New Application of Old Material: Chinese Traditional Ink for Photothermal Therapy of Metastatic Lymph Nodes

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ABSTRACT: Finding a simple and effective strategy to eliminate tumor metastatic lymph nodes is highly desired in clinical tumor treatment. Herein, we reported a Chinese traditional ink (Hu-ink)-based treatment for photothermal therapy (PTT) of tumor metastatic lymph nodes. By simple dilution, stable Chinese traditional ink dispersion was obtained, which presents excellent photothermal effect because of its high absorption in near-infrared (NIR) region. Meanwhile, as revealed by staining and photoacoustic imaging, Hu-ink could transfer to nearby lymph nodes after directly injected into the primary tumors. Under the guidance of dual-modality mapping, the metastatic sentinel lymph nodes could be subsequently eliminated by NIR irradiation. The good biocompatibility of Hu-ink has also been verified by a series of experiments. Therefore, the Hu-ink-based treatment exhibits great potential for PTT of tumor metastatic lymph nodes in future clinical practice.

1. INTRODUCTION

Today, metastasis has become one of the greatest challenges in cancer diagnosis and therapy and is directly or indirectly responsible for ~90% of cancer patient deaths.1,2 In the early stages of cancer metastasis, tumor cells first transfer to sentinel lymph nodes, which are adjacent to the primary cancer, and subsequently flow into deeper lymph nodes via lymphatic vessels.3,4 In clinical practice, physicians generally remove those lymph nodes with potential metastases completely by surgical dissection to prolong the lifetime of cancer patients. However, it inevitably brings about low treatment rate, considerable surgical trauma, and sometimes multiple complications.5,6 Therefore, exploring simple and effective treatments to remove tumor metastatic lymph nodes and improve patients’ quality of life has become very necessary and urgent in clinical tumor therapy.

Photothermal therapy (PTT) is an emerging tumor treatment strategy, which utilizes hyperthermia generated from absorbed near-infrared (NIR) light energy by photoabsorbing agents to kill tumor cells.7−13 Different from chemotherapy, surgical treatment, and radiotherapy, PTT is noninvasive and more efficient.7,14,15 In the past decade, PTT with diverse nanomaterials to eliminate cancer metastases lymph nodes has attracted extensive attention by several groups, including our group.5,16−20 For instance, Liu and his co-workers developed a treatment method based on PEGylated single-walled carbon nanotubes for PTT of tumor sentinel lymph nodes and achieved remarkably improved treatment effect in an animal tumor model.21 To meet the clinical practice, the potential metastasis of deeper lymph nodes was further ablated in our previous work, using magnetic graphene oxide as a theranostic agent.22 However, preparation of these artificial nanomaterials usually requires high cost, complicated synthetic process, and unavoidably toxic catalyst or chemicals,23,24 which impede their future clinical application. For the clinical application, exploring an environment-friendly material with simple preparation procedure, good biocompatibility, and excellent therapeutic efficiency is still highly desired.

Chinese traditional ink, as a conventional writing material with intrinsic color of black, good water stability, and desired...
fl uidity, has been used for a long time in China and still fascinates contemporary artists around the world. It was derived from natural plant, and its main component is carbon. 

Considering the similarities in color and component between Chinese traditional ink and other artificially synthesized carbonaceous materials, such as carbon nanotubes and graphene, which have been extensively investigated for PTT of cancer lymph node metastases, we are thus encouraged to explore whether Chinese traditional ink can also be used for inhibiting cancer metastases by PTT. Herein, a simple and effective treatment method was successfully developed by utilizing one of the most famous Chinese traditional inks, Hu-Kaiwen ink (Hu-ink). The Hu-ink-based treatment can not only provide dual-modality staining and photoacoustic imaging (PAI) of lymph nodes, but also be used for PTT of tumor metastatic lymph nodes in rectal cancer (Figure 1).

2. RESULTS AND DISCUSSION

2.1. Preparation and Characterization of Hu-Ink. To obtain an applicable sample, the condensed Hu-ink was first diluted into aqueous dispersion with a lower concentration.
The obtained Hu-ink dispersion without any further treatment was black in color and stable in physiological environment, including water, phosphate-buffered saline (PBS), and Roswell Park Memorial Institute (RPMI) 1640; furthermore, no aggregation was observed even after keeping undisturbed for 3 days (Figure 2a). The nanoscaled morphology of Hu-ink was examined by transmission electron microscopy (TEM) (Figure 2b), which demonstrates that Hu-ink mainly exist in the form of small aggregates. These small aggregates consist of a few nanoparticles with diameter of about 20–50 nm. Dynamic light scattering (DLS) measurement (Figure 2c) further shows that Hu-ink aqueous dispersion possesses a hydrodynamic diameter of about 186 nm (polydispersity index: 0.18), which was a crucial prerequisite for biomedical applications. In the X-ray diffraction (XRD) pattern, no other characteristic peaks are found except carbon peak (Figure S1, Supporting Information), which confirms that the main component of Hu-ink is carbon. Raman spectroscopy was a common tool to characterize graphene-related materials. D band (~1300 cm$^{-1}$, corresponding to the defects) and G band (~1600 cm$^{-1}$, related to the sp$^2$ carbon sites) peaks could be observed in Figure 2d with the ratio $I_D/I_G = 0.96$, which confirms the existence of graphene sheet-like structure in Hu-ink. The UV-vis-NIR spectra (Figure 2e) also revealed that Hu-ink has high absorption in the NIR region around 650–900 nm, in which hemoglobin and water, the major absorbers of biological tissue, have their lowest absorption coefficient. The high NIR absorption capability of Hu-ink is very important for clinical application. Considering the simple preparation, excellent photothermal properties of Hu-ink are very important for clinical storage.

2.2. In Vitro Photothermal Therapy. A promising nanomaterial must have a low cellular toxicity, so the Cell Counting Kit-8 (CCK-8) assay was used to test the cytotoxicity of Hu-ink in vitro. As shown in Figure 4a, the cell viability of human colon cancer cells (SW-620) and human colorectal cancer cell lines (HCT-116) incubated with different concentrations of Hu-ink for 24 h presented insignificant decrease even at high concentration (200 μg/mL), which indicated that our obtained Hu-ink dispersion had no inherent toxicity. The PTT efficiency of cancer cells was also estimated by CCK-8 assay. From Figure 4b, the relative cell viabilities were 50.2% at the Hu-ink concentration of 25 μg/mL, which is quite higher than that of water (below 5 °C). The photothermal conversion efficiency of Hu-ink was found to be about 39% (Figure 3a) using the reported method. Except nanographene (51.6%), Hu-ink possesses higher photothermal conversion efficiency than commercial gold nanorods (21%) and nanoshells (13%), Cu$_2$S$_2$ nanocrystals (25.7%), MoS$_2$ nanosheets (24.37%), and ICG (15.1%) (Table S1, Supporting Information). Furthermore, we found that the photothermal effect of Hu-ink showed almost no change with increasing storage time for 90 days (Figure 3b), revealing that Hu-ink presented stable photothermal effect. The hydrodynamic diameter and ζ potential were also stable during the experiment (Figure 3c,d). The stable physicochemical properties of Hu-ink are very important for clinical storage. The high NIR absorbance of Hu-ink is carbon.

Figure 3. (a) Temperature-rising and temperature-decreasing curves of Hu-ink aqueous dispersion exposed to an 808 nm laser (2 W/cm$^2$, 50 μg/mL). (b) Photothermal effect of Hu-ink aqueous dispersion on 0th day and 90th day (2 W/cm$^2$, 50 μg/mL). (c) Size distribution of Hu-ink on 0th day and 90th day. (d) ζ Potential of Hu-ink aqueous dispersion on 0th day and 90th day.

Figure 4. (a) Cell viability of human colon cancer cells (SW-620) and human colorectal cancer cell lines (HCT-116) incubated with different concentrations of Hu-ink for 24 h. (b) Relative cell viabilities of Hu-ink aqueous dispersion on 0th day and 90th day (2 W/cm$^2$, 50 μg/mL). (c) Size distribution of Hu-ink on 0th day and 90th day. (d) ζ Potential of Hu-ink aqueous dispersion on 0th day and 90th day.
PTT efficiency, the live cells and dead cells were discriminated by calcein acetoxymethyl ester (calcein AM) and propidium iodide (PI) staining, respectively. As shown in Figure 4c, HCT-116 cells only emitted green fluorescence in NS group for the lack of dead cells. However, in the group of Hu-ink at 25 μg/mL, nearly 50% of cells were dead by thermal treatment and thus emitted red fluorescence. In the group of Hu-ink at 50 μg/mL, almost all HCT-116 cells were dead and emitted red fluorescence with the same NIR laser illumination. Thus, the effect of PTT can be improved by increasing the Hu-ink concentration of the tumor site without enhancing the power of NIR laser.

2.3. Dual-Modality Staining/Photoacoustic Imaging of Lymph Nodes. Lateral lymph node metastasis is the main metastatic way of rectal cancer, especially in low rectal cancer, which is an important factor affecting healing.40 However, locating those lymph nodes accurately and rapidly is difficult in clinical treatment. In clinical practice, magnetic resonance imaging (MRI) combined with T1 or T2 contrast agents or computed tomography (CT) is a favorite imaging technique to diagnose tumor and lymph nodes.41,42 However, its complex and bulky instrument cannot provide real-time visualization during the treatment procedure.43 Photoacoustic imaging (PAI) is a powerful imaging technology based on the illumination of light-absorbing nanoprobes and ultrasound detection.44 Compared with the traditional MRI and CT, PAI combines simple operation, enhanced penetration depth, and high sensitivity, which can provide us real-time information during therapeutic process.45,44 Due to the strong NIR absorption property, the Hu-ink can not only be used for PTT, but also has great potentiality for PAI contrast agent.12,45,46 Besides, it is reported that nanoparticles after

Figure 4. Cell experiments. (a) Cell viability of HCT-116 cancer cells and SW-620 cancer cells cultured with various concentrations of Hu-ink for 24 h. (b) The respective cell viability of HCT-116 cancer cells after treated with normal saline, 25 and 50 μg/mL of Hu-ink (2 W/cm², 5 min). (c) Confocal fluorescence images of HCT-116 cancer cells stained with calcein AM (live cells, green fluorescence) and PI (dead cells, red fluorescence) after treated with normal saline, 25 and 50 μg/mL of Hu-ink (2 W/cm², 5 min).
being injected into the primary tumors could effectively transfer to the sentinel lymph nodes and subsequently flow into deeper lymph nodes along the lymphatic vessels. Therefore, we wondered if we could use Hu-ink as a nanoprobe to locate lymph nodes by PAI. In our experiments, Balb/c mice with HCT-116 tumor inoculation on their right hind pad were allowed to grow 30 days for the growth of primary tumors and the development of metastatic tumors in lymph nodes. Next, NS and Hu-ink were separately injected into the primary tumors on the right hind pad of those mice, and popliteal lymph nodes (the first station lymph nodes or sentinel lymph nodes) and sciatic lymph nodes (the second station lymph nodes) were then imaged under a PAI scanner after 24 h. In the NS group (Figure 5c,d), the popliteal and sciatic lymph nodes were hardly distinguished from the surrounding tissues due to the same signal. On the contrary, in Hu-ink group (Figure 5g,h), remarkably enhanced PAI signals were detected in both popliteal and sciatic lymph nodes after injecting Hu-ink into the primary tumors 24 h later. The relative signal intensities were $4.75 \pm 0.23$ and $2.33 \pm 0.03$ ($n = 3$). Compared with the NS group, the photoacoustic signals in popliteal and sciatic lymph nodes were improved by 15 and 5 times, respectively. At the same time, after exposed by anatomy, popliteal and sciatic lymph nodes were also easy to be discriminated from the surrounding normal tissues owing to the accumulation of black Hu-ink (Figure 5c,f). In a word, the dual-modality staining/PAI of Hu-ink was proved to be an effective method for guiding the treatment of tumor lymph nodes.

2.4. Dual-Modality Lymph-Mapping-Guided PTT. Next, dual-modality mapping was employed to guide the photothermal ablation of metastatic sentinel lymph nodes in rectal cancer. In this study, popliteal lymph nodes (sentinel lymph nodes) were appointed as therapeutic target to elaborate the feasibility of PTT. Hu-ink ($50 \mu L$, $5 \text{ mg/mL}$) was injected into the primary tumors on the right hind pad of those mice manifesting lymph node metastases. After 24 h injection, the popliteal lymph nodes were irradiated by an 808 nm laser ($1 \text{ W/cm}^2$) and the temperature change was recorded by an infrared thermal camera (Figure 6). In the Hu-ink + laser group, the local temperature of the popliteal lymph nodes quickly increased from 26.4 to 58.8 °C in 5 min, which is sufficient to eliminate the tumor tissues. However, the temperature of the NS group under the same laser power exhibited slight change and reach 31.1 °C, which would not induce any damage to the normal tissues.

Subsequently, we evaluate the efficacy of PTT in vivo. Mice manifesting sentinel lymph node metastases were randomly divided into four treatment groups after tumor inoculation for 30 days ($n = 5$ per group): (1) $50 \mu L$ of NS injected into the primary tumors on the right hind without laser irradiation; (2)
Figure 7. In vivo dual-modality-guided photothermal therapy (PTT). (a) Photograph of popliteal lymph nodes after excision from normal saline only, normal saline with 808 nm laser irradiation, Hu-ink only, and Hu-ink with 808 nm laser irradiation. (b) Popliteal lymph node weight of each group (*P < 0.05). Histological analysis of H&E for (c) normal saline only (arrow represents intact morphology of cells), (d) normal saline with 808 nm laser irradiation, (e) Hu-ink only, and (f) Hu-ink with 808 nm laser irradiation (arrow represents coagulative necrosis of cells).

50 μL of NS injected into the primary tumors on the right hind foot pad with laser irradiation (1 W/cm², 5 min); (3) 50 μL of Hu-ink (5 mg/mL) injected into the primary tumors on the right hind foot pad without laser irradiation; and (4) 50 μL of Hu-ink (5 mg/mL) injected into the primary tumors on the right hind foot pad with laser irradiation (1 W/cm², 5 min). PTT was carried out after 24 h injection. On the 7th day, the mice were sacrificed through anesthetization and the popliteal lymph nodes were removed. The weight of the popliteal lymph nodes for groups I, II, III, and IV were 8.92 ± 5.2, 9.72 ± 4.93, 9.28 ± 4.73, 1.02 ± 0.34 mg, respectively (Figure 7b). The remarkable antitumor efficiency was obtained in group IV, which was better than all of the other groups (P < 0.05). To further evaluate the PTT effect, histopathological examination with hematoxylin and eosin (H&E) staining was conducted. Necrosis and pyknosis (typical thermal damage characteristics of the tumors) were presented in group IV (Figure 7f). By contrast, intact morphology of cells existed in the other three groups (Figure 7c−e). Finally, the biological safety of Hu-ink was further evaluated. No pathological damage was detected in the pathological sections of major organs, including brain, heart, lung, spleen, liver, and kidney (Figure S2a−f, Supporting information). In the course of the whole treatment, the weight of mice did not change. Therefore, our obtained Hu-ink had no significant side effect and exhibited good biocompatibility.

3. CONCLUSIONS

In summary, we have utilized one of the famous Chinese traditional inks, Hu-ink, and successfully developed a simple and effective Hu-ink-based treatment for PTT of metastatic lymph nodes in rectal cancer. Both in vitro and in vivo experiments indicated that Hu-ink exhibit good biocompatibility. Benefiting from the intrinsic black and the strong absorbance in the NIR region, Hu-ink could not only serve as dual-modality mapping agents for staining/PAI of tumor lymph nodes, but also act as a photothermal agent to ablate the metastatic sentinel lymph nodes. The dual-modality lymphatic tracing method and the noninvasive PTT of metastatic sentinel lymph nodes have good clinical operability, especially in lateral lymph node metastasis of rectal tumor. Therefore, our work possesses remarkable potential in future clinical practices.

4. EXPERIMENTAL SECTION

4.1. Materials. Hu-ink was obtained from Anhui Jixi Hu-Kaiwen Ink Factory, China. Propidium iodide (PI), calcine acetoxymethyl ester (calcein AM), and Cell Counting Kit-8 (CCK-8) were obtained from KeyGen BioTech. Penicillin-streptomycin solution, Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 cell culture medium, trypsin-ethylene diamine tetraacetic acid solution, and fetal bovine serum (FBS) were obtained from Gibco. Human colon cancer cells (SW-620) and human colorectal cancer cell lines (HCT-116) were acquired from Life Science (Shanghai, China). Male Balb/c nude mice (6- to 8-week old) were purchased from Shanghai Slac Lab Animal Co. Ltd. All procedures for animal experiments were conducted strictly under the operation manual ratified by the Ethics Committee of Fudan University. All of these reagents were used as received without further purification, and deionized water was obtained from a Millipore water depuration system.

4.2. Preparation and Characterization of Hu-Ink.

Condensed Hu-ink was first diluted into aqueous dispersion with a lower concentration, and the obtained Hu-ink was then stored for further use. Transmission electron microscopy (TEM) images were recorded on a Tecnai G2-20 TWIN transmission electron microscope. Dynamic light scattering (DLS) particle size analyzer (Malvern Nano-ZS90) was used to measure ζ potential and hydrodynamic diameters. X-ray diffraction (XRD) characterization was performed on an X’Pert PRO diffractometer. Raman spectrum was recorded with the 532 nm laser source by Raman spectroscopy (XploRA, HORIBA Jobin Yvon). UV–vis–NIR spectra were obtained using a spectrophotometer (PerkinElmer Lambda 750). Temperature elevation assay was conducted by laser irradiation: Hu-ink dispersions with different concentrations of 25, 50, and 100 μg/mL were exposed continuously to an 808 nm laser....
(power density: 2 W/cm², spot size: 6 mm × 8 mm) for 5 min. The temperature of the dispersions was synchronously noted using an infrared thermal camera (VarioCAM HR, InfraTec, Germany).

4.3. Cell Experiment. The cytotoxicity of Hu-ink was assessed by CCK-8 assay. Approximately 1 × 10⁴ HCT-116 and SW-620 cells were plated in 96-well plates and cultured for 24 h at the standard cell culture environment. The cells were cultured with samples (0–200 μg/mL) for 24 h and then washed twice with PBS; 100 μL of RPMI 1640 cell medium and 10 μL of CCK-8 solution were mixed, and the mixture was then added into each plate and continued culture for another 2 h. Next, the OD value at 450 nm was read using a microplate reader (Synergy TM2, BIO-TEK Instruments Inc.). For in vitro PTT, HCT-116 cells were incubated in 96-well plates and cultured for 12 h. Then, the dispersion containing Hu-ink (25 or 50 μg/mL) was added into each plate and incubated for another 2 h, followed by an 808 nm laser irradiation (2 W/cm², 5 min). Next, the CCK-8 assay was used to test relative cell viabilities. For further qualitative assessment of PTT, HCT-116 cells (2.0 × 10⁴ cells per well) were seeded in a 20 mm glass-bottom culture dish (NEST, China), cultured for 2 h with 25 or 50 μg/mL Hu-ink at 37 °C, and subsequently followed by an 808 nm laser irradiation (2 W/cm², 5 min). Finally, the cells were stained for 20 min with PI and calcine AM and observed by a ZEISS LSM710 live cell confocal laser imaging system (Carl Zeiss, Germany).

4.4. In Vivo Dual-Modality Mapping. HCT-116 lymph node metastases animal models were obtained by subcutaneous injection of 3 × 10⁷ HCT-116 cells suspended in 50 μL of PBS via the right hind foot pad of nude mice; 30 days after inoculation, the mice with spherical hard lumps in their popliteal fossa were designated for our study. 22 Mice manifesting lymph node metastases were randomly divided into two groups (three mice for each group). Then, 50 μL of Hu-ink dispersion (5 mg/mL) or normal saline (NS) was injected into the primary tumors on the right hind foot pad of each mouse. Subsequently, PAI was conducted by VisualSonic Vevo 2100 LAZR system after 24 h. At the same time, sciatic lymph nodes and popliteal lymph nodes were slightly dissected and then photographed.

4.5. Photothermal Ablation of Popliteal Lymph Nodes. In this experiment, popliteal lymph nodes (the sentinel lymph nodes) were appointed as therapeutic target to elaborate the feasibility of PTT. A total of 20 mice exhibiting lymph node metastases were randomly divided into four groups (n = 5 per group). One group was injected with 50 μL of NS into the primary tumors on the right hind foot pad without laser irradiation. The second group was injected with 50 μL of NS into the primary tumors on the right hind foot pad and then irradiated with an 808 nm laser (1 W/cm², 5 min). The third group was injected with 50 μL of Hu-ink (5 mg/mL) into the primary tumors on the right hind foot pad and then irradiated with an 808 nm laser (1 W/cm², 5 min). Temperature changes were subsequently recorded using an infrared thermal camera. After 7 days, the mice were sacrificed through anesthesia and then the popliteal lymph nodes, heart, liver, spleen, lung, kidney, and brain tissue were removed for histopathological evaluation. At the same time, popliteal lymph nodes were weighed to further assess therapeutic efficacy.

4.6. Statistical Analysis. Unpaired Student’s t test was used for comparison between two groups, and one-way ANOVA with Fisher’s LSD was used for multigroup analysis. A probability (P) less than 0.05 was considered statistically significant. Results were expressed as mean ± standard deviation (SD) unless otherwise indicated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00993.

XRD pattern of Hu-ink; H&E staining of (a) brain, (b) heart, (c) lung, (d) spleen, (e) liver, and (f) kidney after Hu-ink-based photothermal therapy; photothermal conversion efficiency of Hu-ink and previous reported photothermal agents (Figures S1 and S2, Table S1) (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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