Laser immunotherapy for cutaneous squamous cell carcinoma with optimal thermal effects to enhance tumour immunogenicity

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ABSTRACT

Background: Laser immunotherapy is a new anti-cancer therapy combining photothermal therapy and immunostimulation. It can enhance the tumours by damaging tumour cells directly and promoting the release of damage-associated molecular patterns (DAMPs) to enhance tumour immunogenicity. In this study, we investigated the thermal effects of laser immunotherapy and the potential for enhanced efficacy of laser immunotherapy for cutaneous squamous cell carcinoma (cSCC).

Methods: The cell viability and the DAMPs productions of heat-treated cSCC A431 cells in different temperatures were investigated. Laser immunotherapy with the optimal thermal effect for DAMPs production was performed on SKH-1 mice bearing ultraviolet-induced cSCC and a patient suffering from a large refractory cSCC.

Results: The temperature in the range of 45–50°C killing half of A431 cells had an optimal thermal effect for the production of DAMPs. The thermal effect could be further enhanced by local application of imiquimod, an immunoadjuvant. Laser immunotherapy eliminated most tumours and improved the survival rate of the ultraviolet-induced cSCC-bearing SKH-1 mice (p < 0.05). The patient with cSCC treated by laser immunotherapy experienced a significant tumour reduction after laser immunotherapy increased the amounts of infiltrating lymphocytes in the tumour. No obviously adverse effect was observed in the mice experiment or in the clinical application.

Conclusions: Our results strongly indicate that laser immunotherapy with optimal thermal effects is an effective and safe treatment strategy for cSCC.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common human skin cancer [1]. Left untreated, it may spread to other parts of the body, and it may ultimately lead to death [2]. Surgical excision is the first line of treatment for cSCC, although other treatment modalities have also been used depending upon the nature and site of the tumour and condition of individual patients [3,4]. However, there are significant challenges when treating large, metastatic, and invasive cSCC, using conventional therapies [5].

The ultimate control of cancer has been suggested to lie in the host immune system [6]. Immunotherapy has been considered a promising treatment approach, and various strategies have been proposed, including dendritic cell-based vaccines, immune checkpoint therapy, cytokine therapy and immune-activating antibodies [7–9]. However, these immunotherapy strategies have yielded low response rates and most cancers still avoid or escape from immune control [10,11]. Novel approaches are therefore needed to increase the efficacy of immunotherapy. Ideally, such novel approaches should not only destroy local tumours, but also at the same time achieve a potent systemic, tumour-specific immunological response to eradicate metastases at distant sites, with minimal adverse effects. Laser immunotherapy combining of selective photothermal therapy and a locally administered immunostimulant is a novel anti-tumour method, which has a synergistic effect of destroying the tumour and stimulating the anti-tumour immune responses [12–14]. It has been used for breast cancer, melanoma and Rosai–Dorfman disease with promising outcomes [15–17].

Photothermal therapy is the main component of laser immunotherapy which induces a temperature gradient inside the tumours. Heat can damage tumour cells through direct and indirect mechanism [18–20]. Direct mechanism is known as the necrosis and apoptosis of tumour cells. Indirect mechanism refers to tumour cells are eliminated by the immune response activated by photothermal therapy. The heat in the tumour up-regulate the expression or release of tumour antigens, especially various damage-associated molecular patterns (DAMPs), such as heat shock proteins (HSP) 70, HSP 90 and high mobility group protein B1 (HMGB1), enhancing the...
tumour immunogenicity [21–25]. At the same time, the activity of macrophages, dendritic cells, T lymphocytes, B lymphocytes, and natural killer cells could also be enhanced by the heat [26–29]. Finally, tumour cells are eliminated by the immune responses following the DAMPs expression.

Imiquimod, a unique toll-like receptor (TLR) agonist, was selected as the immunostimulant in the study to facilitate immunological stimulation. It has been approved by the FDA for the treatment of anogenital warts, actinic keratosis and superficial basal cell carcinoma [30]. Although the imiquimod monotherapy has a limited effectiveness for cSCC [31–33], it showed a strong immunological stimulating effect when combined with photothermal therapy for the treatment of melanoma patients, indicating that imiquimod is a good candidate as an immunostimulant in laser immunotherapy for cSCC [16].

Laser immunotherapy has never been used for cSCC. Furthermore, the relationship between heat and the DAMPs expression has not been established. It is still unclear what temperature range has optimal thermal effects on the enhancement of tumour immunogenicity in the laser immunotherapy treatment for cSCC.

In this study, to gain the optimal thermal effects, we investigated the correlation between heat and tumour cell death or DAMPs release/expression, with or without imiquimod. We evaluated the effectiveness of laser immunotherapy with the optimal thermal effects for the ultraviolet (UV)-induced cSCC in SKH-1 mice. Finally, we translated this novel anti-tumour method to treat a patient with a large refractory cSCC.

Materials and methods

Chemicals and reagents

High glucose Dulbecco’s-modified eagle medium (DMEM), phosphate buffer saline (PBS), and penicillin/streptomycin were obtained from HyClone™ (Utah, USA). Foetal bovine serum (FBS) was obtained from Gibco (Billings, MT, USA). Rabbit monoclonal anti-actin and rabbit monoclonal anti-HMGB1 (Cell Signaling Technology, Leiden, Netherlands), mouse monoclonal anti-HSP70, and rabbit polyclonal anti-HSP90 (Abcam, Cambridge, UK) were used. Human HSP70 ELISA Kit (Ebioscience™, San Diego, CA, USA), human HSP90 ELISA Kit (Ebioscience™, San Diego, CA, USA), and human HMGB1 ELISA Kit (Shino-Test, Tokyo, Japan) were also used. In addition, FITC Annexin V Apoptosis Detection Kit I (BD, NJ, USA) and Chromogenic Western Blot Immunodetection Kit (Thermo Scientific, Waltham, MA, USA) were also used. Imiquimod lyophilised powders were purchased from Inivogen (San Diego, CA, USA) and imiquimod creams were purchased from Sichuan Med-shine Pharmaceutical (Chengdu, China) were used.

Cell culture

Human cSCC cell line A431 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were maintained in high glucose DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics at 37°C in an atmosphere of 5% CO₂.

Heat treatment of cells

A431 cells were randomly divided into six heat treatment groups with the following temperature: 37, 40, 45, 50, 55 and 60°C. The A431 cells (2 × 10⁵/dish) growing in 60 mm dish were placed in a water bath with different temperature settings. The heating time was 10 min. Then, the A431 cells were cultured at 37°C in an atmosphere of 5% CO₂ after replacement of equal fresh medium for different durations (the recovery time). At designated time points, supernatants and heat-treated A431 cells were collected for different experiments.

Measurement of cell viability

The heat-treated A431 cells were observed with an inverted microscope at 8 and 24 h to determine the cell morphological change. In addition, the heat-treated A431 cells (2 × 10⁵/well) were seeded in 96-well plates immediately and incubated for 8 and 24 h at 37°C before being subjected to CCK8 assay to detect cell proliferation. Each group had 5 wells and the experiment was repeated three times. Survival rate = (OD treatment group-OD blank group)/(OD 37°C control group-OD blank group) × 100%. The cells (5 × 10⁵ cells/group) were also collected at 8 and 24 h after heat treatment and then washed in PBS, followed by incubation with FITC-AnnexinV and PI for 20 min at room temperature in the dark. After incubation, the cells were analysed with flow cytometer to detect the percentage of cell apoptosis and necrosis.

Quantification of the extracellular release of DAMPs (HSP70, HSP90 and HMGB1)

In order to evaluate the release of HSP70, HSP90 and HMGB1 in response to heat, the supernatants of the A431 cells were collected and centrifuged at different time points (1, 4, 8, 12 and 24 h) after heat treatment with different temperature settings. Then, the supernatants were analysed using ELISA-based HSP70, HSP90 and HMGB1 detection kits according to the manufacturer’s instructions.

Intracellular expression of DAMPs (HSP70, HSP90 and HMGB1)

To determine the effect of heat on intracellular expressions of HSP70, HSP90 and HMGB1, A431 cells were heat-treated with different temperature settings and were measured at 8 h after treatment. The correlation of DAMPs expression with time was also investigated via the measurement in 45°C at different recovery time points (1, 4, 8, 12 and 24 h). The proteins of heat-treated A431 cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (Tris base 50 mM, NaCl 150 mM, NP40 1%, sodium deoxycholate 0.25%, EDTA 1 mM) containing a protease inhibitor cocktail (Roche
Effect of imiquimod on proliferation of A431 cells

Aqueous solution of imiquiod was prepared using the powder of imiquimod. A431 cells (2 × 10⁴/well) were incubated with imiquimod of different concentrations (0, 5, 10, 20, 50, and 100 μg/ml) for 24 h before being subjected to CCK8 assay. Each group had five wells and the experiment was repeated three times.

Effect of imiquimod on release of DAMPs (HSP70, HSP90 and HMGB1) by A431 cells during heat treatment

A431 cells (2 × 10⁶/dish) were seeded in a 60-mm dish and incubated with 10 or 50 μg/ml imiquimod for 24 h before being subjected to heat treatment with different temperature settings. Then, the A431 cells were continuously incubated with 10 or 50 μg/ml imiquimod. The supernatants were collected and analysed using ELISA-based HSP70, HSP90 and HMGB1 detection kits according to the manufacturer’s instructions, at determined time points (1–24 h) after the heat treatment.

Laser immunotherapy with optimal thermal effects for one patient with refractory cSCC

A 63-year-old woman presented with previously untreated bleeding ulcer on the right elbow came to our hospital. The patient suffered 30 years ago from neck pain and numbness of the right upper extremity. The patient was diagnosed as cervical spondylopathy and treated with a surgery. One year after the surgery, the numbness of right upper extremity became more serious due to damage of the nerve with cervical spondylopathy. The skin on her right upper extremity, especially her right forearm, became pale with a low skin temperature. Gradually, her right elbow developed a bleeding ulcer. In the past 30 years, the lesion grew larger and larger without any improvements on bleeding or ulceration. In April of 2014, the patient came to our hospital. Clinical investigation showed a ulcer of about 6 × 9 cm on her right elbow, accompanied with oozing, bleeding and multiple nodules covered by crust. The skin surrounding the ulcer was pale with a low skin temperature. The right elbow joint suffered from stiffness and loss of motion. A blood routine test showed her haemoglobin was only 53 g/l. A biopsy revealed the diagnosis of skin squamous cell carcinoma, grade I. The patient refused surgical excision in consideration of the large area of the ulcer. We treated the patient with laser immunotherapy after a consent form was signed in June 2014.

A 10-week cycle of laser immunotherapy with optimal thermal effects was carried out with the following steps: (1) imiquimod cream was topically applied to the surface of tumour every other day for 10 weeks. (2) The tumour was
irradiated by an 808-nm laser once every two weeks for four times, and the first laser irradiation was carried out at the end of week 2. The power density at the treatment area, which encompassed of 2 cm², the skin lesions, was 1 W/cm² for a treatment duration of 10 min. During laser irradiation, the superficial temperatures of tumour were monitored. If temperatures were higher than 55 °C, the irradiation was discontinued until the temperatures dropped to 45 °C. The patient was treated with the 10-week cycle of laser immunotherapy for three sessions. The treatment interval was one month. A histopathological examination was performed again after two sessions of laser immunotherapy. All the clinical treatment and animal experiments followed the principles of the Declaration of Helsinki.

Statistical analyses

Data were analysed with Spass13.0 software and presented as mean±standard deviation. All statistical analyses were performed using t-test except survival rates were compared using Mantel–Cox log rank test. p < 0.05 was considered statistically significant.

Results

Effects of thermal treatment on A431 cell activity

After different thermal treatments (10 min), the A431 cells were placed at 37 °C. The morphology (Figure 1(A)), the viability (Figure 1(B)), and the percentage of apoptosis/necrosis (Figure 1(C)) were detected at 8 and 24 h. Heat caused death of A431 cells in a temperature-dependent manner. The cells in 37 °C (control group) and 40 °C group grew normally, adhering to the wall with the morphological integrity. The survival rates of cells in 40 °C were 100.7 ± 3.1% at 8 h and 100.8 ± 1.7% at 24 h. Most cells in 45 °C group became spherical and floated up, having a lower survival rate (89.8 ± 0.8% at 8 h, 82.4 ± 3.5% at 24 h) than cells in control group (p < 0.01). Most cells in 50 °C adhered tightly to the wall with white bubbles inside the cells. The survival rates were 91.5 ± 1.0% at 8 h, and 82.4 ± 4.4% at 24 h. All of the cells in 55 and 60 °C adhered the wall tightly with white bubbles. The survival rates were only 24.0 ± 3.2% at 8 h, 7.0 ± 2.1% at 24 h in 55 °C, 3.2 ± 0.1% at 8 h and 3.1 ± 0.7% at 24 h in 60 °C. The percentages of apoptosis/necrosis were shown in Figure 1(C). Most cells were alive in 37 °C and 40 °C group. Only 5.0–10.5% cells appeared apoptotic. Most cells in 55 and 60 °C group died. 84.2–93.8% cells appeared necrotic. Around 30–60% cells died in 45 and 50 °C group, with an apoptosis percentage of 12.3–55.5% and a necrosis percentage of 3.1–43.4%.

Effects of thermal treatment on the extracellular release of DAMPs from tumour cells

The extracellular releases of HSP70, HSP90, and HMGB1 in the supernatant of A431 cell culture were measured by enzyme linked immunosorbent assay (ELISA) after different temperature treatments (37, 40, 45, 50, 55, and 60 °C) for 10 min. As shown in Figure 2, A431 cells released HSP70, HSP90 and HMGB1 after treatment. The productions of HSP70, HSP90 and HMGB1 increased along with the time in the range of 37–50 °C. Cells at 50 °C released the maximum amount of HSP70 while cells in 45 °C released the maximum amount of HSP90 and a high level of HMGB1.

Effects of thermal treatment on the intracellular expression of DAMPs in tumour cells

To determine intracellular changes of DAMPs, the expressions of HSP70, HSP90, and HMGB1 of A431 cells treated by different temperatures (37, 40, 45, 50, 55, and 60 °C) for 10 min were analysed by western blot analysis. As shown in Figure 3, the expressions of both HSP70 and HSP90 at 45 °C were the highest in all temperature groups. And the expressions of HMGB1 at 45 °C was similar to that of 37 or 40 °C group, which was higher than that of 50, 55 °C or 60 °C groups. HSP 70, HSP 90 and HMGB1 were down-regulated in high temperature groups (50, 55, and 60 °C). The expressions of HSP70 and HSP90 increased gradually with time within the 24 h-recovery period.

Effects of imiquimod on proliferation of A431 cells and DAMPs release

Since imiquimod could directly affect keratinocytes viability and it has been used for the treatment of cSCC. We investigated its effects on A431 cells. When the A431 cells were incubated with imiquimod, cell death occurred in a dose-dependent manner (Figure 4(A)). ELISA results (Figure 4(B–D)) showed that imiquimod, especially at a dose of 10 μg/ml increased the total release of HSP70, HSP90 and HMGB1 24 h after heat treatment. Generally, A431 cells treated by the combination of imiquimod and heat treatment released a maximum amount of DAMPs in a temperature range of 45–50 °C.

Anti-tumour activity of laser immunotherapy with optimal thermal effects for UV-induced cSCC mice

UV irradiation on SKH-1 mice lasted for five months until papules of 1–4 mm in diameter appeared and maintained for two weeks. After the UV irradiation, the papules continued to grow to various size of cauliflower-type lesions and some tumours began to develop surface erosion and ulcers, as shown in Figure 5(A). The lesion was proved as cSCC by histopathological examination, showing a large number of atypical cells and keratin pearls in the dermis (Figure 5(B and C)).

During laser irradiation, the superficial temperatures of mouse tumour were monitored to maintain the temperature in the range of 45–50 °C to produce the maximum of DAMPs (Figure 6(A)). The temperature in laser immunotherapy group increased from 36 °C during irradiation and reached a saturation of around 50 °C in about 3 min.
The observation of tumour growth started at the first day of treatments (laser immunotherapy, laser, imiquimod, and untreated control). The results of the tumour volume from day 1 to day 60 are shown in Figure 6(B). On day 27, the tumour volumes of mice in the untreated control group and imiquimod group increased significantly, larger than before treatment ($p < 0.05$). However, there was no significant increase in tumour volumes in laser immunotherapy group and laser group ($p > 0.05$) compared with before treatment. The tumour volumes of mice in laser immunotherapy group and laser group were significantly lower than that in the control group ($p < 0.05$). The tumour volumes of mice in laser group increased, comparable with tumour growth in the imiquimod group and control group after day 27. On day 60, only the tumour volumes of mice in laser immunotherapy group had no difference from before treatment ($p > 0.05$), which were much smaller than laser group ($p < 0.05$).

The representative photographs of mice after treatments are shown in Figure 6(C). The tumours in control, imiquimod and laser groups became larger and more numerous with time.
Figure 2. Heat affected extracellular release of damage-associated molecular patterns (DAMPs) from A431 cells in a temperature-dependent manner. The release of heat shock proteins (HSP) 70 (A), HSP90 (B), and high mobility group protein (HMGB1) (C) from heat-stimulated A431 cells at different time points (1–24 h) after the 10-min heat treatments, at 37, 40, 45, 50, 55, and 60 °C, analysed using enzyme linked immunosorbent assay (ELISA) assay. Statistical analysis was performed by t-test; \( * p < 0.05, \quad ** p < 0.01, \quad *** p < 0.001 \), ns = not statistically significant. Mean ± SD are shown from three independent experiments.
time. In the laser immunotherapy group, the treated tumours were cleared or decreased in volume. Some of the untreated tumours in this group also disappeared. No new tumours or tumour recurrence were observed in the laser immunotherapy group. In the laser only group, the treated tumours decreased in size or grew slowly. The untreated tumours and new tumours also grew slowly. In the imiquimod group, the treated, untreated and new tumours grew more rapidly than laser group, but more slowly than control group. The survival rates of mice in 60 days are shown in Figure 6(D). All mice died within 40 days in the control group, and within 50 days in the imiquimod group. In the laser group, two mice died on day 52 and day 53, respectively. No mice in laser immunotherapy group died within 60 days. The survival rate of laser immunotherapy group was remarkably higher than the control, imiquimod groups and laser group ($p < 0.05$).

Compared to control group, no obvious side effect was observed in imiquimod, laser and laser immunotherapy groups.

**Laser immunotherapy with optimal thermal effects for patient with refractory cSCC**

A 63-year-old woman suffering from refractory cSCC was treated by a 10-week cycle of laser immunotherapy with optimal thermal effects for three sessions (Figure 7(A and B)). During the treatment, the highest temperature on the tumour surface was 45°C (Figure 7(C)). After one laser irradiation, the irradiated region appeared erythema immediately. A week later, the irradiated regions appeared crusts. After one session of laser immunotherapy treatment, the oozing and bleeding stopped. The surface of ulceration became dry and skin was growing from the edge to the centre. The area of tumour was much smaller than before treatment. The skin
Figure 4. Effects of imiquimod on the viability of A431 cells and release of damage-associated molecular patterns (DAMPs). (A) Imiquimod directly killed A431 cell in a dose-dependent manner. Cells were incubated with imiquimod at different concentrations (0–100 µg/ml) for 24 h before being subjected to CCK8 assay. Statistical analysis was performed by t-test; (5, 10, 20, 50 or 100 µg/ml vs. 0 µg/ml). (B–D) Release of heat shock protein (HSP) 70, HSP90, and high mobility group protein B1 (HMGB1) from A431 cells treated under different temperature, with or without imiquimod. A431 cells were incubated with imiquimod at different concentrations (10 and 50 µg/ml) for 24 h, followed by a 10-min heat treatment under different temperatures (37–50 °C), and then recovered at 37 °C with imiquimod. The supernatant was collected and analysed by ELISA assay. Statistical analysis was performed by t-test; (temperature + IMQ (Imiquimod) group vs temperature group). Means ± SD are shown from three independent experiments *p < 0.05, **p < 0.01.
temperature around the lesion returned to normal. After two sessions, the area of tumour became smaller further and the nodules disappeared or became flat (Figure 7(A)). The pale skin surrounding the tumour became ruddy. The motion ability of the right elbow joint recovered. Histopathology showed that there were more infiltrating lymphocytes in the upper and medial dermis than before treatment (Figure 7(B)), indicating that anti-tumour immune responses were enhanced by the laser immunotherapy. After three sessions of laser immunotherapy treatment, the tumour area was reduced further and most nodules disappeared, leaving a superficial ulcer with a dry surface (Figure 7(A)). The updated blood routine showed her haemoglobin increased to 92 g/l. Besides the erythema and crusts, no other adverse reactions, such as oedema, erosion, scarring or pain, was observed.

Discussion

Cutaneous squamous cell carcinoma typically manifests as a spectrum of progressively advanced malignancies, ranging from a precursor actinic keratosis to squamous cell carcinoma in situ, invasive squamous cell carcinoma, and finally metastatic squamous cell carcinoma [36]. However, for large, invasive, and metastatic cSCC, traditional treatments, such as surgery, chemotherapy, and radiotherapy, were often unsatisfactory [37,38]. In addition, the traditional treatments had many disadvantages, including destruction of normal tissue structure and function, dose limitation, and high recurrence rate [39,40].

Laser immunotherapy is a novel approach using a combination of photothermal therapy and immunological stimulation [35]. The heat generated by photothermal therapy has
an important role in killing tumour cells and activating immune response. Different temperatures inside the tumours induce different biological responses, including cell stress, damaged and death, which can expose different quantity of DAMPs as tumour antigens for immunological stimulation [19,41]. We investigated the correlation between temperature and tumour cell death and DAMPs release/expression using the 10-min water-bath heating as hyperthermia in vitro in the study. The results showed that hyperthermia killed A431 cells in a temperature-dependent manner (Figure 1). Temperatures in the range of 45°C-50°C induced 50% of apoptotic or necrotic cells and enhanced the maximum
amount of DAMPs releasing or expressing. Since DAMPs are mainly expressed or released by damaged/dying cells, the above results indicated heat treatment at 45°C-50°C for 10 min induced most cell stress and expressed DAMPs before death. Besides necrosis, apoptosis also contributed to the DAMPs emission. The increasing experiments indicated that certain anticancer therapies (e.g. chemotherapeutic drug, radiotherapy and photodynamic therapy) could induce an immunogenic apoptosis associated with the emission of DAMPs to further activate immunogenic host response [24,25,42,43]. The processes of cell apoptosis or necrosis accompanying the emission of DAMPs to activate the potent anticancer immunoreaction is defined as immunogenic tumour cell death (ICD) [44–47]. ICD requires antigen linked spatiotemporal DAMP signals including the cell surface translocation of HSP70 and HSP90 followed by the passive release of HMGB1 protein from the nucleus during the late apoptotic stage [48–50]. HSPs are a family of conserved chaperones induced by cell stress including oxidative stress, irradiation, chemotherapeutic drugs, and heat and electromagnetic field [51]. Under stress, most of HSPs are expressed within the cellular cytoplasm or organelles (i.e. mitochondria and endoplasmic reticulum). In the cytoplasm, overexpressed HSP70 and HSP90 can inhibit apoptosis and act as a cytoprotector maintaining protein homeostasis [52,53]. However, HSP70 and HSP90 can also be translocated to the plasma membrane or released to extracellular matrix, with tumour-associated antigens, forming HSP-tumour antigen complexes [54]. HSP-tumour antigen complexes either exposed on the surface of cells or released to the extracellular matrix could be acquired by dendritic cell through the surface receptors like CD91, LOX1 and CD40 and to facilitate crosspresentation of
tumour-associated antigen via major histocompatibility complex class I to activate (CD8+) T-cell response [44,55,56]. Therefore, the exposed or released HSPs can boost adaptive immune response [41,57]. HMGB1 represents a late signal of ICD with diverse roles. In the nucleus, it acts as a non-histone chromatin binding protein interacting with the minor groove of DNA and regulatory molecules such as p53, NF-κB, and steroid hormone receptors [46]. Upon cell stress, HMGB1 is released either from necrotic or apoptotic cells [58]. The apoptotic cells might release HMGB1 with DNA together during its late stages [44]. Once released outside, HMGB1 can be a cytokine-like activator of macrophages, a chemotactant for neutrophils and a promoter of dendritic cell maturation through TLR 2/4 [25,46–48], thereby activating innate immunity and leading to acquired immunity responses. In this study, large amount of HMGB1 was released from the damaged/dying cells after the heat treatment at 45°C–50°C. However, HMGB1 was also released when most of cells were viable at 37°C–40°C. It has been detected that various tumour cells could also overexpress and secret HMGB1 actively to enhance tumour cell proliferation and migration. HMGB1 secreted by viable tumour cells without damage might have an opposite effect on activating anti-tumour immune, compared to which released from damaged or dying cells [59]. From the above, the temperature between 45°C and 50°C could induce the maximum DAMPs, having an optimal thermal effect for the ICD of cSCC cells.

Application of imiquimod was also an important component of laser immunotherapy. Our results showed that imiquimod could directly kill A431 cells in a dose-dependent manner (as shown in Figure 4(A)) [60,61]). Besides, low concentration imiquimod could further increase the release of HSP70, HSP90 and HMGB1 during the heat treatment at 45–50°C, which suggests that imiquimod could enhance the optimal thermal effect of heat in promoting DAMPs release (as shown in Figure 4).

We used laser immunotherapy with optimal thermal dose (45–50°C) combining topical imiquimod to treat UV-induced cSCC in immune competent SKH-1 mice. The UV-induced cSCC could mimic the human skin squamous cell carcinoma perfectly [62]. In this study, the imiquimod monotherapy could only slow down the tumour growth and improve survival time slightly, which were consistent with the clinical reports of topical imiquimod treatment for cSCC [31–33]. Photothermal monotherapy could also slow down the tumour growth and significantly improve survival time, which were consistent with the reports that photothermal therapy could kill squamous cell carcinoma and other cancer cells [63]. However, photothermal monotherapy had no effect on the untreated tumours, which suggested that photothermal therapy had a limited systemic effect. Compared to imiquimod or photothermal monotherapy, laser immunotherapy could not only destroy local tumours, but also at the same time induced a potent systemic, tumour-specific immunological response to slow, and sometimes eradicate, untreated tumours at distant sites, as shown in Figure 6(A–D). Our therapeutic effects, along with the lack of noticeable side effects, demonstrated that laser immunotherapy was a safe and effective approach for cSCC, especially for multiple, large, late-stage, metastatic, or other inoperable cSCCs.

In the clinical practice, we used laser immunotherapy with optimal thermal effect to treat an old woman suffering from a large refractory cSCC. The superficial temperatures on the patient were lower than mice. It might be because human had better blood circulation, contributing to a faster heat dispersion than mice. The laser irradiation induces a temperature gradient in the irradiated site. The temperatures inside the tumour usually are higher than the surface of the tumour [21,64]. Though the highest superficial temperature was 45°C, the tissues inside the tumour might still have temperatures between 45°C and 50°C, generating sufficient DAMPs to activate the potent anticancer immunity. As expected, after three sessions of the 10-week cycle of laser immunotherapy, the lesion and symptom of tumour was improved. Meanwhile, the histopathology showed that there were more infiltrating lymphocytes in the upper and medial dermis than before the treatment (Figure 7(B)), indicating that laser immunotherapy successfully activated the anti-tumour immune responses [26]. Unfortunately, the tumour wasn’t eliminated completely by laser immunotherapy during our study period. It might be because the area was too large to be eliminated in a short-term. And the local anti-tumour immune responses of the patient were diminished by the age, anaemia and cervical spondylopathy. No obvious adverse reaction was observed. The above results suggested laser immunotherapy with the thermal treatment at 45–50°C was an effective and safe clinical method for cSCC.

Conclusions

In conclusion, the temperature in the range of 45–50°C could induce the maximum DAMPs, having an optimal thermal effect in inducing the ICD of cSCC cells. Imiquimod could improve the effect of photothermal therapy for generating DAMPs. The laser immunotherapy composing of photothermal therapy with the optimal thermal effect and imiquimod was an effective and safe method for the treatments of cSCC. Our results provided the basic understanding of photothermal therapy in inducing immune response in cSCC treatment, particularly when used in combination with an immunostimulant, paving the way to further develop laser immunotherapy into an effective clinical treatment modality for cSCC, especially for multiple, large, late-stage metastatic, or other inoperable cSCCs.

Disclosure statement
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