Effect of moxibustion combined with cisplatin on tumor microenvironment hypoxia and vascular normalization in Lewis lung cancer mice

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Research Article

Keywords: Moxibustion, lung cancer, chemotherapy, tumor microenvironment

Posted Date: January 16th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2473556/v1

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Abstract

Purpose
This study was developed to evaluate the effects of moxibustion on tumor microenvironmental hypoxia in a murine model of Lewis lung carcinoma (LLC).

Methods
In total, 30 female C57BL/6 mice were randomized into tumor (T), moxibustion (TM), chemotherapy (TC), and moxibustion + chemotherapy (TMC) groups (n = 6/group). A tumor model was established by implanting LLC cells into the right flank of each mouse. Animals in the TM group were subjected to moxibustion treatment at the ST36 (bilateral) and GV4 acupoints on the day after visible tumor formation (8 days after tumor injection). Moxibustion treatment was repeated every other day for 7 total treatments. Animals in the TC group were intraperitoneally injected with cisplatin (3 mg/kg) on day 3 after visible tumor formation (day 10 after modeling), and this treatment was repeated every 3 days for 4 total treatments. Animals in the TMC group underwent a combination of moxibustion and chemotherapy using the same conditions outlined above. After treatment was complete, ELISAs were used to measure serum concentrations of hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), and CD31, while the levels of the former three of these factors were assessed in tumor tissue samples via Western immunoblotting and immunohistochemical staining.

Results
Relative to the T model group, treatment in the TM, TC, and TMC groups resulted in varying reductions in tumor growth (P < 0.001 or P < 0.05), while tumor microenvironmental hypoxia was alleviated as evidenced by the downregulation of HIF-1α, VEGFA, and CD31 (P < 0.001 ~ P < 0.05).

Conclusion
The combination of moxibustion and cisplatin can alleviate intratumoral hypoxia, promote vascular normalization and slow LLC tumor growth in mice.

Introduction
Hypoxia is defined by a lack of access to a sufficient oxygen supply, and it is a common finding in the tumor microenvironment (TME), as the rapid uncontrolled growth of most solid tumors ultimately restricts the availability of oxygen to many of these malignant cells\(^1\). A hypoxic TME is also closely linked to the aggressive growth and metastatic progression of many cancers\(^2\). When tumor cells encounter hypoxic
conditions, they alter their transcriptional programs through mechanisms mediated by oxygen-sensing machinery, altering the expression of the key hypoxia signaling-associated hypoxia-inducible factors (HIFs) \(^3\). As hypoxia is an extremely common finding in solid malignant tumors, HIF activation is almost universal across tumor types. HIF-1 can promote the transcriptional activation of a series of target genes associated with proliferation, survival, glucose metabolism, angiogenesis, invasion, and metastasis such that it functions as the master regulator of cellular responses to hypoxic conditions\(^3\)–\(^5\). HIF-1\(^\alpha\) is a HIF-1 subunit, the stability of which is regulated by tissue oxygen levels. Under normal oxygen levels, a key proline in this protein is hydroxylated by proline hydroxylase (PHD), leading to its subsequent recognition by Hippel Lindau disease gene product (pVHL) and consequent molecular degradation. Under hypoxic conditions, however, PHD activity is reduced such that the HIF-1 \(\alpha\) subunit is not hydroxylated and HIF-1\(^\alpha\) can accumulate\(^6\). VEGF upregulation is associated with angiogenic activity under hypoxic settings and is promoter in an HIF-1\(\alpha\)-dependent fashion\(^5\). This HIF/VEGF signaling activity in response to hypoxia thereby promotes angiogenic activity that can facilitate metastatic progression\(^2\).

Moxibustion is a traditional Chinese medicine (TCM) practice that is common in many Asian nations. This noninvasive procedure entails the burning of the herb Artemisia vulgaris on or above specific acupoints, thereby warming the area and alleviating associated symptoms\(^7\). Moxibustion has been reported to inhibit tumor growth\(^8\), and to help reduce cancer-related fatigue\(^9\) and pain\(^10\), in addition to reducing radiotherapy and chemotherapy side effects\(^11\). The mechanistic basis for this activity, however, remains to be established. The curative efficacy of moxibustion is linked to warming stimulation\(^12\), which also promotes blood circulation, enhances the local blood supply, and mitigates the incidence of ischemia and hypoxia. Whether moxibustion conducted at specific acupoints can alleviate the ischemic hypoxic TME and thereby reduce associated tumor growth warrants further study and has important implications for the clinical treatment of cancer patients.

Moxibustion is commonly applied in cancer patients following surgery or concurrently with or following chemoradiotherapy\(^13\). Accordingly, this study was developed to assess the combined impact of moxibustion and cisplatin chemotherapy on HIF-1\(^\alpha\) and VEGF expression in the TME using a murine model of Lewis lung carcinoma (LLC). These experiments were conducted based on the hypothesis that moxibustion can alleviate intratumoral hypoxia and promote vascular normalization, thereby reducing the aggressive growth of these malignancies.

**Materials And Methods**

**Reagents**

This study utilized Cisplatin (P4394) from Sigma-Aldrich (USA), high-glucose DMEM (10-013-CVRC) and Penicillin-streptomycin (ZY90307) from Corning, 0.25% EDTA (25200-056) and Fetal Bovine Serum (FBS) (10099141) from Gibco, and ELISA kits specific for HIF-1\(^\alpha\) (ab275281), VEGF (ab209882), and CD31
(ab204527) from Abcam. Antibodies specific for HIF-1α (ab228649), VEGFA (ab51745), CD31 (ab281583), Ki67 (ab16667), and beta Actin (ab8226) were also from Abcam.

## Cell culture

LLC cells from the Cell Bank of Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences) were cultured in DMEM containing 10% FBS and penicillin-streptomycin in a 37°C humidified 5% CO₂ incubator in the Department of Acupuncture and Tuina of Shanghai University of Traditional Chinese Medicine.

## Tumor model establishment

Thirty female C57BL/6 mice (6–8 weeks old, 18–22 g) from Beijing Wei Tong LiHua Laboratory Animal Technology Co., Ltd., Beijing, China were housed under controlled conditions (24°C, 40–50% humidity, 12 h light/dark cycle) with ad libitum food and water access. The Guidelines for the Care and Use of Laboratory Animals published by the Ministry of Science and Technology of China were used to conduct all animal studies, which received approval from the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine ( Permit Number: PZSHUTCM220711024).

After a 1-week acclimatization period, mice were subcutaneously implanted in the right flank with $1 \times 10^5$ LLC cells in 100 µl of PBS. The following day, tumors were measured with digital calipers and the volume (mm$^3$) of these tumors were measured with the following equation: $(\text{length} \times \text{width}^2)/2$. On day 21, mice bearing LLC tumors were weighed prior to euthanasia following deep anesthetization with 4% isoflurane in oxygen. Tumors were subsequently excised, weighed to allow for the calculation of net body weight (body weight-tumor weight), and imaged alongside a ruler. We also assessed it by Tumor Growth Inhibition Rate (TGI). TGI was calculated as follows: $\text{TGI, } \% = (V_T - V_{TM} \text{ or } V_{TC} \text{ or } V_{TMC}) \times 100/V_T$, where $V_T$, $V_{TM}$, $V_{TC}$, and $V_{TMC}$ mean tumor volume (mm$^3$) in T, TM, TC and TMC groups, respectively.

## Moxibustion and Cisplatin treatment

At one week after tumor implantation, tumor-bearing mice were randomized into control (Ctrl), tumor (T), cisplatin (TC), moxibustion (TM), and cisplatin + moxibustion (TMC) groups (n = 6/group). Mice in the Ctrl and T groups were handled for 30 minutes per day. Mice in the T group underwent tumor implantation but no other treatments, and were handled for 30 minutes on the second day after modeling. On the day after visible tumor formation (8 days after tumor implantation), mice in the TM and TMC groups underwent moxibustion treatment of the bilateral "Housanli" acupoint (ST36, 2 mm lateral to the anterior tubercle of the tibia in the anterior tibial muscle and 4 mm distal to the bottom of the knee joint ) and the "Mingmen" acupoint (GV4, on the subspinous process of the second lumbar spine on the middle dorsal line). Stimulation was performed for 10 minutes per acupoint once every other day for 14 days (7 rounds of stimulation). Briefly, mice were immobilized using a homemade moxibustion fixer, and the distance between the skin overlying the target acupoints and the lit end of the moxa sticks (length: 120 mm, diameter: 12 mm, Nanyang Hanyi Moxibustion Technology Development Co., Ltd., China) was controlled to within 2–3 cm. The distance was adjusted using an electronic temperature meter to maintain the
temperature in the 40 ± 2°C range. Treatment was performed at room temperature, and control mice were placed in the moxibustion fixer under identical conditions without further manipulation.

**ELISAs**

Following centrifugation serum samples were stored at -80°C. Commercial ELISA kits were used to quantify serum HIF-1α, VEGF and CD31 concentrations based on provided instructions.

**Western immunoblotting**

Lysis buffer supplemented with a protease inhibitor cocktail (Pierce) was used to extract the protein from tumor tissue samples. These lysates were then separated via 10% SDS-PAGE, transferred to PVDF membranes (Millipore, MA, USA), and the blots were blocked for 60 min using 5% non-fat milk at room temperature. Blots were then probed overnight with antibodies specific for VEGF, CD31, or HIF-1α at 4°C, followed by treatment with secondary antibodies conjugated to HRP. A Western blotting detection system was subsequently used in accordance with provided direction to detect protein bands.

**Immunohistochemical staining**

Paraffinized tissue sections (4–5µm) were used to conduct immunohistochemical (IHC) staining. Briefly, these sections were deparaffinized, rehydrated, blocked, and probed overnight with antibodies specific for VEGF, CD31, Ki67, or HIF-1α at 4°C prior to probing with streptavidin HRP. DAB (Santa Cruz Biotechnology, CA) was then used for color development and slides were counterstained with hematoxylin prior to imaging with an Olympus IX81 microscope.

**Statistical Analysis**

GraphPad Prism 8 was used to analyze all data, which are reported as the ±s. Results were compared with two-sided Student’s t-tests or one-way ANOVAs, with $P<0.05$ as the threshold for statistical significance.

**Results**

**Moxibustion potentiates the inhibitory effects of cisplatin on the in vivo growth of LLC tumors**

For this study, C57BL/6 mice were leveraged to model the impact of cisplatin and/or moxibustion on lung cancer treatment outcomes. Significant differences in tumor growth were observed when comparing the T model group to the three treatment groups (TM, TC, and TMC). Specifically, beginning on day 13, tumor growth in the three treatment groups began to slow with a clear reduction in tumor size relative to the T group that persisted until the end of treatment. This effect was most pronounced in the TMC group (Fig. 1a). Significant differences in tumor size at study end were also observed among these three groups (Fig. 1b). Tumor weight values in the three treatment groups were significantly reduced relative to the T group, and this effect was most significant in the TMC group (Fig. 1c). Lastly, relative to the T group,
tumor volumes in the TM, TC, and TMC groups were reduced such that the respective calculated degrees of tumor inhibition were 42.44%, 49.36%, and 58.72% (Table 1, Fig. 1d).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Before therapy (mm³)</th>
<th>After therapy (mm³)</th>
<th>Inhibitory rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>6</td>
<td>53.341</td>
<td>798.368</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>6</td>
<td>55.589</td>
<td>654.394*</td>
<td>42.44%</td>
</tr>
<tr>
<td>TC</td>
<td>6</td>
<td>56.108</td>
<td>532.642*</td>
<td>49.36%</td>
</tr>
<tr>
<td>TMC</td>
<td>6</td>
<td>56.172</td>
<td>354.706**</td>
<td>58.72%</td>
</tr>
</tbody>
</table>

Compared with T group, *P < 0.05, **P < 0.01.

Ki67 staining was next used to detect tumor cell proliferation based on brown staining, revealing that tumor cells expressed high levels of Ki67 but that such expression was markedly reduced in the TM, TC, and TMC groups following the 14-day treatment period (Fig. 1e-f).

**Moxibustion and cisplatin alter relative serum cytokine levels**

Next, ELISAs were used to quantify serum HIF-1α, VEGF, and CD31 concentrations, revealing that all three were significantly upregulated in the T group relative to Ctrl mice. After treatment for 14 days, however, these three proteins were significantly downregulated in the TM, TC, and TMC groups, with significant declines in HIF-1α and VEGF levels in the TMC group relative to the TC and TM groups (Fig. 2a-c).

**Moxibustion and cisplatin synergistically suppress LLC tumor angiogenic activity**

Western immunoblotting revealed that there were marked reductions in CD31, HIF-1α, and VEGFA levels in the TM, TC, and TMC groups relative to the T group, with these reductions also being significant in the TMC group as compared to the TM and TC groups (Fig. 3a-d). Moxibustion, cisplatin, and the combination of the two thus exhibited beneficial activity that was more pronounced when mice underwent moxibustion + cisplatin treatment. Consistently, IHC staining revealed significant decreases in intratumoral HIF-1α, VEGFA, and CD31 levels in the TM, TC, and TMC groups relative to the T group (Fig. 4a-d).

**Discussion**
The present results highlight the therapeutic value of moxibustion, cisplatin, and the combination of the two given the significant suppression of tumor growth in the TM, TC, and TMC groups as compared to the T model group. Consistently, the tumor weight and volume values in these three treatment groups were significantly lower and there was a concomitant drop in the levels of Ki67, which serves as a biomarker of tumor cell proliferative activity\(^{18}\). This indicates that both moxibustion and cisplatin are capable of constraining target tumor growth through the suppression of proliferation, in line with prior evidence\(^{8,19,20}\). Cisplatin is a broad-spectrum antineoplastic agent that is widely used in clinical settings to suppress the growth of lung cancer and many other types of malignancies\(^{21}\). While effective, the clinical application of cisplatin is limited by its side effects, inherent toxicity\(^{22}\), and issues of drug tolerance\(^{23}\). Moxibustion offers the combined benefits of suppressing tumor growth \(^{8,19,20}\) while also alleviating chemotherapeutic drug side effects\(^{13}\). These data thus indicate that moxibustion can suppress lung tumor growth. Strikingly, moxibustion and cisplatin treatment can synergize to improve therapeutic outcomes.

These results demonstrated that there were significant increases in serum HIF-1\(\alpha\) and VEGF levels in these LLC tumor-bearing mice. This supports the hypoxic TME associated with these solid tumors as has been reported previously\(^1,2,24\). This intratumoral hypoxia can increase HIF-1\(\alpha\) expression, thereby promoting VEGF upregulation and ultimately promoting angiogenic activity that is conducive to further tumor growth\(^{25–27}\). This malignant activity is also directly related to a range of adverse effects including metastatic progression and therapeutic drug resistance\(^{6,8,9}\). Angiogenesis is controlled by angiogenic factors, with one of the most important being VEGF\(^{28}\). CD31 is highly expressed on the surface of endothelial cells and is well established as a marker for the monitoring of vessel density in malignant tissue\(^{29}\). VEGF and CD31 are well defined markers of angiogenesis. Accordingly, efforts to alleviate intratumoral hypoxia and promote vascular normalization are central to the effective treatment of cancer.

Following moxibustion, cisplatin, or combined treatment, serum and tumor HIF-1\(\alpha\) and VEGF levels fell significantly in these mice relative to those in the T model group. Strikingly, significantly decreased serum HIF-1\(\alpha\) expression and intratumoral HIF-1\(\alpha\) and VEGF levels were evident in the TMC group as compared to the TM and TC groups. Here, mice in the three treatment groups exhibited significantly lower serum and intratumoral CD31 expression as compared to mice in the T group. Notably, CD31 levels in these target tumors were significantly lower in the TMC group as compared to the TM and TC groups. Cisplatin and moxibustion can thus interfere with vascular development within tumors by counteracting the hypoxic nature of the TME, with these two therapies effectively synergizing to better improve this anti-hypoxic effect. Cisplatin is a broad-spectrum antitumor chemotherapeutic agent, and its efficacy in this LLC model system is presumably the result of its ability to directly kill target tumor cells and to thereby slow overall tumor growth. While moxibustion lacks the ability to directly kill malignant cells, the associated warming stimulation and the promotion of blood circulation may account for its anti-hypoxic efficacy\(^{20}\). Despite potential concerns that moxibustion-mediated enhancement of tumor oxygenation and the associated blood supply might ultimately accelerate tumor progression, the moxibustion treatment of the ST36 and GV4 acupoints in these LLC tumor-bearing mice did not have such an effect,
instead significantly suppressing tumor growth. Overall, moxibustion thus exhibits excellent potential for its application as a treatment that specifically targets the hypoxic TME.

**Conclusions**

In summary, these results demonstrate that the combination of moxibustion and cisplatin can effectively suppress tumor growth through the alleviation of intratumoral hypoxia. All three treatment groups in this study exhibited reductions in HIF-1α, VEGF, and CD31 levels consistent with the suppression of angiogenesis, and these results thus confirm that combining cisplatin with moxibustion can more robustly inhibit HIF-1α expression within the TME, thereby disrupting new blood vessel formation and inhibiting tumor growth. The combination of these two therapeutic interventions in a clinical setting may thus represent a promising means of improving lung cancer patient outcomes.

**Declarations**

**Funding:** This research is funded in part by grants from the National Natural Science Foundation of China (grant number 82274640) and the National Natural Science Foundation of Shanghai (grant number 21ZR1463300).

**Conflicts of interest:** All authors declare that they have no conflict of interests.

**Availability of data and material:** The data that support the findings of this study are available from the corresponding author upon reasonable requested.

**Code availability:** Not applicable.

**Authors’ contributions:** Conception and design by J.W. The development of the method was done by J.W and H.M. Animal experiments were done by N.M., X.W. and C.W. Data analysis was performed and interpreted by J.W. and N.M. The manuscript was written by H.M. and N.M. The reviewed and revised manuscript was written by J.W. and H.M..

**Ethics approval:** Approval from the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Permit NumberPZSHUTCM220711024).

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**References**


Figures
Figure 1

The effect of combinatorial moxibustion and cisplatin treatment on tumor growth in LLC bearing mice. (a) Tumor growth curve analysis of the LLC-bearing mice in each group at the corresponding time points. Compared with T group, *P < 0.05, **P < 0.01, ***P < 0.001 (n = 6). (b) Images of the dissected tumors from each group (n=6). (c) Tumor weight in vitro after 14 days of treatment. Compared with T group, *P < 0.05, **P < 0.01 (n= 6). (d) Tumor volume in vitro after 14 days of treatment. Compared with T group, **P < 0.01 (n=6). (e-f) Expression of Ki67 in tumor cells. Compared with T group, ***P < 0.001 (n=6).
Figure 2

Expression levels of relative serum cytokine in peripheral blood. (a) HIF-1α expression level in serum. Compared with T group, ***$P<0.001$; compared with Ctrl group, ###$P<0.001$; compared with TM group, $$$P<0.001$; compared with TC group, ΔΔΔ$P<0.001$ (n=6). (b) VEGF expression level in serum. Compared with T group, **$P<0.01$, ***$P<0.001$; compared with Ctrl group, ###$P<0.001$ (n=6). (c) CD31 expression level in serum. Compared with T group, **$P<0.01$, ***$P<0.001$; compared with Ctrl group, ##$P<0.01$ (n=6).
Figure 3

Moxibustion inhibited HIF-1, VEGFA and CD31 protein levels in tumor tissue. (a-d) Representative blots showing HIF-1, VEGFA and CD31 protein levels. Compared with T group, \( *P<0.05 \), \( **P<0.01 \), \( ***P<0.001 \); Compared with TM group, \( §§P<0.01 \); Compared with TC group, \( ΔΔP<0.01 \) (n=6).
Figure 4

Moxibustion inhibited HIF-1α, VEGFA and CD31 expression in tumor cells. (a) Representative tumor cross-sections from T, TM, TC, and TMC. The sections were stained with antibodies against HIF-1α, VEGFA and CD31. 400×. (b-d) Representative Immunohistochemistry showing levels of HIF-1, VEGFA and CD31 in tumor cells. Compared with the T group, *P<0.05, **P<0.01, ***P<0.001 (n =6).