

Increased CD56 expression after photodynamic therapy indicates an increased natural killer cell count following early photodynamic therapy for cutaneous squamous cell carcinoma

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Abstract. Cutaneous squamous cell carcinoma (cSCC) is the second most common type of skin cancer. Photodynamic therapy (PDT) is a promising therapeutic method for managing cSCC due to its proven ability to target specific areas over time and its low risk of side effects. PDT may cause tissue damage and vascular shutdown, and may regulate local immunological responses. The present study aimed to investigate and compare the early lymphocyte modifications before and after PDT for SCC. A total of 10 patients with SCC were identified by pathological investigation. Initially, all wounds were treated with 20% aminolevulinic acid (ALA)-PDT as the initial stage in the therapeutic procedure. The wounds were treated by exposing them to red LED light with a wavelength of 635 nm, an energy density of 100 J/cm² and an intensity of 80 mW/cm². The tumor tissue was surgically removed 24 h later, and another round of PDT therapy was administered. Immunohistochemistry for CD3 and CD56 was conducted on the wound tissue post-surgery. If the wound showed granulation, necrosis or secretion, debridement was added to the therapy. All patients were monitored for 0.6-1.0 year post-treatment. ALA-PDT combination surgery fully controlled the tumor tissue in all 10 patients. The immunohistochemical analysis of the wound tissues showed that the expression of CD56 increased, while the expression of CD3 was not different after photodynamic therapy. These results also indirectly indicated that the overall count of NK cells in the 10 patients increased, nevertheless, there was no alteration in the T lymphocyte count. In

conclusion, the ALA-PDT combination surgical therapy for cSCC demonstrates favorable results. An increase in CD56 expression may be a mechanism for the effective treatment of cSCC with PDT.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common type of non-melanoma skin cancer, after basal cell carcinoma. cSCC constitutes ~20% of all skin malignancies and is responsible for >75% of deaths related to non-melanoma skin cancer (1). The prevalence of this illness is increasing rapidly, mostly due to the aging population and the focus on screening for skin cancer. cSCC develops from the aberrant proliferation of keratinocytes in the epidermis, perhaps as a result of an extended period of intraepidermal dysplasia (2). Tumor development is a known progressive process that involves numerous histological and pathologically defined stages, starting with actinic keratosis (AK) and progressing to invasive cSCC (3,4). cSCC has a tendency to cause local cutaneous damage affecting the soft tissues, cartilage and bone; however, metastasis is uncommon (5). Generally, the prognosis for cSCC is positive, with a 5-year survival rate of ≥90%. The etiopathogenesis of the condition is mainly impacted by risk factors such as exposure to UV radiation, chronic photoaging, increasing age, the male sex, immunosuppression, smoking and certain genetic variables (6,7).

Surgery remains the main treatment option for cSCC (8). Lesions on the eyelid, lip or ear need particular attention for tissue preservation. Surgery may not be an appropriate choice as it may lead to disappointing cosmetic outcomes. Hence, it is essential to explore non-surgical approaches. The use of cryotherapy, imiquimod and 5-fluorouracil has been associated with negative outcomes and a high chance of the condition coming back (9). By contrast, photodynamic therapy (PDT) has significant effectiveness, producing pleasing cosmetic outcomes and showing a low rate of recurrence (10).

PDT is a treatment method for skin cancer that relies on the combined effect of a photosensitizer, light and oxygen (11). Tumor cells and vascular endothelial cells inside the body have a greater attraction to photosensitizers than other cell

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types. Specifically, tumor cell surface proteins, or receptors, may interact better with certain substances and they may absorb photosensitizers better (12). Vascular endothelial cells line bodily blood vessels, and they are necessary for blood flow, immunological reactions and other functions (13). Cancer and inflammation increase tumor surface molecule expression and blood arterial permeability. These changes may increase vascular endothelial cell photosensitizer attraction. This means tumor and vascular endothelial cells are more likely to absorb photosensitizers, making them more photodynamically vulnerable. Photosensitizers may capture light energy and transmit it to nearby triplet oxygen molecules. This mechanism results in the generation of reactive oxygen species (ROS). ROS may harm tumor cells and vascular endothelial cells by directly triggering the necrosis and apoptosis pathways (14). An antitumor immune response is initiated due to the harmful consequences caused by ROS. This immune response aims to efficiently eliminate tumor cells and hinder the development of tumor blood vessels, hence starving the tumor of necessary nutrition. Recently, there has been a greater emphasis on studying the immunological response in PDT for cancer treatment, exceeding the attention previously devoted to the vascular effects (14,15). Cell death induced by PDT has been well researched in laboratory settings and living organisms (16,17). However, there is a lack of information about the immunological changes after PDT for cSCC, and the particular differences in the inflammatory infiltrates after PDT are yet unknown (18). The major aim of the present study was to investigate and compare the immune cell composition before and after PDT therapy for cSCC to assess the changes in immune cells caused by the treatment.

Patients and methods

Patients enrolled. A total of 10 patients aged between 65 and 70 years who were diagnosed with cSCC, as determined by histological examination at Daping Hospital of Army Medical University (Chongqing, China), were randomly selected for the investigation (Table I). Patients were recruited between November 2023 and March 2024. The 10 included individuals remained in the study for >4 months. The inclusion criterion was a histological diagnosis of cSCC and the exclusion of any other tumor tissue. The exclusion criteria were the presence of non-cSCC, anogenital SCC, Marjolin ulcers and genetic abnormalities that predisposed patients to cSCC. All patients were provided with comprehensive information on aminolevulinic acid (ALA)-PDT, including its indications, treatment principles, therapeutic effects and potential problems. Patients provided written informed consent to engage in this research and for the publication of their relevant information.

Histopathological biopsy and immunohistochemistry. Patients meeting the inclusion criteria and suspected of malignancy were managed by the Department of Plastic and Cosmetic Surgery, which conducted necessary surgical interventions. Subsequently, biopsy or resected specimens were analyzed by the Department of Pathology. The surgical procedure was carried out following recognized guidelines, and histology slides were generated.

The surgical specimens were processed for hematoxylin and eosin staining. Briefly, the obtained tissue was fixed overnight in a 4% paraformaldehyde solution at room temperature. Fixed tissue samples were dehydrated using a series of graded alcohol solutions (70, 95 and 100% ethanol) to remove water from the tissues. Dehydrated tissues were cleared using xylene to remove the alcohol and make the tissues transparent, and then the tissue was placed in paraffin after embedding. Thin sections (4- to 5- μ m thick) were cut from the paraffin block using a sharp blade and transferred onto glass slides. Rehydrated tissue sections were stained with hematoxylin for 5 min and then counterstained with eosin for 3 min at room temperature. The approach is concisely summarized as follows: The investigation included analyzing the tissue specimen at a magnification of x40 using a fluorescence microscope, and then doing a more detailed review at a magnification of x100 using a fluorescence microscope. Two pathologists, both skilled and working independently, examined the tissue specimens on the slides.

For immunohistochemistry, first, the tissue slices prepared as aforementioned were blocked with a 10% bovine serum albumin-purified solution (cat. no. 37520; Thermo Fisher Scientific, Inc.) at room temperature for ~1 h. Next, incubation was conducted using the following primary antibodies: Rabbit anti-CD3 (cat. no. 78588; 1:200; Cell Signaling Technology, Inc.) and rabbit anti-CD56 (cat. no. 99746; 1:200; Cell Signaling Technology, Inc.). Tissue slices were deparaffinized and then incubated with antibodies overnight at 4°C. The samples were washed with PBS and then incubated with donkey anti-rabbit IgG antibodies (cat. no. A-21206; 2 μ g/ml; Thermo Fisher Scientific, Inc.) at room temperature for 1 h.

ALA-PDT procedure. Once the patient consent for treatment was obtained, the tumor tissue underwent one session of PDT. The tumor tissue was surgically removed 24 h after confirming the patient had no apparent adverse effects, followed by immediate PDT therapy on the afflicted region. Specifically, the lesions that were positioned in close proximity to the region of interest, extending 0.5 cm beyond the apparent lesions, were exposed to cleaning using benzalkonium bromide. A solution containing 20% ALA (0.5 ml: 118 mg; Shanghai Fudan Zhangjiang Biomedical, Co., Ltd.) in saline was applied to the wound. The specified area was covered with a plastic film and protected from any light exposure for 4 h. After removing the plastic layer, a diode laser type XD-635AB (Xingda Photoelectricity Medical Equipment Corp.) generated laser beams with a wavelength of 635 nm. The laser beams were aimed toward the therapeutic region, ensuring a constant energy density of 120 J/cm². The exposure period for each spot size of 3 cm² was set at 15 min. For big lesions, numerous spots were employed to light the afflicted region. The power output was set at 100 mW/cm². The light exposure was adjusted to provide a constant vertical distance of 5 cm from the lesions. The PDT treatment protocol remained consistent both pre- and post-operatively.

Visual analogue scale. The Visual Analog Scale (VAS) (19) was used to assess patient discomfort throughout the PDT session. VAS is a technique used to measure pain severity. The process uses distance in centimeter on a 10-centimeter line to

Table I. Clinical data of 10 patients with cutaneous squamous cell carcinoma.

Characteristic	Patient no.									
	1	2	3	4	5	6	7	8	9	10
Age, years	89	74	69	80	66	70	70	70	70	70
Sex	M	F	F	M	F	F	M	F	M	F
Tumor location	Head	Face	Vulva	Face	Scrotum	Face	Face	Face	Head	Vulva
Size, cm	0.5x0.5	3.5x5	5x5	2.5x3	6x5	0.5x0.5	0.5x0.5	0.5x0.5	0.5x0.5	0.5x0.5
Number of follow-up visits	1	2	2	3	1	2	1	2	1	1
Length of follow-up time, years	1.2	0.8	1.5	1.1	1.2	0.7	1.1	1.2	0.9	1.0
VAS score of patients ^a										
1st PDT session	4	4	3	4	5	3	4	3	3	5
2nd PDT session	4	5	4	3	3	4	4	3	5	3

^aVAS score: 0-1, no pain; 2-3, slight pain; 4-6, moderate pain; 7-10, unbearable pain. M, male; F, female; VAS, Visual Analog Scale; PDT, photodynamic therapy.

represent pain, where each centimeter corresponds to one unit, between the 'no pain' reference point and the mark reported by the patient. The approach produces ratings that range from 0 to 10.

Results

All 10 patients with cSCC were identified by histological investigation, with additional MRI or X-ray scans conducted as needed. Tumors in all patients had diameters ranging from 4-6 cm. No major wound infection was apparent in the patients, and no drug-resistant bacteria such as *Pseudomonas*, *Citrobacter freundii*, *Staphylococcus aureus* or *Serratia marcescens* were found in the secretions.

All 10 patients received surgical excision of the tumor tissues, and adjuvant PDT was provided before and after surgery. All surgical wounds healed well without any cases of tumor recurrence or wound infections. Fig. 1 shows two representative cases of cSCC, one on the head and the other on the face. The pathological results showed that there were no cells with heterotrophic hyperplasia after use of PDT on the tumors (Fig. 2). Therefore, the cases that underwent the described technique showed positive treatment outcomes with significant effects. It is well known that CD56 serves as a distinctive cell surface marker of natural killer (NK) cells (20), while CD3 is recognized as a typical cell-surface marker of T lymphocytes (21). The immunohistochemistry results from the tissues in the present study indicated that the expression of CD56 increased and that the expression of CD3 was not different after PDT. The results also indirectly indicated that the overall count of NK cells in the 10 patients increased, while there was no difference in T lymphocyte count (Fig. 3).

Following ALA-PDT, some patients may have temporary local side effects, such as skin burning and heightened wound exudation, often linked to the execution of the procedure. Table I displays the VAS scores of all patients after each ALA-PDT therapy. A flurbiprofen axetil (5 ml:50 mg) injection was administered if the VAS score exceeded 3.

Discussion

cSCC includes AK and keratoacanthomas (KA), and is a type of non-melanoma skin cancer that develops from keratinocytes in the epidermis. AK is often considered a precursor to cSCC. KA may be classified as a variant of cSCC or a benign tumor with histological resemblances to well-differentiated cSCC. KA typically regresses spontaneously in most cases, unlike cSCC. Dermatologists generally consider that KA does not have the potential to metastasize (22-25). The variables that are causing the differences in clinical behavior are not yet understood. One such discrepancy might be the participation of T cells. In the present study, a comparative investigation was performed on the clinical data from 10 cSCC lesions.

In the present study, the patients with cSCC showed no difference in T cell number after PDT within 24 h, which seems to be a contradicting result. Studies have shown that PDT may trigger immune responses that contribute to its therapeutic effectiveness (26,27). PDT is linked to several important immunological effects. PDT often triggers an inflammatory response within the specific area it targets (28). Inflammation may help attract immune cells like macrophages and T lymphocytes to a particular site, aiding in the removal of abnormal cells and leftover photosensitizers (29). PDT may directly activate immune cells, increasing their ability to respond aggressively. This intervention helps strengthen the ability of the immune system to recognize and eliminate abnormal cells, such as those linked to cancer. PDT may induce tumor cell death, resulting in the release of tumor-specific antigens. The immune system may recognize antigens as foreign substances, triggering both humoral (including antibodies) and cellular (using T-cells) immune responses (30). PDT may also enhance immunological memory. Successful PDT may help improve the capacity of the immune system to recognize and respond to abnormal cell growth in the future. Based on the parameters discussed, we consider that the decrease in the total number of T lymphocytes in cSCC after early PDT may be due to a number of reasons, including the precise location of tumor growth, the degree of tumor penetration, the different

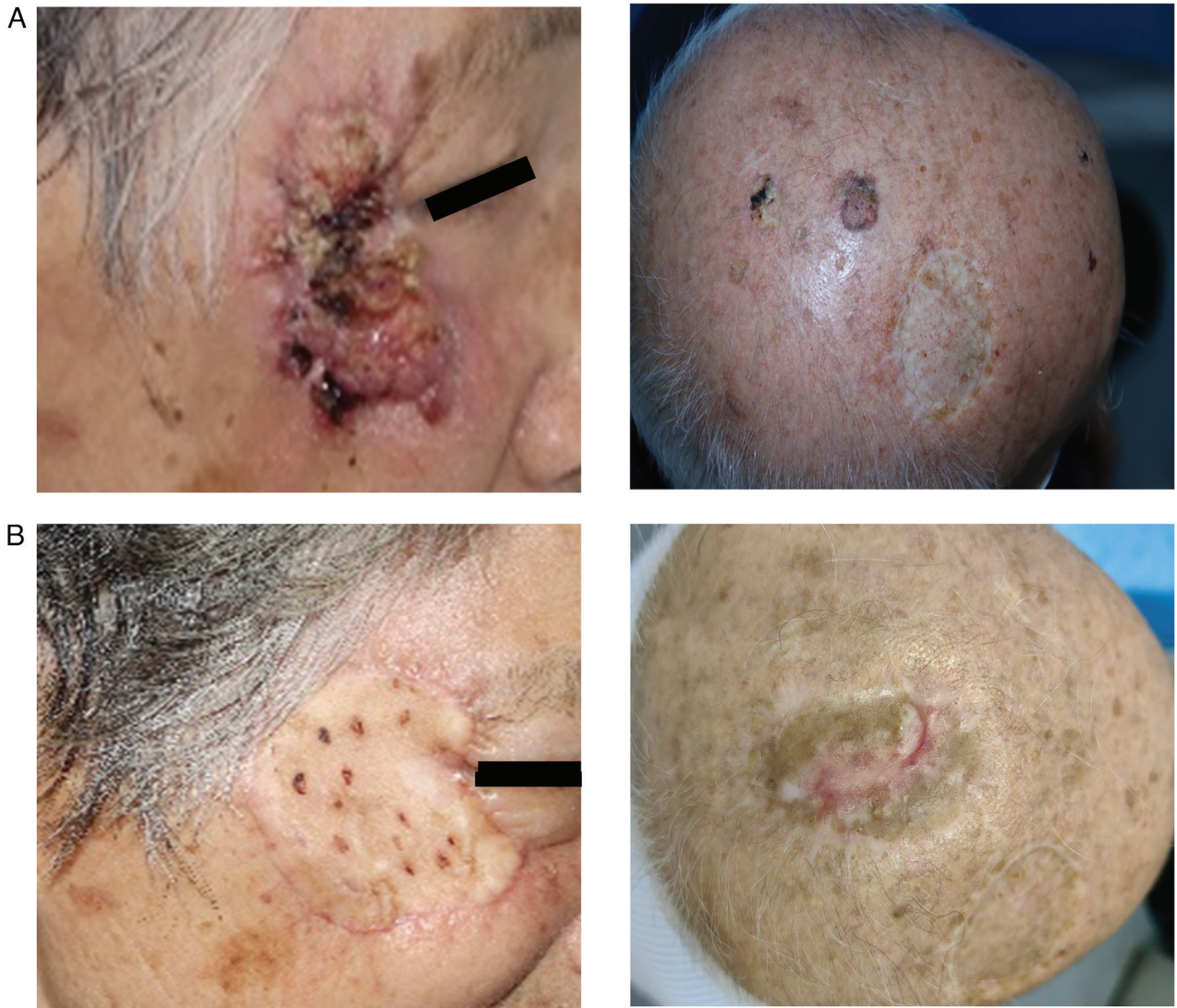


Figure 1. Representative images showing the effects of the combination of aminolevulinic acid-PDT and surgical excision on cutaneous squamous cell carcinoma. (A) Patients before PDT. (B) Patients at the 1-year follow-up after treatment showing no local recurrence. PDT, photodynamic therapy.

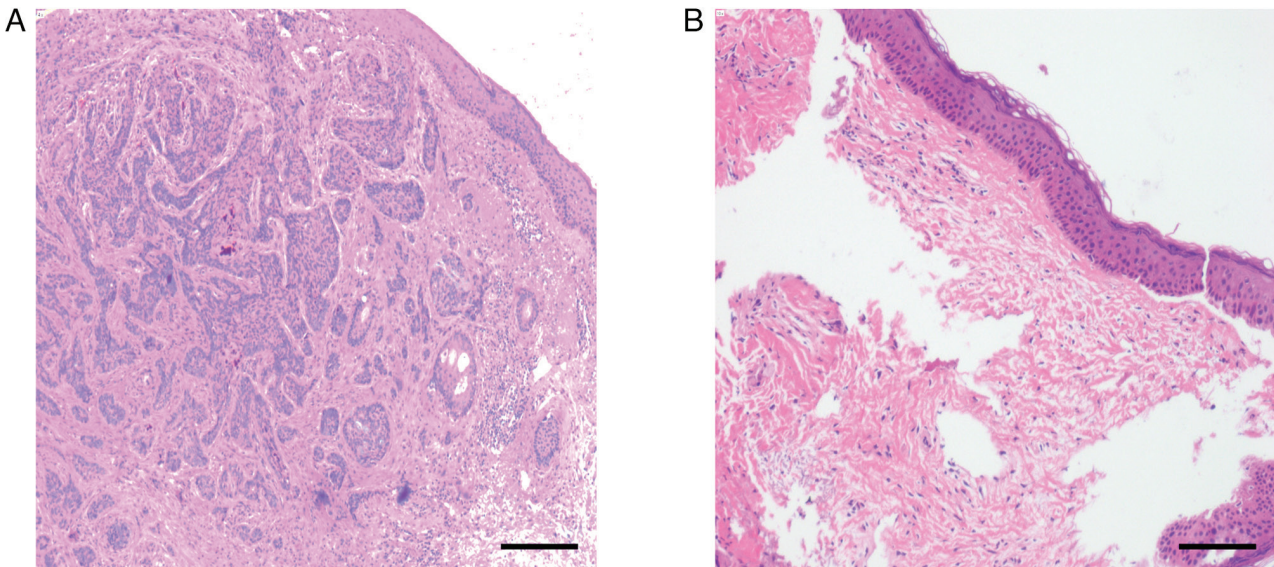


Figure 2. Histological analysis by hematoxylin and eosin staining (x100 magnification). (A) Before PDT, a large number of tumor cells with heterotrophic hyperplasia were apparent in the dermis (scale bar, 100 μ m). (B) After PDT sessions, there were no heteromorphous cells (scale bar, 100 μ m). PDT, photodynamic therapy.

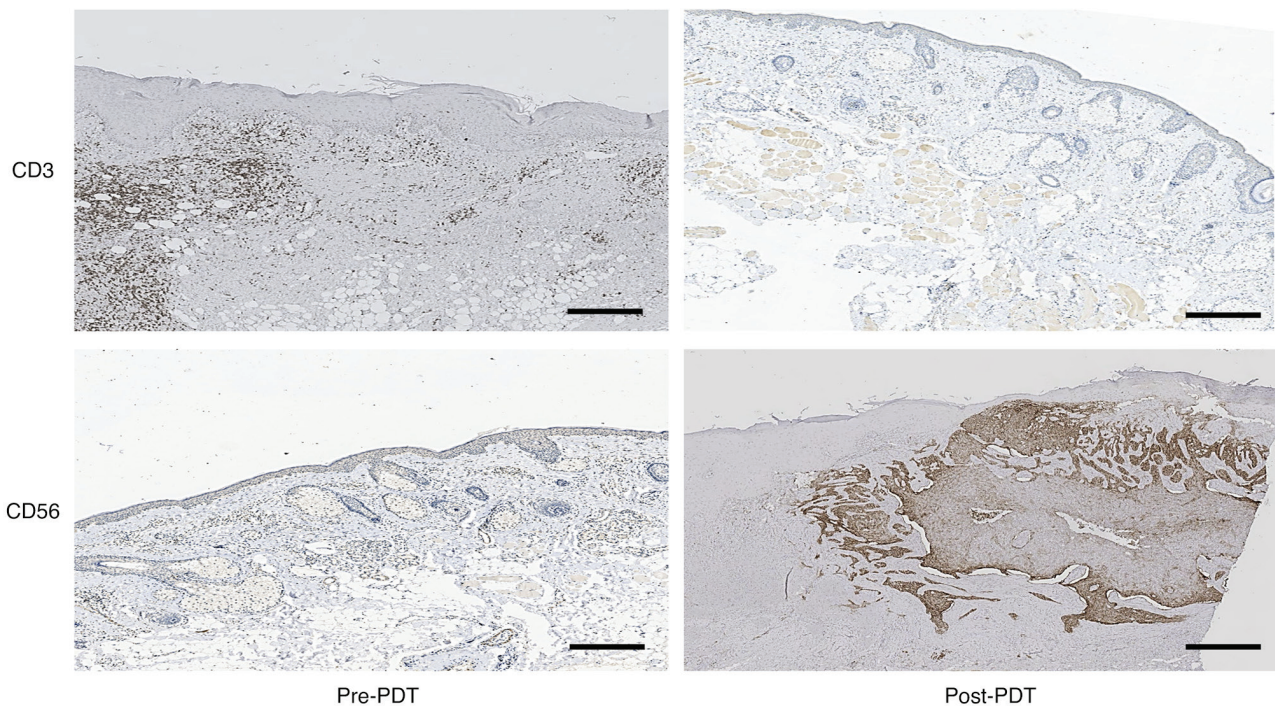


Figure 3. Immunohistochemical staining. Pre-PDT and post-PDT immunohistochemical staining results revealed similar levels of CD3⁺ and increased levels of CD56⁺ cells in the wound tissues (scale bar, 500 μ m). PDT, photodynamic therapy.

reactions of people to photosensitizing drugs and the possible existence of immunosuppressive diseases in patients. In a previous study, although there was a decrease in the total T lymphocyte count, the ratio of CD4⁺/CD8⁺ T lymphocytes varied across the patient group, with some showing an increase and others a decrease (31).

The possibility of utilizing PDT just for treating cSCC should be considered. PDT appears to be a feasible alternative to surgery. Both PDT and PDT with surgery are effective modalities for managing cSCC. The choice between the two treatments should be based on individual patient-specific characteristics. PDT is a non-invasive treatment that uses a photosensitizer and precise light wavelengths to target and destroy tumor tissue by producing active chemicals such as oxygen-free radicals, which eliminate cancer cells (32,33). PDT offers benefits such as minimum trauma, faster recuperation and fewer side effects, while its effectiveness may not match that of surgery. PDT is used initially to target and kill some cancer cells, followed by surgical excision of the tumor. This combined method offers powerful therapeutic effectiveness but comes with the drawbacks of increased trauma, delayed recovery and a higher likelihood of adverse effects (34,35). The present study aimed to investigate the impact of PDT on NK cells in patients with cSCC, since, to the best of our knowledge, little information is available on this topic. Additionally, it was observed that the white blood cell count of each patient had increased following PDT. Possibly due to the inflammation from wound healing, infection and the patients' underlying conditions (data not shown). In the future, we aim to expand our patient recruitment efforts for this research study. The results necessitate a larger dataset for validation. Over 4 months, only 10 patients were enrolled in the present study due to several factors: Primarily, the disruptive impact

of the COVID-19 pandemic; secondly, a dearth of eligible candidates within the local vicinity; and lastly, an increasing trend of patient preference for alternative treatment facilities. Therefore, the next step will be to design an animal experiment to further explore the specific mechanism of PDT for skin SCC, especially the change in characteristics of lymphocytes in the peripheral blood.

The present results showed an increase in the total number of NK cells after early PDT for cSCC. NK cells are a type of innate cytotoxic lymphoid cell that plays a crucial role in tumor surveillance (36). Activated NK cells destroy tumor cells by releasing cytotoxic cytokines and producing granules containing perforin and granzyme B. Perforin and granzyme B are recognized for their ability to compromise the structural integrity of tumor cell membranes and trigger the onset of apoptosis. Unlike T cells (37), NK cells do not need prior antigen sensitization to start cytolytic activity (38). NK cells possess cytotoxic capabilities, rendering them a valuable asset for immunotherapy (39). The partnership of NK cells and Langerhans cells has been observed to successfully prevent the growth of 1,12-dimethylbenz(a)anthracene-induced cSCC tumors in mice (40). NK cells significantly impede tumor growth, especially in the initial phases of cSCC (41). As the disease progresses, there is a possibility of functional impairment of the NK cells. The reduced activity of NK cells in patients with advanced cSCC may be caused by the tumor microenvironment (42). Prolonged exposure of NK cells to the cSCC tumor environment leads to reduced NK cell activity (43). NK cells have shown great effectiveness in both laboratory studies and medical experiments, establishing them as a valuable treatment strategy for preventing tumor growth (44).

A previous study has shown the significant potential of PDT in treating cSCC; PDT selectively kills cancer cells using

a photosensitizing chemical and light, and when combined with surgical intervention, it is the most ideal treatment for cSCC. Cancer cells preferentially absorb the photosensitizing drug, which is triggered by certain wavelengths of light. This specific activation destroys malignant cells without harming healthy tissue. PDT offers fewer side effects and scars than radiation therapy (45). Further research is needed to confirm this assertion; however, it is important to highlight the fact that NK cells have shown potential in both preclinical studies and clinical trials, establishing them as a crucial strategy for inhibiting cSCC. Specifically, these results showed that NK cells may directly kill cSCC cells and suppress tumor development, supporting therapeutic trials (46). Meanwhile, clinical studies using NK cell-based treatments for cSCC have shown tumor shrinkage, disease stability and better patient outcomes (47), so the innate cytotoxicity and tumor-targeting ability of NK cells may be a selective and effective therapy.

In conclusion, the present study demonstrated that the combination of ALA-PDT and surgery is efficacious in the treatment of cSCC. The upregulation of NK cells, as evidenced by increased expression of CD56, may represent one of the mechanisms underlying the effectiveness of PDT in treating cSCC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HZ and JZ conceived and designed the experiments. XZ and YR contributed new reagents and conducted the experiments. HZ and HK wrote the manuscript. HZ, JZ, XZ, YR and HK analyzed and discussed the results, and reviewed the manuscript. HZ and XZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Human skin tissue samples were collected from consenting patients at Daping Hospital, Army Medical University (Chongqing, China). The research was conducted in accordance with the Helsinki Declaration and the Guidelines for the Care and Use of Laboratory Animals of the Chinese Institute of Health. The study was authorized by the Research Committee and Ethics Committee of the General Hospital (Daping Hospital) of the Army Medical University (approval no. DP2019-46). All patients who took part in this study provided written informed consent.

Patient consent for publication

All patients consented to the publishing of this paper, and provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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