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<sup>pcdm</sup>) 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).
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 (IκKα and IκKβ) and one regulatory subunit [NF-κB essential modulator (NEMO)], is activated, allowing the phosphorylation of the IκBκκ 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 IκBκκ, 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.
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 \[(30 \times 1) + (70 \times 3) = 240\] out of a maximum of 300. This system was performed as described previously by Bollag \cite{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...
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 $\chi^2$ 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.
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      |
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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 forear | 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 forear  | 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      |
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

| 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.
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 pdpm 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
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.

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.As39Gly        | 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.
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