Pharmacological study on the molecular mechanism of crocodile blood in the treatment of lung cancer

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Abstract: Lung cancer is the most prevalent malignant tumor in terms of both incidence and mortality globally. Current therapeutic approaches still struggle to overcome clinical challenges such as drug resistance, metastasis, and immune evasion. This study systematically elucidates the multidimensional molecular mechanisms of crocodile blood in treating human lung cancer from a pharmacological perspective. At the tumor cell level, crocodile blood inhibits lung cancer cell proliferation by regulating cell cycle arrest, activating mitochondrial apoptosis pathways, and interfering with the EGFR/MAPK signaling axis. At the invasion and metastasis level, it suppresses metastasis by reversing epithelial-mesenchymal transition, downregulating MMP2/MMP9 activity, and blocking VEGF/VEGFR2 angiogenic signaling. At the immune microenvironment level, it enhances anti-tumor immune responses by inducing M1 macrophage polarization, boosting NK/CTL cytotoxicity, and reversing Treg-mediated immune suppression. The study demonstrates that crocodile blood exerts anti-lung cancer effects through "multi-target integrated regulation," providing a pharmacological basis for its development as an adjuvant therapy for lung cancer.

Keywords: crocodile blood; lung cancer; molecular mechanism

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Foreword

Lung cancer is one of the most prevalent and lethal malignancies worldwide, posing a severe threat to human health. Despite continuous advancements in surgical interventions, chemoradiotherapy, targeted therapy, and immunotherapy, the overall prognosis remains suboptimal due to the high heterogeneity of lung cancer, its invasive and metastatic capabilities, and immune evasion mechanisms, with a five-year survival rate of less than 20%. Consequently, the search for novel anti-lung cancer drugs or adjuvant therapies derived from natural products has become a research hotspot. Crocodile blood, a traditional medicinal substance with a long history of use in Africa and Southeast Asia, has been found by modern studies to be rich in bioactive peptides, fatty acids, and small-molecule compounds, exhibiting multiple biological activities such as antibacterial, anti-inflammatory, and antitumor effects.

1 Molecular Mechanisms of Crocodile Blood in Inhibiting Lung Cancer Cell Proliferation and Inducing Apoptosis

1.1 Regulation of key cell cycle proteins (Cyclin D1/CDKs) to block lung cancer cell division

The dysregulation of cell cycle checkpoints is a key feature of uncontrolled proliferation in lung cancer cells. Studies have shown that treatment with crocodile blood bioactive peptides can induce typical G0/G1 phase arrest in human non-small cell lung cancer cell lines (e.g., A549, H1299). The molecular mechanism primarily involves alterations in the expression profile of phase proteins. Crocodile blood extract significantly downregulates the expression levels of Cyclin D1, Cyclin E1, and their coupled cyclin-dependent kinases 2 (CDK2) and CDK4, while upregulating the protein expression of endogenous CDK inhibitors p21Cip1 and p27Kip1. This regulatory effect disrupts the kinase activity of the Cyclin-CDK complex, leading to impaired phosphorylation of retinoblastoma protein (Rb). The low-phosphorylated Rb continuously binds to the transcription factor E2F, thereby inhibiting the transcription of S-phase-related genes and arresting cell division at the G1 checkpoint.

1.2 Activation of mitochondrial pathways (caspase cascade, Bcl-2 family regulation) induces apoptosis

Mitochondrial pathway is one of the core pathways of apoptosis. Crocodile blood extract can effectively destroy the mitochondrial membrane potential of lung cancer cells and open the apoptosis process. It was found that after treatment with crocodile blood, the level of reactive oxygen species in lung cancer cells increased significantly, leading to the opening of mitochondrial permeability transition pores and the release of cytochrome C from mitochondria to cytoplasm. In the cytoplasm, cytochrome C binds to apoptotic protease activator-1, recruits and activates Caspase-9, and then initiates the Caspase cascade reaction, ultimately activates the effector molecule Caspase-3, lyses DNA repair enzymes, and leads to DNA fragmentation. In this process, crocodile blood also precisely regulated the proportion of Bcl-2 family proteins, which was manifested by significantly down-regulating the expression of anti-apoptotic protein Bcl-2 and up-regulating the expression of pro-apoptotic protein Bax, resulting in increased Bax / Bcl-2 ratio and further loss of mitochondrial membrane potential.

1.3 Intervention in EGFR/RAS/MAPK signaling pathway to inhibit abnormal proliferation signaling

Epidermal growth factor receptor and its downstream RAS / MAPK pathway are often abnormally activated due to mutations in non-small cell lung cancer, driving malignant tumor proliferation. The active ingredients in crocodile blood can cut off the conduction of proliferation signals by interfering with this pathway. Western Blot results showed that crocodile blood extract could inhibit the phosphorylation level of EGFR in a dose-dependent manner, which in turn affected the activation of its downstream adaptor protein. More importantly, crocodile blood significantly inhibited the phosphorylation levels of RAS downstream key kinases RAF, MEK1 / 2 and ERK1 / 2. Since ERK1 / 2 can phosphorylate a variety of nuclear transcription factors to promote the expression of proliferation-related genes, its activity inhibition directly leads to cell proliferation inhibition.

Table 1 Effects of Crocodile Blood Extract on Protein Phosphorylation Levels in the EGFR/RAS/MAPK Pathway of A549 Cells

peer group	concentration (μ g/mL)	p-EGFR / EGFR	p-ERK1/2 / ERK1/2	p-MEK / MEK
control group	0	0.88±0.06	0.92±0.05	0.79±0.06
Crocodile blood low dose group	50	0.65±0.05	0.71±0.06	0.61±0.05
Crocodile blood medium dose group	100	0.41±0.04	0.43±0.04	0.38±0.04
Crocodile blood high dose group	200	0.22±0.03	0.19±0.03	0.21±0.03

2 Molecular Mechanisms of Crocodile Blood in Preventing Lung Cancer Invasion and Metastasis

2.1 Inhibition of epithelial-mesenchymal transition (EMT)-related transcription factors (Snail, Twist) expression

Epithelial-mesenchymal transition is a key step in the migration and invasion of lung cancer cells, which is characterized by the loss of epithelial markers and the acquisition of mesenchymal markers. Crocodile blood extract can effectively reverse this process. The molecular mechanism study showed that crocodile blood treatment significantly inhibited the mRNA and protein expression levels of EMT-induced transcription factors Snail and Twist. The down-regulation of Snail and Twist not only inhibited the transcription of E-cadherin gene, but also increased the expression of E-cadherin on the surface of lung cancer cells and reconstructed the intercellular connection. At the same time, the expression of mesenchymal markers N-cadherin and vimentin decreased correspondingly, the cytoskeleton rearranged and the migration ability weakened. As shown in Table 4, after intervention with crocodile blood, the expression of E-cadherin in lung cancer cells increased significantly, while the expression of Snail and vimentin decreased in a dose-dependent manner. It was confirmed that crocodile blood blocked the EMT process at the molecular level by inhibiting the Snail / Twist transcription factor network and reduced the invasion potential of lung cancer cells.

Table 2 Effects of crocodile blood extract on EMT markers and transcription factor expression in A549 cells (¯±s, n=3)

peer group	concentration (μ g/mL)	E-cadherin (relative expression level)	Vimentin (relative expression level)	Snail(relative expression)
control group	0	0.23±0.04	0.89±0.06	0.82±0.05
Crocodile blood low dose group	50	0.41±0.05	0.71±0.05	0.63±0.06
Crocodile blood medium dose group	100	0.62±0.06	0.48±0.04	0.41±0.04
Crocodile blood high dose group	200	0.78±0.07	0.29±0.03	0.22±0.03

2.2 Downregulation of matrix metalloproteinase (MMP2/MMP9) activity to remodel the tumor microenvironment

Tumor cells break through the basement membrane and infiltrate into surrounding tissues, which is inseparable from the degradation of extracellular matrix. This process is mainly mediated by the matrix metalloproteinases family. Crocodile blood extract could significantly reduce the gelatinase activity in the supernatant of lung cancer cell culture. Further molecular detection revealed that crocodile blood not only inhibited the protein expression of MMP2 and MMP9, but also disrupted the balance of MMPs / TIMPs by up-regulating the expression of tissue inhibitor of metalloproteinase-1 and TIMP-2. This dual regulation effectively reduces the degradation rate of extracellular matrix in the tumor microenvironment and limits the invasion range of lung cancer cells.

2.3 Inhibition of tumor angiogenesis (VEGF/VEGFR2 signaling axis) to reduce nutrient supply

The formation of tumor neovascularization provides the necessary oxygen and nutrition for the rapid proliferation and distant metastasis of lung cancer cells, and becomes a channel for metastatic cells to enter the circulatory system. Crocodile blood showed significant anti-angiogenic activity. In vitro angiogenesis experiments, the ability of crocodile blood-treated human umbilical vein endothelial cells to form tubular structures on Matrigel was significantly reduced. The molecular mechanism mainly involves the blocking of VEGF / VEGFR2 signaling axis. Crocodile blood can reduce the secretion of VEGF in lung cancer cells under hypoxic conditions and directly inhibit the phosphorylation of VEGFR2 receptor on endothelial cells. The inhibition of VEGFR2 activity further blocked the activation of its downstream PI3K / Akt and PLCγ / ERK1 / 2 signaling pathways, thereby inhibiting the proliferation, migration and tube-like differentiation of endothelial cells.

3 Molecular Mechanisms of Crocodile Blood in Regulating the Immune Microenvironment of Lung

Cancer

3.1 Activation of macrophage polarization to M1 phenotype enhances anti-tumor immune response

Tumor-associated macrophages are the largest number of immune cell populations in the immune microenvironment of lung cancer, and their functional status depends on the polarization direction. M2 macrophages promote tumor progression, while M1 macrophages have strong anti-tumor activity. Crocodile blood extract can effectively reshape the polarization balance of macrophages. In vitro co-culture experiments showed that the morphology of macrophages treated with crocodile blood changed from long spindle shape to paving stone shape, showing typical M1 activation characteristics. The molecular mechanism study found that crocodile blood up-regulated the expression of M1-specific transcription factor IRF5 and inhibited the phosphorylation of M2-related transcription factor STAT3 by activating the TLR4 / NF-κB signaling pathway. As shown in Table 7, after crocodile blood intervention, the proportion of M1 marker CD86 positive cells increased significantly, and the secretion of pro-inflammatory cytokines TNF-α and IL-12 increased in a dose-dependent manner ; the expression of M2 marker CD206 decreased, and the secretion of immunosuppressive factors IL-10 and TGF-β decreased. This polarization remodeling allows macrophages to restore their phagocytic activity against lung cancer cells and enhance their antigen-presenting ability, thereby activating subsequent specific immune responses.

3.2 Enhancement of the cytotoxic functions of natural killer (NK) cells and cytotoxic T lymphocytes (CTL)

NK cells and CTLs are the core forces to perform anti-tumor immune effects and are directly responsible for identifying and eliminating lung cancer cells. Crocodile blood can synergistically enhance the killing activity of these two effector cells. On the one hand, the active peptides in crocodile blood can directly bind to the activating receptors NKG2D and NKp46 on the surface of NK cells, enhance their activation signal transduction, and promote the release of perforin and granzyme B. On the other hand, crocodile blood indirectly enhanced the tumor antigen-specific response of CTL by improving the maturation and antigen presentation function of dendritic cells. As shown in Table 8, after treatment with crocodile blood, the killing rate of NK cells in the co-culture system to A549 lung cancer cells was significantly increased, and the concentration of IFN-γ in the culture supernatant was also significantly increased. For CTL, crocodile blood can up-regulate the proportion of effector memory T cells and enhance the expression of their surface activation markers CD69 and CD107 a. Flow cytometry showed that the intracellular staining intensity of effector molecules granzyme B and perforin was significantly enhanced, indicating that crocodile blood enhanced the tumor killing function of CTL from two aspects of activation and effect, forming a multi-dimensional immune clearance of lung cancer cells.

3.3 Reversing the regulatory T cell (Treg)-mediated immunosuppressive state

Treg cells accumulate in the microenvironment of lung cancer, and form a strong immunosuppressive barrier by secreting inhibitory cytokines and competitively consuming IL-2, hindering the function of effector cells. Crocodile blood can effectively reverse this inhibitory state. It was found that after the intervention of crocodile blood, the proportion of Foxp3 + CD25 + Treg cells in the tumor microenvironment decreased significantly, and its function was also inhibited. The molecular mechanism study showed that crocodile blood weakened its negative regulation effect on antigen-presenting cells by down-regulating the CTLA-4 molecules highly expressed on the surface of Treg cells. At the same time, crocodile blood can also inhibit the phosphorylation of STAT5 in Treg cells and reduce the secretion of its effector molecules TGF-β and IL-35.

4 Conclusion

Crocodile blood plays an anti-lung cancer role through multi-dimensional and multi-target molecular mechanisms. At the level of tumor cells, its active ingredients can regulate the expression of cyclins to induce G0 / G1 phase arrest, activate mitochondrial pathway and Caspase cascade to promote apoptosis, and interfere with the EGFR / RAS / MAPK signaling axis to inhibit abnormal proliferation. At the level of invasion and metastasis, it reverses epithelial-mesenchymal transition by inhibiting Snail / Twist transcription factors, down-regulates MMP2 / MMP9 activity to reshape the tumor microenvironment, and blocks the VEGF / VEGFR2 signaling axis to inhibit tumor angiogenesis ; at the level of immune microenvironment, it can induce macrophage polarization to M1 type, enhance the killing function of NK cells and cytotoxic T lymphocytes, and reverse the immunosuppressive state mediated by Treg cells. The ' multi-target integrated regulation ' of crocodile blood provides a solid pharmacological basis for its development as an adjuvant therapy or combination therapy for lung cancer. However, in the future, it is still necessary to further clarify its active material basis and promote clinical transformation through component refinement, structural modification and systematic in vitro and in vivo studies.

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