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Effects of Geraniin on Platelet Aggregation and Interactions between Platelets and Neutrophils

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[**Abstract**] Objective To investigate the effects of geraniin on platelet aggregation and platelet-neutrophil interactions. Methods Platelet aggregation, *in vitro* and *ex vivo*, was determined by use of Born's method, and the binding of thrombin-stimulated platelets to neutrophils was observed based on the rosette assay. Intracellular calcium concentration of platelets was measured by using Fura-2-AM. Results Geraniin *in vitro* significantly inhibited arachidonic acid (AA)-, adenosine diphosphate (ADP)-, or platelet activating factor (PAF)-induced platelet aggregation, in a concentration-dependent manner. The medium inhibitory concentrations (IC_{50}) were 2.4, 0.4 and 1.1 μmol/L, respectively. Intragastric geraniin at 5 mg/kg markedly suppressed platelet aggregation induced by AA, ADP, or PAF. Geraniin decreased the total rise of $[Ca^{2+}]_i$, Ca^{2+} release, and Ca^{2+} influx, in a concentration-dependant manner. The IC_{50} values were 71.9, 84.9, and 62.9 μmol/L, respectively. Geraniin decreased the binding of thrombin-stimulated platelets to neutrophils, and significantly inhibited washed platelet aggregation stimulated by fMLP-activated neutrophils. The IC_{50} values were 3.2 and 10.2 μmol/L, respectively. Conclusion It is suggested that geraniin inhibited platelet aggregation *in vitro* and *ex vivo*, decreased the calcium mobilization of platelets, and suppressed the interactions between platelets and neutrophils.

[**Key words**] Geraniin; Platelet aggregation; Calcium; Platelet-neutrophil interactions

Previously, platelets were considered to be the main cells involved in the pathogenesis of thrombosis, whereas only a minor role was attributed to neutrophils. However, many recent investigations realized that neutrophils not only participate in thrombus formation but may also exert a direct action on the extension of myocardial infarction by releasing several cytotoxic factors[1]. The interactions of platelet and neutrophil may be one of the key factors in the development of thromboembolic diseases [2]. It is more valuable, therefore, to develop an antithrombotic drug especially at an angle of influencing multiple cellular interactions. Geraniin was extracted and isolated from *Phyllanthus urinaria*, a Chinese medicinal herbal plant rich in Yunnan Province. In our previous study, geraniin was found to show anti-osteoporotic effect due to its inhibition of osteoclastic bone resorption [3]. Interestingly, geraniin showed potent inhibition of platelet aggregation in the *in vitro* screening of antiplatelet agents. In the present study, the effects of geraniin were investigated on arachidonic acid (AA)-, adenosine diphosphate (ADP)-, or platelet activating factor (PAF)-induced platelet aggregation *in vitro* and *ex vivo*, platelet calcium mobilization, platelet-neutrophil adhesion, and washed platelet aggregation induced by N-formyl-methiongl-leucyl-phenylalanine (fMLP)-activated neutrophils.

1 Materials and Methods

1.1 Animals

Healthy rabbits weighing 2.0-3.0 kg, were offered

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by the Experimental Animal Center of Kunming Medical University (SCXK2009008). The protocol of this study was approved by the Ethics Committee of Kunming Medical University.

1.2 Drugs and chemicals

Geraniin was extracted from *Phyllanthus urinaria*, a Chinese medicinal herbal plant rich in Yunnan Province of China (purity: 99%) (Fig.1). It was dissolved in dimethyl sulphoxide (DMSO), pH 7.0. Crystalline aspirin was purchased from Sigma Chemical Co. It was dissolved in 100 mmol/L Na_2CO_3 before use. ADP, AA, PAF, human thrombin, Fura-2-AM, and fMLP were all from Sigma Chemical Co.ADP, AA, and PAF were dissolved in phosphate buffer solution (PBS), 100 mmol/L Na_2CO_3, and Tris-NaCl buffer solution containing 0.25% bovine serum albumin, respectively. fMLP was dissolved in DMSO.

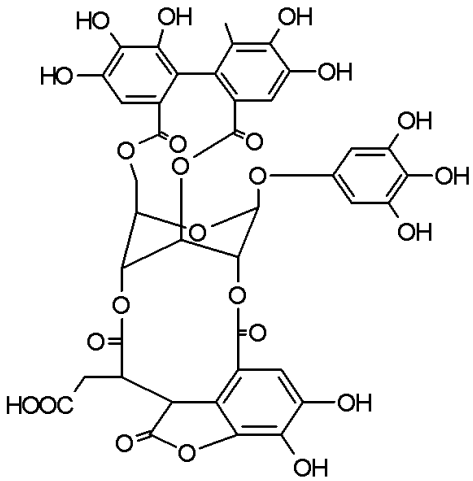

Fig. 1 Structure of Geraniin

1.3 Preparation of platelets

Blood sample from rabbit carotid artery was collected into plastic tubes, anticoagulated with 2.7% edetic acid (for the binding of platelets to neutrophils) or 3.8% sodium citrate acid (for platelet aggregation). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by the centrifuging the blood at 180 × *g* and 1 000 × *g*, respectively, for 10 min. PRP was further spun to pellet platelets at 1 000 × g for 10 min. Platelet pellets were washed three times and resuspended in PBS (containing 1.0 % bovine serum albumin and 1 mmol/L $CaCl_2$). Cell viability by Typan blue exclusion was above 95% and cell counter was adjusted to 10^8 cell/mL.

1.4 Preparation of neutrophils

Neutrophils were isolated from the remaining blood by dextran sedimentation and followed by Ficoll-Hypaque (specific density 1.077 g/mL) and hypotonic lysis of erythrocytes. The cell pellet was resuspended in an erythrocyte lysis buffer composed of 155 mmol/L NH_4Cl, 2.96 mmol/L $KHCO_3$, and 3.72 mmol/L edetic acid. The tube was gently inverted and after 5 min the suspension was centrifuged at 350 × *g* for 10 min, and the cell pellet was washed in PBS lacking calcium; then resuspended in Hanks' solution containing 1 mmol/L $CaCl_2$. Cells were adjusted to a count of 2 × 10^6 cell/mL. Cells prepared in this manner contained 98 % neutrophils and were 96% viable.

1.5 Platelet aggregation

1.5.1 Platelet aggregation *in vitro* Platelet aggregation in PRP was measured as described by Born[4]. The maximal aggregation was monitored (final concentration of ADP 3 μmol/L, AA 0.35 mmol/L, and PAF 7.2 nmol/L) in a Lumiaggregometer. Percentage inhibition by drugs was calculated by use of the following equation:

$$\text{Inhibition of aggregation}\ (\%) = \frac{A - B}{A} \times 100$$

Where A is the maximum change of tubidity when the control (saline) is added and B is the maximum change of tubidity when the drug (geraniin or aspirin) is added.

1.5.2 Platelet aggregation *ex vivo* Rabbits were randomly divided into 3 groups of six. Group A was given intragastrically the same volume of saline. Group B was administered intragastrically 5 mg/kg geraniin, and group C 5 mg/kg aspirin. PRP and PPP were prepared before administration and at 60, 120, 180, and 240 min after administration, respectively. Platelet aggregation induced by ADP, AA or PAF was monitored as in vitro test, respectively.

1.6 Measurement of cytosolic Ca^{2+}

The above prepared washed platelets were then loaded with 3 μmol/L Fura-2-AM by incubating the suspension at 37 ℃ for 45 min. The external calcium was adjusted by addition of 1 mmol/L $CaCl_2$ or egtazic acid, respectively. Intracellular Ca^{2+} concentration of platelets was measured by using Fura-2-AM[5] with a spectrofluorophotometer (Model RF-5000, Shimazh-

u，Japan） at 37 ℃ and magnetically stirred.

AA（200 μmol/L）–stimulated rise in $[Ca^{2+}]i$ was measured separately in the presence of 1 mmol L $CaCl_2$ or egtazic acid. The rise in $[Ca^{2+}]i$ in the presence of $CaCl_2$ 1 mmol/L represents a combination of Ca^{2+} release and influx of Ca^{2+}. The rise in the presence of egtazic acid 1 mmol/L reflects Ca^{2+} release. The difference between these two measurement reflects the influx of external Ca^{2+}.

1.7 Rosette assay

The method of Hamburger[6] was modified. Briefly, 50 μL aliquots of platelet suspension were placed in microtiter wells and exposed to 10 μL of 1.2 U/mL human thrombin（final concentration: 0.2 U/mL） for 15 min at room temperature without stirring. The platelets were then fixed with 50 μL of 2% paraformaldehyde for 30 min at room temperature. When required, 50 μL of 0.9% saline or drug solution was added before thrombin activation. Platelets were washed three times with PBS and resuspended in 50 μL PBS. Then 100 μL of neutrophils was added to the platelet suspension and incubated for 30 min at 4 ℃ under rocking condition. One hundred neutrophils were scored for the presence （two or more platelets per neutrophil） or absence （zero or one platelet per neutrophil） of platelets. Neutrophils bearing two or more platelets were thus defined as rosettes. For each assay, done in triplicate, the rosetting score was assessed by two different observers.

1.8 Platelet aggregation induced by the supernatant from activated neutrophils

Activated neutrophil supernatant was obtained by adding fMLP（2 μmol/L） into neutrophil suspension for 10 min at 37 ℃, and then centrifuged at 3 000 × g for 1 min. Washed platelets were incubated with 0.9% saline or drug solution for 10 min at 37 ℃, then added 5 μL of the cell-free supernatant of activated neutrophils. Platelet aggregation was determined as above described.

1.9 Statistical analysis

The data were analyzed by the software bag of SPSS（Version 13, SPSS Inc., USA）.All the data were expressed as $\bar{x} \pm s$, and analyzed by one way ANOVA and least-significant difference（LSD） test, respectively. Differences were considered statistically significant when $P < 0.05$.

2 Results

2.1 Effects of geraniin on platelet aggregation *in vitro*

Geraniin significantly inhibited AA-induced platelet aggregation in a concentration-dependent manner. The IC_{50} value was 2.4 μmol/L. On ADP-or PAF-induced platelet aggregation, geraniin also had significant influence, obtaining 0.4 and 1.1 μmol/L of IC_{50} values, respectively. Aspirin concentration-dependently suppressed platelet aggregation induced by AA（IC_{50} = 6.3 μmol/L）, but not by ADP or PAF（Table 1）.

Tab. 1 Effects of geraniin on AA-, ADP-, or PAF-induced rabbit platelet aggregation *in vitro* [$n = 6, (\bar{x} \pm s)$]

Drug（μmol/L）	Platelet Aggregation（%）					
	AA		ADP		PAF	
	Geraniin	Aspirin	Geraniin	Aspirin	Geraniin	Aspirin
0.5% DMSO	78.2 ± 6.7	73.7 ± 7.5	65.3 ± 7.1	69.2 ± 3.4	69.6 ± 2.3	67.2 ± 3.7
0.16	74.7 ± 6.1	65.3 ± 5.2	42.7 ± 2.5*	66.3 ± 5.5	52.7 ± 5.8*	67.3 ± 2.4
0.8	49.2 ± 5.4*	51.5 ± 3.3*	22.3 ± 4.4**	67.2 ± 4.1	31.5 ± 1.8**	60.7 ± 4.2
4	31.8 ± 3.6**	37.4 ± 2.9**	13.7 ± 1.6**	60.6 ± 6.1	20.9 ± 5.7**	62.9 ± 1.8
20	10.4 ± 2.2**	28.5 ± 4.2**	4.6 ± 1.1**	67.9 ± 5.7	9.6 ± 2.7**	59.8 ± 6.6
100	1.7 ± 0.2**	15.6 ± 3.6**	0.8 ± 0.2**	62.9 ± 4.9	1.8 ± 1.1**	64.9 ± 4.1

*$P < 0.05$, **$P < 0.01$, compared with 0.5 % DMSO.

2.2 Effect of intragastric geraniin on platelet aggregation *ex vivo*

5 mg/kg of geraniin and aspirin showed marked inhibitory effect on AA-induced platelet aggregation during 60 ~ 180 min after administration. Geraniin also had markedly inhibitory effects on platelet aggregation induced by ADP as well as PAF, during 60-180 min and 60-120 min after administration, respectively. Aspirin, however, had no significant effect either by ADP or by PAF (Table 2).

2.3 Effect of geraniin on platelet cytosolic calcium

Geraniin decreased the total rise of $[Ca^{2+}]_i$, Ca^{2+} release, and Ca^{2+} influx, in a concentration-dependant manner. The IC_{50} values were 71.9, 84.9, and 62.9 μmol/L, respectively. Varapamil significantly lowered $[Ca^{2+}]_i$ elevation (Table 3).

Tab. 2 Effect of intragastric 5 mg/kg geraniin on AA-, ADP-, or PAF-induced rabbit platelet aggregation [n = 6, ($\bar{x} \pm s$)]

Drug	Dose(mg/kg)	Platelet Aggregation (%)				
		0 min	60 min	120 min	180 min	240 min
AA						
Saline	–	75.6 ± 5.9	72.9 ± 6.7	78.2 ± 3.7	74.2 ± 5.1	73.8 ± 6.4
Aspirin	5	73.6 ± 6.1	33.5 ± 3.8**	19.8 ± 4.7**	42.5 ± 6.2**	70.4 ± 6.6
Geraniin	5	77.1 ± 5.5	30.7 ± 4.2**	10.5 ± 2.8**	29.5 ± 4.4**	72.8 ± 1.4
ADP						
Saline	–	62.3 ± 3.9	66.4 ± 5.7	60.8 ± 4.9	65.0 ± 4.6	59.8 ± 3.3
Aspirin	5	60.8 ± 4.1	62.8 ± 1.5	63.7 ± 4.8	60.4 ± 5.5	63.7 ± 2.9
Geraniin	5	65.0 ± 3.7	50.4 ± 4.6*	30.7 ± 2.4**	36.9 ± 2.7**	55.9 ± 6.7
PAF						
Saline	–	61.3 ± 5.1	64.8 ± 2.4	62.4 ± 1.9	60.8 ± 6.9	61.7 ± 2.5
Aspirin	5	60.5 ± 2.2	63.9 ± 7.1	69.6 ± 6.5	62.2 ± 41	62.9 ± 5.5
Geraniin	5	64.8 ± 5.5	46.9 ± 4.3*	38.7 ± 2.1**	59.6 ± 3.3	63.7 ± 3.8

*$P < 0.05$, **$P < 0.01$, compared with saline or 0 min.

Tab. 3 Effect of geraniin on cytosolic calcium induced by arachidonic acid (200 μmol/L) in washed rabbit platelets [n = 6, ($\bar{x} \pm s$)]

Drug	Concentration (μmol/L)	Total rise of $[Ca^{2+}]i$ (nmol/L)	Ca^{2+} release (nmol/L)	Ca^{2+} influx (nmol/L)
0.5% DMSO	0	282.3 ± 46.8	143.8 ± 29.4	169.4 ± 31.2
	0.16	293.5 ± 51.2	156.7 ± 30.4	172.6 ± 40.2
	0.8	273.6 ± 32.5	131.8 ± 31.5	155.4 ± 24.1
	4	219.6 ± 38.5*	120.6 ± 19.5*	135.2 ± 27.6*
	20	208.9 ± 28.6*	115.8 ± 27.5**	120.3 ± 21.7*
	100	182.9 ± 22.2**	112.8 ± 20.7**	129.6 ± 24.6*
Varapamil	100	153.8 ± 23.4**	99.6 ± 19.4**	95.3 ± 18.5**

*$P < 0.05$, **$P < 0.01$, compared with 0.5 % DMSO.

2.4 Effect of geraniin on the binding between platelets and neutrophils

Geraniin and aspirin significantly decreased the binding of platelets to neutrophils with IC_{50} of 3.2 and 25.5 μmol/L, respectively (Table 4).

2.5 Effect of geraniin on washed platelet aggregation induced by activated neutrophils

Geraniin markedly inhibited washed platelet aggregation induced by the supernatant from fMLP-acti-

vated neutrophils. The IC_{50} value was 10.2 μmol/L. Aspirin had no inhibitory activity on platelet aggregation stimulated by fMLP –activated neutrophils (Table 5).

Tab. 4 Effect of geraniin on the binding of thrombin–activated platelets to neutrophils [n = 6, ($\bar{x} \pm s$)]

Drug (mol/L)	Adhesion (%)	
	Geraniin	Aspirin
0.5% DMSO	70.5 ± 12.3	72.9 ± 10.5
0.16	65.8 ± 8.2	60.3 ± 9.7
0.8	41.3 ± 5.1**	52.3 ± 4.9*
4	22.8 ± 4.3**	45.6 ± 2.4**
20	15.9 ± 1.8**	36.5 ± 4.5**
100	10.2 ± 3.4**	30.8 ± 2.6**

*$P < 0.05$, **$P < 0.01$, compared with 0.5 % DMSO

Tab. 5 Effect of geraniin on washed platelet aggregation stimulated by the supernatant of fMLP–activated neutrophils in rabbits [n = 6, ($\bar{x} \pm s$)]

Drug (μmol/L)	Platelet Aggregation (%)	
	Geraniin	Aspirin
0.5 % DMSO	55.2 ± 3.6	54.3 ± 3.7
0.16	56.2 ± 2.7	53.3 ± 3.1
0.8	42.6 ± 3.9*	51.6 ± 2.2
4	20.4 ± 3.7*	59.6 ± 3.7
20	12.7 ± 2.9*	52.7 ± 1.8
100	8.6 ± 1.4*	56.9 ± 4.6

*$P < 0.05$, compared with 0.5 % DMSO

3 Discussion

ADP, AA, and PAF can cause platelet activation and result in platelet aggregation through different pathways[7–9]. In this study, geraniin in vitro significantly inhibited AA–, ADP–, or PAF–induced platelet aggregation, in a concentration–dependent manner. Intragastric geraniin had a significant inhibitory effect on AA–induced aggregation 60–120 min after administration. Differing from aspirin, geraniin also significantly inhibited ADP–or PAF–induced platelet aggregation 60–180 min and 60–120 min after administration, respectively. Platelet hyperfunction is believed to be associated with thrombotic disorders. It is suggested that geraniin showed a nonspecific inhibition on platelet aggregation and might be useful for antithrombosis.

After activation of platelets by thrombin, platelet selectin is rapidly redistributed to the cell surface during granulation, and mediates the binding of activated platelets to neutrophils[10]. Aspirin was reported not to inhibit expression of platelet selectin[11]. However, it strongly inhibited ADP–stimulated platelet release of both granule and dense granule contents. Our data indicated that aspirin inhibited platelet–neutrophil adhesion. Aspirin may suppress secondary expression of other adhesion molecule on platelet surface and reduce platelet–neutrophil adhesion. Geraniin significantly decreased the binding of platelets to neutrophils obtaining a lower IC_{50} value to that of aspirin. Platelet–neutrophil adhesion is obviously increased in some cardiovascular diseases[2]. The adhesion between these two kinds of blood cells are involved in the process of thrombomodulation[12]. Activation of platelets increases neutrophil adhesion to foreign surfaces, neutrophile aggregation, lysosomal enzyme release, etc. Platelet–derived products are able to promote neutrophil chemotaxsis, enzyme release, and phagocytosis and to inhibit oxidative burst. On the other hand, neutrophil–derived products can promote platelet aggregation, serotonin release and generation of thrombotic materials[13]. Thrombus formation is mediated by the platelet–neutrophil interactions including cell binding and platelet aggregation[2]. Clinical studies showed that platelet–neutrophil rosette in patients with thrombotic disease significantly increased[11]. Our results suggest that geraniin showed the ability to suppress the binding of activated platelets to neutrophils, and may be beneficial to the treatment of thromboembolic disorders.

Bioactive substances derived from activated neutrophils can challenge platelet aggregation, thromboxane A_2 formation, and cytoplasmic Ca^{2+} movement[14]. Aspirin, however, had no influence on platelet aggregation stimulated by activated neutrophils, suggesting that cyclooxygenase may not be directly involved in neutrophil–induced platelet aggregation. Toxic oxygen radicals, cathepsin G, elastase and PAF have been suggested to act as mediators of neutrophil–dependent platelet activation[10]. Geraniin exerted a concentration–dependent inhibitory effect on platelet aggregation induced by the cell–free supernatant from fMLP –activated neutrophils. Briefly, geraniin suppressed

platelet–neutrophil interaction through different mechanism from aspirin.

Calcium is an important mediator in platelet activation as well as in the process of platelet – neutrophil interactions[14]. In this study, the increase of $[Ca^{2+}]i$ induced by AA in the presence of $CaCl_2$ was much larger than that in the absence of extracellular Ca^{2+}, suggesting that the major component of the AA–induced increase in $[Ca^{2+}]i$ is caused by the influx of Ca^{2+} ions across the plasma membrane. Geraniin concentration–dependently lowered the increase of $[Ca^{2+}]i$ stimulated by AA in the presence of $CaCl_2$ or EGTA, respectively. This demonstrated that geraniin inhibited both the influx of extracellular calcium and the mobilization of calcium from intracellular stores. Decreasing cytosolic calcium is one of the mechanisms of geraniin in suppressing platelet hyperfunction and the interactions between platelets and neutrophils.

In conclusion, geraniin inhibited platelet aggregation *in vitro* and *ex vivo*, decreased platelet – neutrophil adhesion, and suppressed platelet aggregation induced by activated neutrophils. Geraniin may be developed as an antithrombotic agent due to its antiplatelet effect and suppression of platelet–neutrophil interactions.

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老鹳草素对血小板聚集和血小板－中性粒细胞之间相互作用的影响

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［摘要］**目的** 研究老鹳草素抗血小板聚集作用及其对血小板与中性粒细胞之间相互作用的影响. **方法** 采用Born方法观测老鹳草素在体内和体外对家兔血小板聚集功能的影响，运用玫瑰花结实验测定血小板与中性粒细胞的黏附反应，以Fura-2-AM为荧光指示剂，检测老鹳草素对激活的血小板内钙水平的影响. **结果** 老鹳草素在体外呈浓度依赖性明显抑制花生四烯酸（AA）、腺苷二磷酸（ADP）及血小板活化因子（PAF）诱导的血小板聚集功能. 其半数抑制浓度（IC_{50}）分别为2.4、0.4和1.1 μmol/L；5 mg/kg的老鹳草素灌胃亦明显抑制AA、ADP和PAF诱导的血小板聚集；老鹳草素显著降低洗涤血小板内钙离子浓度、减少细胞外钙内流及内钙释放，其IC_{50}分别为71.9、84.9、62.9 μmol/L. 老鹳草素明显阻抑凝血酶激活的血小板与中性粒细胞之间的黏附作用，并抑制肉豆蔻佛波醇（fMLP）激活的中性粒细胞上清液诱导的血小板聚集功能，其IC_{50}分别为3.2和10.2 μmol/L. **结论** 老鹳草素明显抑制血小板聚集功能，降低血小板内钙水平，同时阻抑血小板与中性粒细胞之间的相互作用.

［关键词］老鹳草素；血小板聚集；细胞内钙；血小板－中性粒细胞相互作用
［中图分类号］R973［文献标识码］A［文章编号］1003－4706

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（上接第3页）

除术的化疗，其目的是通过化疗减少微转移而提高生存率.

4.3 肺癌的分子靶向治疗

肺癌的分子靶向治疗（molecular targeted therapy）是指针对肿瘤发生发展过程中的细胞信号传导和其它生物学途径的治疗手段. 其作用靶点可以是细胞表面的生长因子受体或细胞内信号传导通路中的蛋白质. 广义的分子靶点则包括参与肿瘤细胞分化周期、凋亡、迁移、浸润，淋巴转移，全身转移等过程中从DNA到蛋白或酶水平的任何亚细胞分子. 主要包括表皮生长因子受体（epidermal growth factor receptor，EGFR）家族抑制剂、血管生成抑制剂、多靶点的酪氨酸激酶抑制剂等几类药物.

EGFR和其配体通常在非小细胞肺癌（no small cell lung cancer，NSCLC）中过度表达，但在小细胞肺癌（small cell Lung cancer，SCLC）中几乎不表达. 成为分子靶向治疗的重要目标. 目前靶向作用于EGFR的因子主要包括酪氨酸激酶抑制剂（TKI），如吉非替尼（Gefitin ib；Iressa）和埃罗替尼（Erlotinib；Tarceva），单克隆抗体如西妥昔单抗（Erb ituk；C etuxim ab）等.

血管内皮生长因子（VEGF）家族包含6个生长因子（VEGFA、B、C、D、E以及胎盘生长因子）和3个受体[VEGFR1（Flt1）、VEGFR2（KDR/Flk1）和VEGFR3（Flt4）]. VEGF的过度表达与肿瘤进展及不良预后相关. 目前针对VEGF途径的治疗包括抗VEGF单克隆抗体和VEGFR-TKI两大类. 代表药物包括贝伐单抗（Bevacizumab; Avastin）、西妥昔单抗（Cetuximab）和血管内皮抑制素（Endostar）.

肺癌分子靶向治疗的出现，为肺癌患者带来新的希望，而且为肺癌的治疗提供了一种全新的思路. 靶向治疗药物与当前手术、放疗、化疗和其他靶向治疗药物联合治疗的疗效及安全性需进一步评估. 笔者应该坚信，随着分子生物学及分子病理学等基础研究、临床试验技术和其他相关技术的不断发展，肺癌分子靶向治疗药物的开发和临床应用会达到更加成熟的阶段，对肺癌的治疗可能产生深远的意义. 以手术为主的综合治疗/个体化治疗是肺癌治疗的主要手段.

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