTT	AAGAGGACTGACCGCGCGCC
2	ATTTCTTTGCTCCTCGTGCG	CTTCCCAGACATCCAGCAGT
3	CAGCACAGCACACCCATATC	GGGACTATCTGCTATCCCCG
4	AGCAGATAGTCCCCAGTGGT	GTGGGTTCAGGGATGGATGG
5	CATCAGGTGAGGCCTGGG	CCGAGCTCTGAGAACACCTG
6	ATCACCTGCCCTGATGCTC	GTGGAGCTCAGGACTGTGG
7	CACAGTCCTGAGCTCCACC	GTTGCTTCCCTGCTCTTTCC
8	CAGGGAAGCAACAACTGTCT	ATTCCTGTGGATTCTGCCCT

Table II. PCR amplification thermocycler conditions of Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein gene.

Touchdown PCR			Ordinary PCR		
Steps	Temperature, ˚C	Duration	Steps	Temperature, ˚C	Duration
1	94	5 min	1	94	5 min
2	94	30 sec	2	94	30 sec
3	63, EX[a] -0.5	30 sec	3	60/56/57[b]	30 sec
4	72	20 sec	4	72	20 sec
5	Back to step 2	16 times	5	Back to step 2	35 times
6	94	30 sec	6	72	7 min
7	54	30 sec	7	4	Until use
8	72	20 sec	-	-	-
9	Back to step 5	20 times	-	-	-
10	72	7 min	-	-	-
11	4	Until use	-	-	-

PCR, polymerase chain reaction; E, exon. E3 was amplified by Touchdown PCR. E1, E2, E4, E5, E6, E7 and E8 were amplified by ordinary PCR. [a]EX indicates that the annealing temperature decreased by 0.5˚C per cycle. [b]Annealing temperature of E1 was 60˚C, annealing temperature of E2, E4, E5, and E8 was 56˚C and annealing temperature of E6 and E7 was 57˚C.

Histologic scoring and analysis. Samples were evaluated using a standard light microscopic technique (magnification, x200) as performed by two pathologists (Shenzhen Hospital in Southern Medical University). Staining for the SHARPIN protein was evaluated in the tumors and in the normal skin tissues, which were invariably SHARPIN-positive and served as positive controls. Each tumor sample was scored by the cross-product (H score) of the percentage of tumor cell staining at each of the 3 staining intensities. Degrees of staining were divided into four levels: None, 0; weak, 1; moderate, 2; and strong, 3. For example, a particular tumor may have 30% cell staining at intensity=1 and 70% of cell staining at intensity=3, for a combined H score of 240 [(30x1)+(70x3)=240] out of a maximum of 300. This system was performed as described previously by Bollag *et al* (25). Concordance was observed between the scores given by the two pathologists (81% of the scores were in agreement within a 40-point range). Cases with discrepancies of <50 points were recorded and reconciled on a two-headed microscope. Final H scores for each case were averaged by each pathologist. The expression scale of SHARPIN was graded by H score as follows: Low, H score 1-100; moderate, H score 101-200; and high, H score 201-300.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation. Differences in SHARPIN expression levels between normal skin and SCC, BCC and KA samples were analyzed using one-way analysis of variance and Tamhane's T2 post hoc test. The Broder grading system of SCC is commonly utilized to assess prognosis. It divides SCC into four categories based on histological grade. Grade I is composed of well-differentiated tumors, in which 75-100% of squamous cells are differentiated. Grade II is composed of moderately differentiated tumors in which 50-75% of squamous cells are differentiated. Grade III is composed of

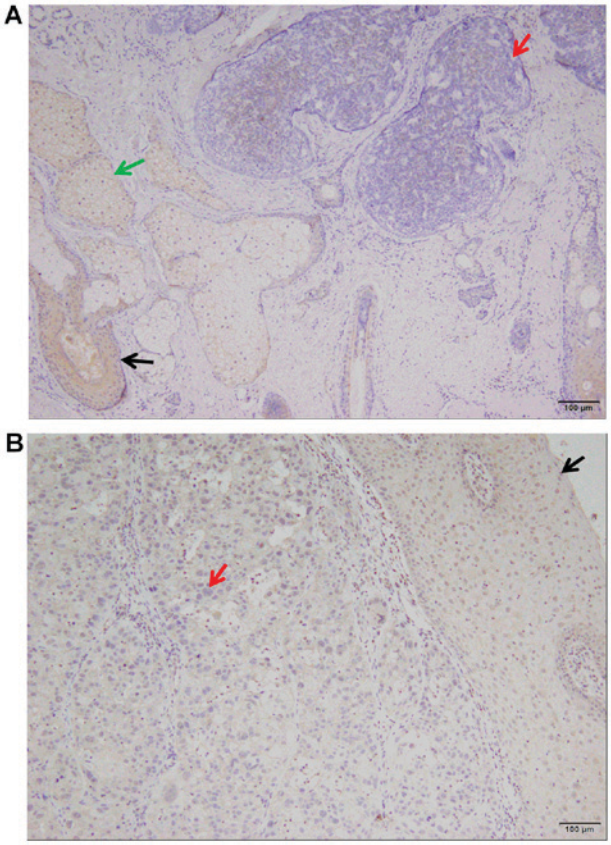

Figure 1. Expression of SHARPIN in nonmelanoma skin cancer. (A) The expression of SHARPIN in BCC at magnification, x100. The red arrow indicates BCC cells. The black arrow indicates sebaceous glands. (B) The expression of SHARPIN in SCC at magnification, x100. The red arrow indicates SCC cells. The black arrow indicates epithelia. SHARPIN, Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

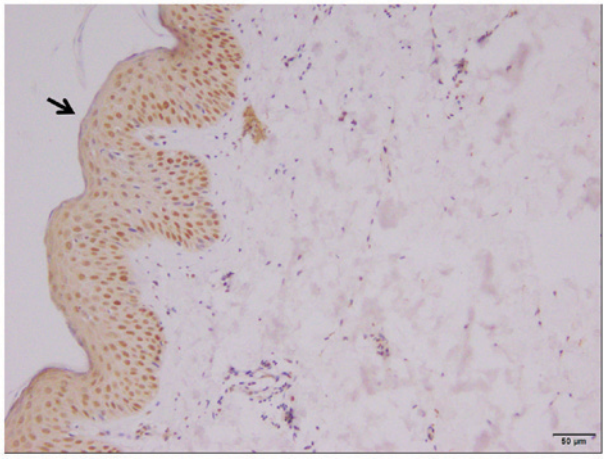

Figure 2. Expression of Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein in normal skin at magnification, x100. The black arrow indicates the epithelia in normal skin.

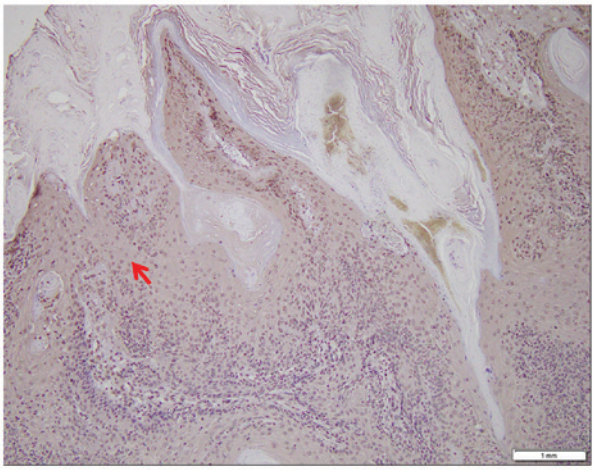

Figure 3. Expression of Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein in KA at magnification, x200. The red arrow indicates tumors cells in KA. KA, keratoacanthoma.

poorly differentiated tumors in which only 25-50% of cells are differentiated. Grade IV is an anaplastic tumor in which 0-25% of cells are differentiated (26). Main histologic variants of BCC include nodular type, adenoidal type, superficial type and sclerosing type (27). Associations between SHARPIN expression levels and aforementioned clinicopathological parameters were analyzed using the χ^2 test for categorical variables. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SHARPIN is aberrantly decreased in human NMSC. The SHARPIN protein was absent in the tumor nests and significantly decreased in precancerous lesions of SCC and BCC (Fig. 1) when compared to normal epithelium (Fig. 2). In addition, SHARPIN was moderately to highly expressed in KA samples (Fig. 3).

In BCC, SHARPIN expression was low in 63 cases (74.5%) and moderate in 22 cases (25.5%). In SCC, SHARPIN expression was low in 52 cases (68.1%) and moderate in 25 cases (31.9%). Furthermore, the difference in SHARPIN expression levels between BCC and normal skin, SCC and normal skin, and SCC and KA were all significant ($P<0.05$) (Fig. 4). However, no significant association was observed between SHARPIN expression and tumor grading of SCC. Demographics of all the patients and their H scores are summarized in Tables III-VI.

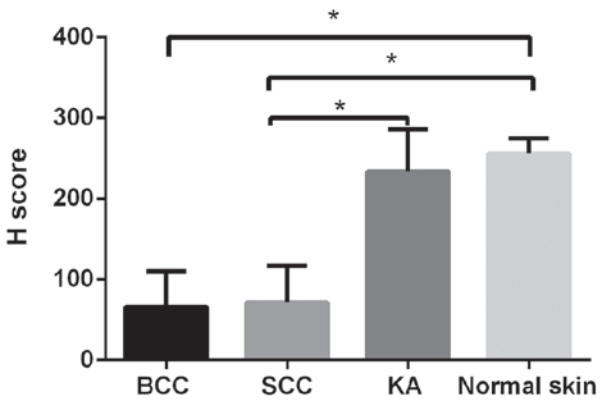

Figure 4. H scores of BCC, SCC, KA and normal skin. Values are mean ± standard deviation. *$P<0.05$ using one-way analysis of variance and Tamhane's T2 post hoc test. BCC, basal cell carcinoma; SCC, squamous cell carcinoma; KA, keratoacanthoma.

Table III. Demographics and H scores of patients with basal cell carcinoma.

ID	Sex	Age, y	Location	Type	H score
B01	M	74	Nose	Adenoidal type	10
B02	F	52	Right ear	Adenoidal type	10
B03	M	72	Trunk	Superficial type	10
B04	F	70	Right hand	Superficial type	10
B05	M	68	Right shoulder	Superficial type	10
B06	M	62	Upper lip	Nodular type	10
B07	M	63	Nose	Adenoidal type	10
B08	M	44	Lower lip	Sclerosing type	10
B09	F	82	Lower lip	Sclerosing type	10
B10	F	45	Head	Adenoidal type	10
B11	F	70	Nose	Adenoidal type	15
B12	F	63	Right hip	Adenoidal type	20
B13	F	49	Right thigh	Sclerosing type	20
B14	F	73	Left forearm	Superficial type	20
B15	M	69	Upper lip	Nodular type	20
B16	M	69	Nose	Adenoidal type	20
B17	M	65	Left hand	Sclerosing type	20
B18	M	67	Nose	Adenoidal type	20
B19	F	47	Nose	Adenoidal type	20
B20	M	65	Neck	Superficial type	20
B21	F	73	Head	Nodular type	25
B22	M	22	Nose	Nodular type	30
B23	M	58	Lower lip	Adenoidal type	30
B24	F	58	Nose	Superficial type	30
B25	F	57	Left cheek	Adenoidal type	35
B26	M	59	Upper lip	Adenoidal type	35
B27	F	68	Right tempus	Superficial type	40
B28	F	80	Upper lip	Pigmented type	40
B29	F	95	Back	Nodular type	40
B30	M	78	Back	Nodular type	40
B31	F	65	Left tempus	Nodular type	40
B32	F	76	Back	Pigmented type	40
B33	M	64	Left leg	Superficial type	40
B34	M	43	Head	Sclerosing type	45
B35	F	41	Left forehead	Pigmented type	50
B36	F	68	Chest	Pigmented type	50
B37	F	83	Right hand	Nodular type	50
B38	M	89	Nose	Superficial type	50
B39	F	75	Left hand	Pigmented type	50
B40	F	75	Lower lip	Superficial type	55
B41	M	63	Nose	Sclerosing type	55
B42	M	45	Upper lip	Nodular type	60
B43	F	64	Left thigh	Adenoidal type	60
B44	M	50	Back	Nodular type	60
B45	M	59	Lower lip	Nodular type	60
B46	M	46	Upper lip	Adenoidal type	70
B47	M	81	Head	Nodular type	70
B48	F	60	Lower lip	Nodular type	70
B49	M	58	Left thigh	Adenoidal type	70
B50	F	43	Anus	Nodular type	70
B51	F	78	Left thigh	Nodular type	75

Table III. Continued.

ID	Sex	Age, y	Location	Type	H score
B52	F	61	Nose	Nodular type	80
B53	M	56	Nose	Nodular type	80
B54	M	53	Left thigh	Nodular type	80
B55	F	45	Right cheek	Pigmented type	80
B56	F	29	Left check	Superficial type	80
B57	F	44	Left thigh	Adenoidal type	80
B58	F	58	Left thigh	Nodular type	80
B59	F	73	Back	Nodular type	85
B60	M	58	Nose	Superficial type	90
B61	F	73	Head	Nodular type	90
B62	M	73	Left cheek	Sclerosing type	90
B63	F	57	Nose	Nodular type	90
B64	F	34	Lower lip	Superficial type	100
B65	F	41	Nose	Nodular type	100
B66	F	34	Lower lip	Superficial type	100
B67	M	73	Lower lip	Nodular type	100
B68	M	82	Nose	Nodular type	100
B69	M	63	Back	Nodular type	100
B70	F	38	Upper lip	Superficial type	105
B71	F	53	Right tempus	Nodular type	105
B72	F	64	Back	Nodular type	110
B73	F	53	Lower lip	Nodular type	120
B74	F	63	Back	Nodular type	120
B75	M	35	Upper lip	Superficial type	120
B76	F	44	Nose	Superficial type	130
B77	F	60	Right tempus	Nodular type	135
B78	F	70	Nose	Superficial type	140
B79	M	37	Nose	Nodular type	140
B80	F	36	Nose	Nodular type	140
B81	F	64	Right tempus	Adenoidal type	150
B82	M	56	Lower lip	Sclerosing type	150
B83	M	73	Back	Superficial type	160
B84	F	47	Right hand	Nodular type	160
B85	M	58	Lower lip	Nodular type	190

F, female; M, male.

SHARPIN mutation analysis. A total of 8 exons and exon-intron adjacent sequences of *SHARPIN* were analyzed using DNA extracts from FFPE blocks of BCC, SCC and KA samples and healthy skin specimens, and DNA extracts from peripheral blood samples of 100 normal controls. Complete descriptions of the mutations detected in BCC and SCC are presented in Table VII. Total mutation rates were 21.8% in BCC and 17.0% in SCC samples. The C>T substitutions were 5.5% in BCC and 6.4% in SCC. Additionally, no mutations of *SHARPIN* were detected in DNA extracts from one benign skin tumor, 12 healthy skin tissues and blood samples from 100 normal individuals.

Discussion

The present study evaluated the expression of SHARPIN protein and analyzed the sequences of *SHARPIN* in NMSC. To the best of our knowledge, this is the first study to comprehensively investigate the expression and mutations of SHARPIN in a large series of patients with NMSC.

The essential contribution of SHARPIN to the activation of NF-κB supports the possibility that SHARPIN promotes tumorigenesis, as NF-κB signaling possesses well-demonstrated tumorigenic properties (12). This is supported by the SHARPIN-mediated suppression of apoptosis in the

Table IV. Demographics and H scores of patients with squamous cell carcinoma.

ID	Sex	Age, y	Location	Broder grading system	H score
S01	M	57	Left tempus	I	3
S02	M	56	Right tempus	I	5
S03	F	43	Right cheek	I	10
S04	M	28	Nose	II	10
S05	F	78	Perioral	II	10
S06	M	72	Left tempus	I	10
S07	M	79	Left thigh	II	20
S08	F	78	Upper lip	I	20
S09	M	88	Lower lip	I	20
S10	M	52	Nose	I	20
S11	F	59	Trunk	II	20
S12	M	68	Right eyebrow	III-IV	20
S13	M	48	Left eyelid	III	25
S14	M	72	Left forehead	II	25
S15	F	42	Right forearm	II	25
S16	M	65	Right cheek	I	30
S17	F	76	Anus	II	30
S18	F	30	Left cheek	II	30
S19	M	61	Perioral	II	35
S20	M	45	Left forearm	II	35
S21	M	63	Lower lip	IV	40
S22	M	71	Left cheek	IV	40
S23	F	73	Right tempus	I	40
S24	F	50	Lower lip	III	40
S25	F	68	Lower lip	I	40
S26	F	30	Right tempus	I	40
S27	M	41	Right thigh	I	40
S28	F	30	Left eyelid	I	45
S29	M	32	Left eyelid	I	50
S30	F	20	Nose	I	50
S31	F	81	Nose	I	50
S32	F	61	Left eyelid	I	50
S33	F	60	Left cheek	II	55
S34	F	29	Nose	I	55
S35	M	86	Right thigh	II-III	60
S36	M	82	Nose	III	60
S37	F	82	Right cheek	I	60
S38	F	75	Right cheek	I	60
S39	M	71	Left hand	II	60
S40	M	71	Right tempus	I	60
S41	F	40	Right hand	I	70
S42	M	53	Perioral	I	70
S43	F	80	Perioral	I	70
S44	M	71	Perioral	I	70
S45	M	60	Left eyelid	II	80
S46	F	43	Left cheek	I	80
S47	F	21	Left cheek	III	80
S48	M	79	Lower lip	I	80
S49	F	80	Neck	III	90
S50	M	90	Nose	II-III	90
S51	F	78	Left hand	IV	95

Table IV. Continued.

ID	Sex	Age, y	Location	Broder grading system	H score
S52	M	64	Right cheek	II	95
S53	M	57	Neck	I	100
S54	F	42	Left hand	I	100
S55	M	81	Right hand	I	100
S56	M	68	Left eyelid	I	100
S57	M	53	Right hand	III	100
S58	F	82	Left eyelid	III	100
S59	F	71	Upper lip	I	100
S60	M	32	Right cheek	I	110
S61	F	82	Right cheek	I	110
S62	F	75	Right hand	II	110
S63	F	74	Upper lip	I	110
S64	M	62	Left eyelid	II	110
S65	M	47	Nose	II	110
S66	M	26	Right hand	III	120
S67	F	57	Right hand	I	120
S68	F	66	Left tempus	I-II	130
S69	M	57	Left thigh	I	135
S70	F	81	Left cheek	I	135
S71	M	64	Trunk	II	150
S72	M	65	Trunk	I	150
S73	M	64	Left tempus	I	160
S74	M	60	Left eyelid	II	160
S75	M	74	Upper lip	II	160
S76	F	62	Upper lip	I	180
S77	F	66	Right cheek	III	190

F, female; M, male.

keratinocytes and hepatocytes of *cpdm* mice (18). Additionally, SHARPIN promotes the migration of Chinese hamster ovary cells *in vitro* and lymphocytes *in vivo*, and increases the lung metastasis of osteosarcoma *in vivo* in immunocompromised mice (19). In addition, the upregulation of *SHARPIN* has been observed in different types of internal solid cancer, including ovarian cancer, renal cell carcinoma, and cervical and prostate cancer (20,21). Furthermore, SHARPIN induces PTEN polyubiquitination independently of the K48 linkage. This process requires the UBL domain, which mediates SHARPIN's association with PTEN and its ability to bind ubiquitin via the NZF motif (28). Rantala *et al* (16) demonstrated that SHARPIN inactivates integrins in a number of different cell types and affects integrin-dependent cellular functions. Bii *et al* (22) identified SHARPIN as a metastasis gene in breast cancer using a replication-incompetent gammaretroviral vector, suggesting the potential of SHARPIN as a biomarker for stratifying patients with breast cancer. Additionally, Haris *et al* (23) identified that SHARPIN was significantly upregulated in U87 glioblastoma cells upon treatment with Aloe-emodin. Collectively, substantial evidence has demonstrated the role of SHARPIN in promoting tumorigenesis. Despite these data, a PubMed search did not identify any studies examining the expression of SHARPIN in NMSC. Therefore, the present study explored the expression of *SHARPIN* in three types of skin tumors, including the malignant forms BCC and SCC.

Firstly, the expression of SHARPIN was detected via immunohistochemistry. Contrary to the results of examination of internal solid tumors (17), SHARPIN expression was downregulated or absent in the majority of NMSC samples compared with normal skin tissues and KA. KA is commonly diagnosed clinically as it rapidly appears and develops as a raised lesion; however, as a non-pigmented lesion with a central keratin plug, SCC may also exhibit the same appearance. Furthermore, cases of KA with SCC arising from the base have been identified (29). Differential diagnosis between KA and SCC is challenging due to their similarities and the lack of reliable diagnostic criteria to distinguish them. Therefore, whether KA is a separate benign entity, or a variant of SCC, is controversial. At present, no biomarkers exist to distinguish SCC from KA, and KA lesions are commonly

Table V. Demographics and H scores of patients with keratoacanthoma.

ID	Sex	Age, y	Location	H score
K01	M	68	Nose	110
K02	M	55	Left cheek	120
K03	F	69	Trunk	160
K04	F	68	Right arm	175
K05	M	62	Upper lip	180
K06	F	69	Nose	240
K07	F	85	Trunk	240
K08	M	18	Forehead	245
K09	M	48	Upper lip	245
K10	F	69	Nose	250
K11	M	69	Right cheek	250
K12	M	40	Right arm	250
K13	F	55	Nose	260
K14	M	55	Nose	260
K15	M	50	Left arm	265
K16	M	50	Upper lip	270
K17	F	72	Forehead	275
K18	F	70	Left arm	275
K19	M	63	Trunk	280
K20	M	50	Forehead	280
K21	M	53	Right tempus	280

F, female; M, male.

Table VI. Demographics and H scores of negative control patients.

ID	Sex	Age, y	H score
N01	M	30	220
N02	F	22	260
N03	F	57	260
N04	F	48	275
N05	M	32	280
N06	F	50	240
N07	M	40	280
N08	M	18	245
N09	M	39	245
N10	F	28	250
N11	F	69	270
N12	M	55	250

F, female; M, male.

treated in the same way as SCC. However, SCC has a poorer prognosis than KA and is treated more aggressively; therefore, distinguishing between these two malignancies would be advantageous in order to implement the appropriate treatment, thereby decreasing unnecessary surgeries, the burden on the healthcare system and, importantly, the anxiety of the patients (30). Based on the results of the present study, that SHARPIN is absent or exhibits low expression in SCC but a high expression in KA, we hypothesize that SHARPIN may allow early differentiation and *in situ* treatment of SCC and KA to avoid metastasis and tissue destruction of SCC and the overtreatment of KA.

At present, the mechanism of how the downregulation of SHARPIN promotes skin tumorigenesis remains to be elucidated. Ikeda *et al* (11) identified that the absence of *SHARPIN* in *cpdm* mice caused dysregulation of NF-κB and increased apoptosis and necrosis of mouse embryonic fibroblasts. The data from the present study suggested that the downregulation of SHARPIN in NMSC may impair the function of LUBAC, and subsequently, the activation of NF-κB. In the majority of tumors, the aberrant activation of NF-κB signaling stimulates tumor cell proliferation, invasion and metastasis (31). Counterintuitively, van Hogerlinden *et al* (32) demonstrated that selective inhibition of Rel/NF-κB signaling in the skin leads to disturbed epidermal homeostasis and hair follicle development, increased frequency of apoptotic keratinocytes and spontaneous development of SCC. Notably, a number of data have challenged the view that apoptotic signaling solely serves to inhibit cancer, arguing instead that apoptosis is responsible for various effects that may be tumor-promoting (33-36). Apoptotic cell death is a cell-autonomous event, but its effects are not; dying cells affect their surrounding environments in various ways, which may include stimulating the proliferation of neighboring cells, affecting intra-tumoral cell competition and exerting paracrine effects on tumor microenvironments. Various data support the hypothesis that apoptosis may promote tumorigenesis through the recruitment and activation of phagocytic macrophages at the tumor sites (37). Taken together, we hypothesized that decreased SHARPIN expression may promote NMSC through the impaired activation of NF-κB and increased apoptosis and necrosis of epidermal cells, which may disrupt the homeostasis of the epidermis and lead to tumorigenesis.

Traditional Sanger sequencing has been the gold standard for identifying mutations for a number of years due to its low false-positive rate and high specificity (21). Therefore, in the present study, DNA was extracted from NMSC FFPE blocks and mutations in the exons of *SHARPIN* were detected. The results indicated that high proportions of BCC and SCC contained mutations of the *SHARPIN* gene. Mutations in *SHARPIN* exons were identified in 21.8% of BCC and 17.0% of SCC in the present study. The proportions of C>T substitutions were 5.5% in BCC and 6.4% in SCC samples, which were identified as characteristic of mutations associated with exposure to UV exposure (38). In addition, the mutations were not only located in the UBL domain of SHARPIN but also in the PH and NZF domains, thereby potentially affecting other functions of SHARPIN besides the formation of LUBAC. Furthermore, SHARPIN has been indicated to inactivate integrins in a number of cell types and affect integrin-dependent cellular functions independent of LUBAC (16). Approximately one-half of the cellular SHARPIN is not associated with the

Table VII. Distribution of Src homology 3 and multiple ankyrin repeat domains protein-associated RH domain-interacting protein gene mutations in patients with BCC and SCC.

Tumor type	Exon	Mutation	Modified protein	Frequency	Domain
BCC	E1	c.10 C>T	p.Pro4Leu	1/55	-
	-	c.68 C>T	p.Ala23Val	1/55	-
	-	c.146 A>G	p.Asp49Gly	1/55	-
	E2	c.329 T>C	p.Gln110Arg	1/55	PH
	E5	c.733 C>A	p.His245Thr	1/55	UBL
	E7	c.937 C>T	p.Pro313Ser	1/55	-
	-	c.944 A>G	p.His315Arg	1/55	-
	-	c.992 T>C	p.Leu332Ser	3/55	-
	E8	c.1109 T>C	p.Met370Thr	1/55	NZF
	-	c.1137 G>A	p.Trp379Gln	1/55	-
SCC	E1	c.53 C>A	p.Ala18Asp	1/47	-
	E2	c.214 T>C	p.Trp72Arg	1/47	PH
	E3	c.421 C>T	p.Pro141Ser	1/47	-
	-	c.466 C>T	p.Pro156Ser	1/47	-
	-	c.469 C>T	p.Pro157Ser	1/47	-
	-	c.478 G>A	p.Ala160Thr	1/47	-
	E5	c.709 T>C	p.Ser237Pro	1/47	-
	E8	c.1007 G>T	p.Gly336Val	1/47	-

BCC, basal cell carcinoma; SCC, squamous cell carcinoma; PH, pleckstrin homology domain; UBL, ubiquitin-like; NZF, Npl4 zinc finger.

LUBAC complex (28). Therefore, the present study concluded that SHARPIN is a multifunctional molecule and may promote the pathogenesis of NMSC through different mechanisms.

Overall, the present study contributes to a growing body of evidence supporting the importance of *SHARPIN* in NMSC. The results suggest an association between NMSC and low to absent SHARPIN expression and SHARPIN mutations.

It was identified that SHARPIN protein expression was absent in cancer nests and significantly decreased in precancerous lesions of SCC and BCC, but was high in normal skin or in KA. The total mutation rates of *SHARPIN* were 21.8% in BCC and 17.0% in SCC. These data indicated that SHARPIN may serve a tumor-suppressing role and act as a promising diagnostic biomarker in NMSC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding authors on reasonable request.

Authors' contributions

YL designed and supervised the study. YZ performed the histological examination of the samples and prepared the draft. YY performed the DNA extraction, polymerase chain reaction and sequencing. JW conducted data analysis and interpreted the data.

Ethics approval and consent to participate

Blood specimens of 100 normal individuals and skin tissues from 12 healthy volunteers who received cosmetic surgeries, and formalin-fixed paraffin-embedded (FFPE) samples from the Department of Dermatology of Shenzhen Hospital in Southern Medical University (Shenzhen, China), were obtained according to a protocol approved by the Southern Medical University Shenzhen Hospital Subject Review Board (2016-016). All samples were fixed for 24 h in 10% formalin solution at room temperature. The thickness of the sections was 4 μm. Informed consent was obtained from all participants.

Patient consent for publication

Consent was gained from the participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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