Effects of Transfection of Human Ribonuclease Inhibitor Gene on B16 Melanoma Cells and Tumor Metastasis*

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Abstract Human ribonuclease inhibitor (RI) is an acidic cytoplasmic glycoprotein with molecular mass of 50 ku. RI can inhibit the activity of ribonuclease A (RNase A). Angiogenin (Ang) is a member of RNase A superfamily. RI also can inhibit Ang activities by tight combination. Angiogenesis is an essential condition for the development of tumors and their metastatic dissemination. So anti-angiogenesis will be an efficient method in the inhibition of the growth and metastasis of tumor. The experiment demonstrated that RI might effectively block the angiogenesis that was induced by angiogenin. RI is constructed almost entirely of leucine-rich repeats that might involve in other unclear biological effect. In order to understand further the potential function of RI and investigate the role of RI in invasion and metastasis. The study established a transfection of human RI cDNA into B16 melanoma cells by the retroviral packaging cell line PA317 carrying the pLNCX-RI *in vitro*. Transfected B16 cells by PA317 carrying the pLNCX and untransfected B16 cells were used as control. The B16pLNCX-RI cell line with a stably high expression of RI was identified by PCR, RT-PCR, Western blot and immunofluorescence assay respectively. The results showed that the transfected RI gene might significantly inhibit cell proliferation, migration, and enhance cell adhesion, as well as, make morphological changes *in vitro*. Cell doubling time were (24.98 ± 0.16) h, (25.62 ± 0.28) h, (32.64 ± 1.11) h in B16 cells, B16 pLNCX and B16 pLNCX-RI cells respectively. Cell adhesion rate was significantly increased by 19.5% and 17.8% as well as cell migration was reduced by 60% and 61.4% in B16 pLNCX-RI cells compared with pLNCX B16 cells and B16 cells respectively. B16 pLNCX-RI cells became flatter, less nucleoli, less division phases and weaker alkalophilic quality of cytoplasm compared with control groups, which should imply that cell proliferation viability was decreased and malignant phenotype was improved on the cell transfected RI. Mice injected with B16 pLNCX-RI cells show a significant inhibition of the metastasis of tumor with lighter lung weight, fewer metastasis nodules, a lower incidence rate, a lower density of blood vessels and longer survival with respect to the control groups, which implied that RI might be involved in metastasis of melanoma. The results of experiments show RI has a significant antitumor metastasis effect and suggest that it is partially responsible for inhibiting angiogenesis, decreasing cell proliferation, reducing cell migration and enhancing cell adhesion.

Key words B16 cells, ribonuclease inhibitor, angiogenesis, metastasis

Ribonuclease inhibitor (RI) is an acidic cytoplasmic glycoprotein. RI is constructed almost entirely of leucine-rich repeats. Such repeats have been identified in more than 100 proteins that exhibit a wide range functions, including cell-cycle regulation, DNA repair, extracellular matrix interaction, and enzyme inhibition[1]. RI can inhibit the activity of ribonuclease A (RNase A). The amino acid sequence of angiogenin (Ang) is high conservative compared with RNase A. Ang is a member of RNase superfamily. RI can inhibit RNase A and angiogenic activities by tight combination with them[2,3]. Angiogenesis, the formation of new blood vessel is an essential condition for the development of tumors with a diameter greater than 1 ~ 2 mm and also for their metastatic dissemination. So anti-angiogenesis will be an efficient prospective method in the inhibition of the growth and metastasis of tumor[4,5]. The experiment showed that RI might effectively block the angiogenin induced angiogenesis[6]. RI was recognized as a regulating element on formation of new blood vessels in the embryo developing, wound healing, and tumor happening. RI should be used in some angiogenesis dependent diseases as an inhibitor, also is a potential antitumor medicine[7]. RI located on the chromosome 11p15.5, nearly *ras* gene, 11p15.5 at chromosome often has change in cancer patients, therefore, RI may have relation with the cell growth and differentiation[8]. Polakowski *et al.*[9] demonstrated the ability of RI to inhibit tumor growth in mice when administered the recombinant molecule. Nowadays, whether ribonuclease inhibitor has other functions but enzyme inhibition are reported rarely and relationship between gene expression of RI and tumor metastasis remains unclear. This study established a transfection of human RI cDNA into B16 murine melanoma cells by the retroviral packaging cell line PA317 carrying the pLNCX-RI *in vitro*, transfected B16 murine melanoma

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cells by the retroviral packaging cell line PA317 carrying the pLNCX and untransfected B16 murine melanoma cells were used as control. The effects of RI on cell proliferation, adhesion, migration, as well as morphological changes were explored *in vitro* and the tumor metastasis with lung weight and metastasis nodules, a density of blood vessels and survival of mice bearing tumor were observed by transplantation of three kinds of B16 melanoma cells including B16 pLNCX-RI cells, the B16 pLNCX cells and B16 cells in order to understand further the potential function of RI and investigate the role of RI in invasion and metastasis.

1 Materials and methods

1.1 Materials

PA317 cells and C57BL/6 mice were obtained from Tianjin Hemopathy Institute of China. pLNCX-RI was constructed by our group[10]. PA317 carrying the pLNCX-RI and PA317 carrying the pLNCX cells were constructed by our laboratory. B16 cells were from Institute of Biochemistry and Cell Biology, The Chinese Academy of Sciences (Shanghai). Goat anti-rabbit IgG-FITC was a product from Santa Cruz Biotech. PCR primers, PCR Kit and One Step RNA PCR Kit were from TaKaRa Biotech. Goat anti-rabbit IgG-AP was from Huamei Inc. Polyclonal rabbit anti-human RI IgG was produced by our laboratory. Polyclonal antibody of factor Ⅷ related antigen, goat anti-rabbit antibody (IgG/Bio), SP (Streptavidin/Peroxidase) Histostain™-Plus Kits were bought from Beijing ZhongShang Biotech.

1.2 Methods

1.2.1 Cell culture and transfection: The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/L streptomycin and incubated in 5% CO_2 at 37℃. B16 melanoma cells were then seeded into six well plates and allowed to grow until 50% ~ 70% of confluence, the medium/well was replaced with 3 ml of virus containing supernatant of the retroviral packaging cell line PA317 carrying the pLNCX-RI and supplemented 8 mg/L polybrene (Sigma). B16 cells were transfected with virus containing supernatant of PA317 carrying the pLNCX (empty vector) and untransfected B16 melanoma cells were used as control. Repeated the infection process every 12 h for 3 times. After infection, the virus supernatant was replaced with fresh complete medium and was incubated for a further 24 h. After selection with 2 000 mg/L of G418 (Gibco BRL) for 3 weeks, 1 000 mg/L of G418 for another 2 weeks, medium was changed every 72 h, individual G418-resistant clones were obtained by limiting dilution then proliferated and expanded.

1.2.2 PCR and RT-PCR assay: Genomic DNA was extracted as the standard protocol of "Molecular Cloning: A Laboratory Manual" (Cold Spring Harbor Laboratory Press), and total cellular RNA was isolated using the Trizol reagent (Invitrogen) according to the instruction of the manufacturer from the tumor cells including transfected B16 pLNCX-RI cells, B16 pLNCX cells and untransfected B16 cells. To verify the insertion of RI cDNA into B16 cells genome and assay the result of transfection, a pair of primer was used to amplify a part of vector sequence and a part of RI sequence. The following primers were used as PCR and RT-PCR: 5′ CCA AGC TTG TTT ATG AGC CTG GA 3′ (upstream primer) and 5′ GCC GAG AGG CTG CAA TAC T 3′ (downstream primer). PCR amplification was performed as following parameters: 94℃ for 5 min for one cycle; 94℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min total 30 cycles, then 72℃ for 7 min. For RT-PCR, each reaction solution contains total volume 50 μl (5 × buffer 5 μl, Taq 5 U, each primer 1 μl, RNA 1 μg). RT-PCR was proceeded as following parameters: 50℃ for 30 min, 94℃ for 2 min for RT reaction, then 94℃ for 30 s, 55℃ for 30 s and 72℃ for 1.5 min total 30 cycles for PCR. The PCR products were loaded on a 2% agarose gel, stained with ethidium bromide, autographed under UV illumination by Kodak Digital Science ID soft system scanning.

1.2.3 Western blotting analysis: For Western blotting analysis, the protein was extracted using Trizol after total cellular RNA and stored at −20℃. 20 μg equal amounts of protein were loaded into each lane, samples were resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocked with 3% BSA in PBS overnight at 4℃, the membrane was probed with the primary antibody rabbit anti-human RI IgG (1∶200) for 1 h at 37℃, washed thoroughly 3 × 10 min with TTBS, incubated with goat anti-rabbit IgG-AP secondary antibodies (1∶500) for 1 h at 37℃. The bands were detected by NBT/BCIP as a substrate.

1.2.4 Immunofluorescent assay: Cells were grown on narrow coverslips in six well plates and were washed with PBS. After fixation with ice cold 80% acetone, samples were air dried, preserved at −20℃. The samples were washed three times with PBS. The coverslips were treated with 3% BSA in PBS for 20 min at 37℃ to block the non-specific staining, then incubated in the rabbit anti-human RI polyclonal antibody (1∶300) in PBS containing 3% BSA at 37℃ for 2 h. After rinsing three times with 3% BSA in PBS for 3 × 5 min, the cells were incubated in goat anti-rabbit IgG-FITC (1∶100) for 30 min at 37℃, washed thoroughly with PBS. Observation was performed under Olympus multifunction microscope.

1.2.5 Cell morphological observation: Cells were grown on narrow coverslips in six well plates and were

washed with PBS. After fixation with methanol, samples were air dried, stained with Giemsa for 10 min. Observation was performed under Olympus multifunction microscope.

1.2.6 Cell proliferation assay: Cells were seeded in 24 well plate at a concentration of 0.5×10^4 in triplicate wells in DMEM with 10% fetal bovine serum. After 24 h, three well of every kind of cell were trypsinized and counted with a hemocytometer per 24 h for 5 days to make a growth curve and calculate the doubling time.

1.2.7 Cell adhesion and migration assay: Cells were seeded in 96 well plate, 100 μl medium with 10% fetal bovine serum containing 5 000 cells/well, the plate was incubated for 2 h at 37℃ in a 5% CO_2 atmosphere. Cells were rinsed three times with PBS, and nonadherent cells were removed, unwashed well as control. Each well with 100 μl medium was added 20 μl CellTiter 96^R AQueous One Solution Reagent (Promega), the plate was incubated for 2 h at 37℃ and recorded the absorbance at 490 nm with a 96 well plate reader. The percentage of A_{490} of well washed and A_{490} of well unwashed represent adhesion rate of cells[11]. Cell migration was performed by a wound assay that was slightly revised as described previously[12]. Briefly, when cells grew in fully monolayers with coverslips in six well plates, coverslips were drawn a 2 mm channel by 200 μl tip in the center of coverslips and washed with PBS. After the plate was incubated 48 h, fixed with methanol, and stained with Giemsa for 10 min, cells numbers of 10 fields that had advanced into the cell-free space in the scratch were counted under microscope at × 100. Assays were repeated for three times.

1.2.8 Tumor metastasis model: Three kinds of a single cell suspension of 0.1 ml containing 2×10^5 tumor cells including the B16 pLNCX-RI cells, the B16 pLNCX cells and untransfected B16 cells were injected into the vein of the socket of eye of syngeneic male C57BL/6 mice (8 ~ 12 weeks old, SPF degree, (20 ± 3) g). Each group has eight mice. Three weeks after injection, mice were sacrificed, lungs were removed and weighed, and the number of metastasis nodules was counted by two blind observers under a dissecting microscope. At the same time, in a separate set of experiments, mice were inoculated with 2×10^5 tumor cells and traced until 60 days to observe and compare their survival.

1.2.9 Histological and immunohistochemical examination: Lungs were fixed in 10% buffered formalin and embedded in paraffin, and 5 μm sections were stained with H&E. (Hematoxylin-Eosin). Microvessels were randomly counted from 10 different fields under microscope (10 × 40) corresponding to areas with the highest density of vessels. Immunohistochemical assays were done to further confirm impact of RI on angiogenesis. The antigen retrieving steps were taken by means of boiling in the citrate buffer for 15 min at 92 ~ 98℃ and trypsinizing with 0.125% for 15 min at 37℃, tissue sections were incubated for 90 min in polyclonal primary antibody of factor Ⅷ related antigen (1∶50) at 37℃, the sections were incubated in goat anti-rabbit secondary antibodies (IgG/Bio) for 15 min at 37℃, using SP (Streptavidin/Peroxidase) Histostain™-Plus Kits with DAB staining. The nucleus was counterstained by hematoxylin. The rest of the procedures were performed as manufacturer's instruction.

1.2.10 Statistical analysis: All data were dealt with SPSS10.0. Survival periods were analyzed by Mann-Whitney test, compares of ratio were adopted by Chi-square test and the Fisher's Exact Test, the rest values were expressed as $(\bar{x} \pm s)$, Student's t tests were used for statistical analysis. $P < 0.05$ was considered significant.

2 Results

2.1 Identification of ribonuclease inhibitor transfection

In order to study the biological functions of ribonuclease inhibitor (RI), we utilized the retroviral packaging cell line PA317 carrying the pLNCX-RI to transfect B16 melanoma cells. Stable transfected cell lines were isolated by G418 selection, then cloned, proliferated and identified by PCR, RT-PCR, Western blot and immunofluorescence respectively. The products of PCR and RT-PCR were 479 bp. Western blot indicated RI was overexpressed and immunofluorescence also showed RI was efficiently expressed in transfected B16 pLNCX-RI cells compared with B16 pLNCX cells and B16 cells. Results demonstrated that RI has integrated into genomic DNA of B16 melanoma cells and has a stable high expression, as shown in Figure 1 ~ 4.

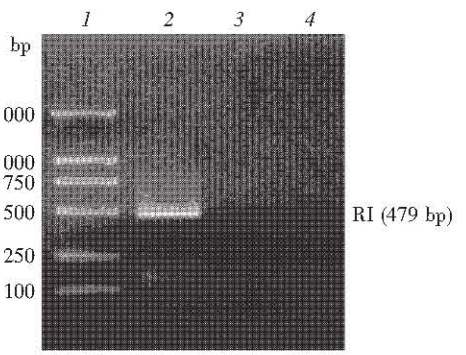

Fig. 1 Identification of transfection RI gene by PCR

1: DL2000 DNA marker; *2*: PCR product of B16 pLNCX-RI cells; *3*: PCR product of B16 pLNCX cells; *4*: PCR product of B16 cells.

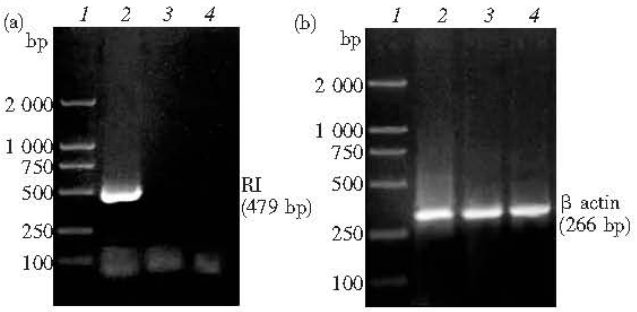

Fig. 2　Transfection detection of RI mRNA expression by RT-PCR

(a) RI mRNA expression; (b) β-actin mRNA expression as an internal control. *1*: DL2000 DNA marker; *2*: B16 pLNCX-RI cells; *3*: B16 pLNCX cells; *4*: B16 cells.

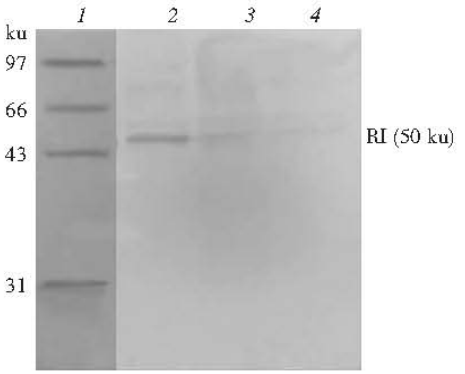

Fig. 3　Western blotting assay of RI expression

1: Protein marker; *2*: B16 pLNCX-RI cells; *3*: B16 pLNCX cells; *4*: B16 cells.

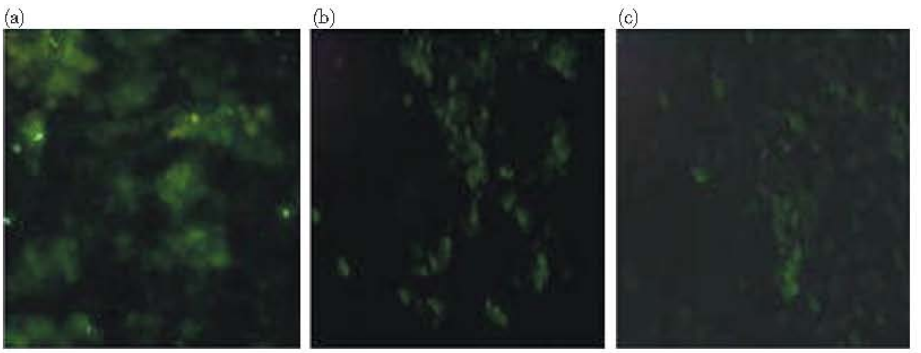

Fig. 4　Demonstration of RI expression by immunofluorescent staining

(a) B16 pLNCX-RI cells; (b) B16 pLNCX cells; (c) B16 cells. Observation was performed under Olympus multifunction microscope(200 ×).

2.2　Cell morphological variety

Giemsa staining showed that B16 cells and B16 pLNCX cells insufficiently spread, overlapped and had more division phases. B16 pLNCX-RI cells become flater, less nucleoli, less division phases and weaker alkalophilic quality of cytoplasm, which should imply that cell proliferation viability was decreased and malignant phenotype was improved as shown in Figure 5.

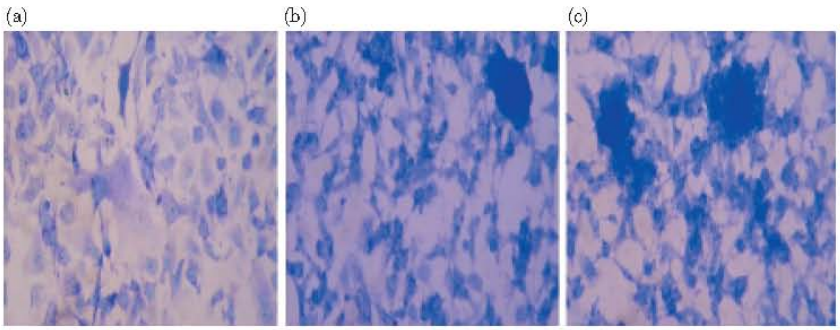

Fig. 5　Cell morphological variety by Giemsa staining

(a) B16 pLNCX-RI cells; (b) B16 pLNCX cells; (c) B16 cells. B16 cells and B16 pLNCX cells spread insufficiently, overlapped and had more division phases. B16 pLNCX-RI cells become flatter, less nucleoli, less division phases and weaker alkalophilic quality of cytoplasm (200 ×).

2.3　Effects of RI transfection on the cell proliferation

Transfected B16 pLNCX-RI cells showed significantly lower cell proliferation rates than those of B16 cells and B16 pLNCX *in vitro*. Doubling time was (24.98 ±0.16) h, (25.62 ±0.28) h, (32.64 ±1.11) h in B16 cells, B16 pLNCX and B16 pLNCX-RI cells,

respectively. Both B16 cells and B16 pLNCX cells had significant difference compared with B16 pLNCX-RI cells, $P < 0.01$. B16 pLNCX-RI cells were 61%, 64%, 52%, 58% of B16 cells at day 3, 4, 5, 6, respectively, $P < 0.01$, as shown in Figure 6.

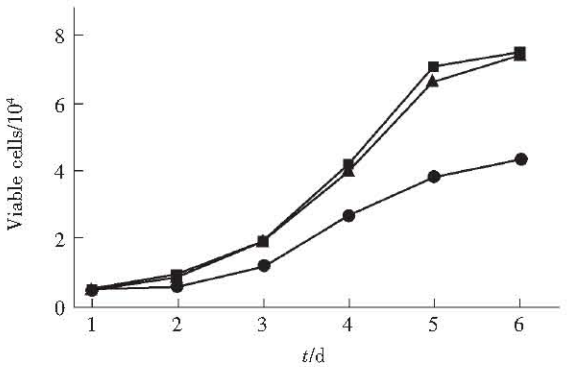

Fig. 6 Effects of RI-transfection on the cell proliferation

Cells were seeded in 24 well plate at a concentration of 0.5×10^4 in triplicate wells. After 24 h, three well of every kind of cell were trypsinized and counted with a hemocytometer per 24 h for 5 days to make a growth curve. Each point represents the mean of three independent samples. Transfected B16 pLNCX-RI cells showed significantly lower cell proliferation rates than those of B16 cells and B16 pLNCX cells. B16 pLNCX-RI cells were 61%, 64%, 52%, 58% of B16 cells respectively at day 3, 4, 5, 6, $P < 0.01$. ●——●: B16pLNCX-RI; ▲——▲: B16pLNCX; ■——■: B16.

2.4 Effects of RI transfection on the cell adhesion and migration

Transfected B16 pLNCX-RI cells showed higher cell adhesion and lower migration capacity than those of B16 cells and B16 pLNCX cells. Cell adhesion rate was significantly increased by 19.5% and 17.8%; cell migration was reduced by 60% and 61.4% in B16 pLNCX-RI cells compared with pLNCX B16 cells and B16 cells, as shown in Figure 7 and Figure 8.

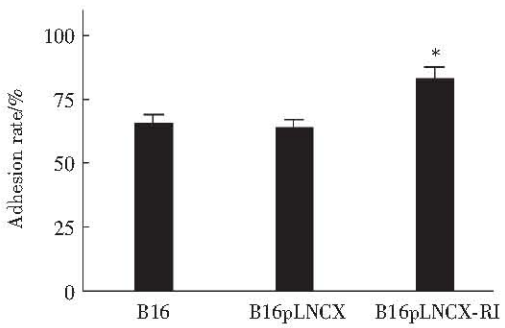

Fig. 7 Effects of RI-transfected on the cell adhesion

The adhesion was assayed as shown in the percent adhesion to each of their unwashed control. Each data point represents ($\bar{x} \pm s$) of 6 repeats, both B16 cells and B16 pLNCX cells had significant difference compared with B16 pLNCX-RI cells, $*P < 0.01$.

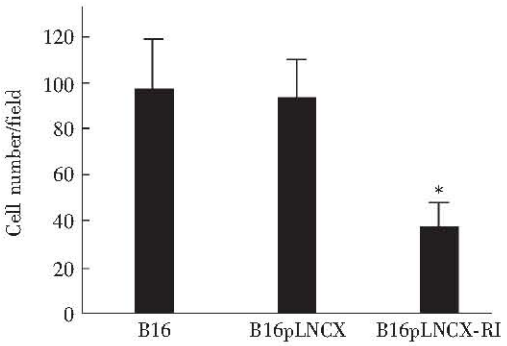

Fig. 8 Effects of RI-transfected on the cell migration

Cell migration assays were performed when cells grew in fully monolayers with coverslips in six well plates, drew a 2 mm channel by 200 μl tip in the center of coverslips and washed with PBS. After the plate was incubated 48 h, fixed with methanol, and stained by Giemsa for 10 min, cell numbers of 10 fields were counted in the scratch under microscope at ×100. Assays were repeated for three times, both B16 cells and B16 pLNCX cells had significant difference compared with B16 pLNCX-RI cells, $*P < 0.01$.

2.5 Tumor metastasis studies

In order to study the role of RI gene in the development of lung metastases, 2×10^5 tumor cells in 100 μl of PBS were implanted into vein of mice. Twenty-one days after injection, two mice had already

Table 1 Effects of RI-transfected tumor lung metastasis

Cell group	Sample	Lung mass/g	Incidence	metastatic nodule
B16	8	0.35 ± 0.10[1)]	100%[1)]	64.75 ± 7.44[1)]
B16 pLNCX	8	0.29 ± 0.04[1)]	100%[1)]	62.63 ± 7.20[1)]
B16 pLNCX-RI	8	0.16 ± 0.01	87.5%	2.13 ± 1.13

[1)] $P < 0.01$. B16 pLNCX-RI group compared with B16 or B16 pLNCX group 2×10^5 tumor cells in 100 μl of PBS were implanted into vein of mice. Twenty-one days after injection, two mice had already died in wild B16 cells group. At this time, all mice were sacrificed. Lungs were removed and weighted; the number of metastasis nodules was counted by two blind observers under a dissecting microscope. Control group showed a remarkable higher number of metastases by lung mass as well as tumor nodules.

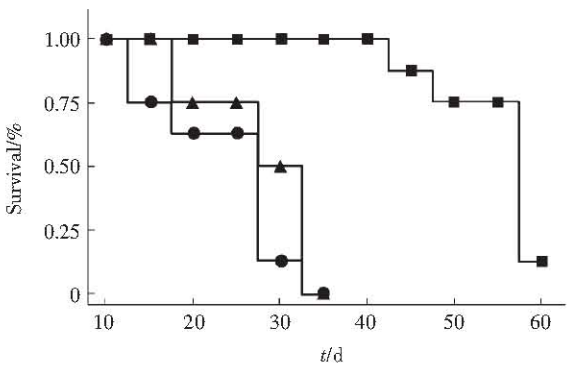

Fig. 9 Survival time and survival rate of mice bearing tumor

Three kinds of a single cell suspension of 0.1 ml containing 2×10^5 tumor cells including B16 pLNCX-RI cells, B16 pLNCX cells and B16 cells were injected into the vein of the socket of eye of syngeneic male C57BL/6 mice and traced until day 60 to observe and compare their survival time and survival rate, both B16 pLNCX cells group and B16 cells group were statistical significant compared with B16 pLNCX-RI cells group, $P < 0.01$. ●——●: B16; ▲——▲: B16pLNCX; ■——■: B16pLNCX-RI.

died in wild B16 cells group. At this time, all mice were sacrificed. Mice injected with B16 pLNCX-RI cells show a significant inhibition of the metastasis of tumor with lighter lung mass, fewer metastases nodules, a lower incidence rate, a lower density of blood vessels and longer survival with respect to control groups, as shown in Table 1 and Figure 9, 10.

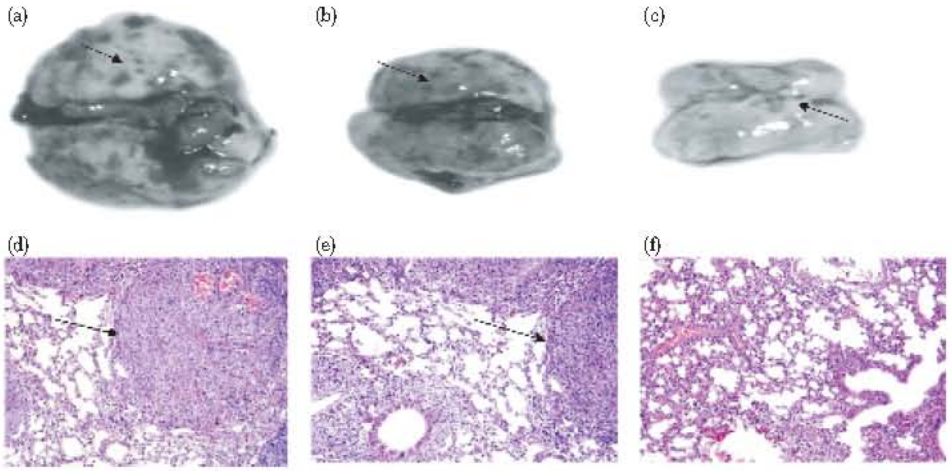

Fig. 10　Lung and microscopic picture of lung by H&E staining *in vivo* metastasis model

(a) and (d) B16 cells group; (b) and (e) B16 pLNCX cells group; (c) and (f) B16 pLNCX-RI cells group. Lungs were removed and photographed, as shown in (a), (b) and (c), B16 cells group and B16 pLNCX cells group were covered by a lot of nodules, but there was a few of nodules in B16 pLNCX-RI cells group. Then lungs were fixed in 10% buffered formalin and embedded in paraffin, 5 μm sections were stained with H&E (hematoxylin-eosin), as shown in (d), (e) and (f). The sample observed under microscope. B16 cells and B16 pLNCX cells have grown into the surrounding lung tissues. A remarkably larger number of metastases with confluent tumor cells can be appreciated in control groups than that in B16 pLNCX-RI cells group (100 ×).

2.6　Histological and immunohistochemical analysis

To confirm whether the inhibition was correlated with the inhibition of blood vessel formation in tumor, pathological analysis was performed. Microvessels were randomly counted from 10 different fields under microscope (10 × 40) corresponding to areas with the highest density of vessels on H&E sections. Immunohistochemical assays were done to further confirm impact of RI on angiogenesis. Mice transfected with RI gene resulted in apparent inhibition of angiogenesis in tumor tissue, whereas more microvessel density in tumor can be seen in mice in control groups (Figure11, 12).

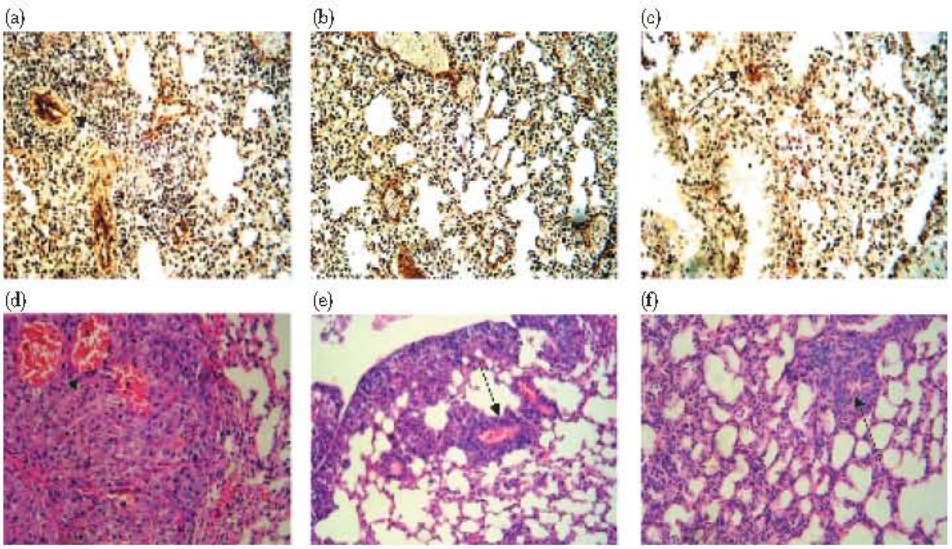

Fig. 11　Histological and immunohistochemical observation of microvessels among the tumors with a lung metastasis

(a) and (d) B16 cells; (b) and (e) B16 pLNCX cells; (c) and (f) B16 pLNCX-RI cells. (a), (b), (c) show immunohistochemical sections stained with an antibody against factor Ⅷ related antigen of vascular endothelial cells; (d), (e), (f) are tissue sections stained by H&E. Histological and immunohistochemical study demonstrated that numerous microvessels with larger cavity and better integrity can be seen among the tumor cells with a lung metastasis of mice injected with B16 cells and B16 pLNCX cells; In contrast, microvessels were a few or absent in the lung metastatic nodes of mice injected with B16 pLNCX-RI cells. (200 ×). Arrows indicate microvessels.

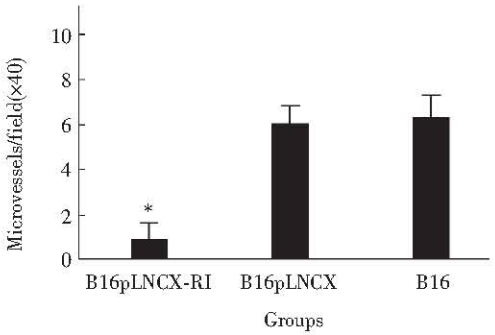

Fig. 12 The microvascular density of the tumor cells with a lung metastasis

Microvessels were randomly counted from 10 different fields under microscope (10 ×40) corresponding to areas with the highest density of vessels on H&E sections. Values are expressed as ($\bar{x} \pm s$), B16pLNCX-RI vs B16 cells or B16 pLNCX cells, $*P < 0.01$, $n = 10$.

3 Discussion

The retroviral vector enable target gene to have a high stable and long expression. It is a good model to study gene function and gene therapy. Since retroviral vectors require target cell division for gene integration, preferential insertion rapidly dividing cells. The target selectivity of this approach exploits the fact that the tumor cells are typically rapidly dividing cells[13]. Invasion and metastasis of tumor cells were the main causes of cancer patients' death[14]. In various experimental system, the expression of a considerable amount of genes affect metastatic ability. Angiogenesis plays an important role in tumor growth and metastasis. Growth of solid tumors depends on induction of angiogenesis to provide adequate oxygen and nutrients to proliferating cells and avoid necrosis. In addition, neovasculature provides a physical route for tumor metastasis. At present, antiangiogenesis is a hot point in tumor therapy. Experimental evidence suggests that avascular tumors rarely grow to over 1 mm^3 in size, and lack metastatic potential[15]. Ribonuclease inhibitor (RI) is an acidic glycoprotein present in cytoplasm, with a molecular mass of 50 ku. RI is an important protein with many functions. The earliest hypothesis was that RI is involved in the regulation of intracellular levels of RNA. The second possible role for RI is to serve as a "sentry" to protect cells against non-cytosolic RNases that gain entry to the cytosol. Another function of RI might be to regulate the potent biological effects of some RNase family members. Finally, this protein may play some additional roles unrelated to inhibition of RNases[1]. RI can inhibit the RNase activity by binding with RNase at a ratio of 1∶1. RI can also bind with angiogenin, inhibiting its angiogenic activity. The affinity of RI with Ang is about 100 folds higher than that with RNase[16,17]. The experiment demonstrated RI can effectively block the angiogenesis induced by angiogenin[18]. The animal experiments showed that RI can inhibit some kinds of transplant tumor cell growth in the animal body, including Ca761 breast carcinoma, S-180 sarcoma, SHG44 and C6 glioma[9,19,20]. However, despite these studies, the function of ribonuclease inhibitor (RI) and the relationship between RI and tumorigenesis had not been well understood yet.

B16 melanoma is a tumor growth and lung metastasis model accepted[21,22]. The study of the impact of RI on cancer cell adhesion and migration *in vitro* have not been reported, and tumor metastasis-suppressive effects of RI could be associated to other its functions besides its anti-angiogenic role also have not been reported. In order to explore further function of RI, in this study, RI was transfected into the mice B16 melanoma. The cell proliferation, adhesion, migration, as well as, morphological changes were examined *in vitro*. The metastasis of tumor with a density of blood vessels, incidence rate, lung mass, metastases nodule, as well as, survival period in tumor bearing mice were observed *in vivo*. Proliferation, adhesion, migration and microvessel number play an important role in tumor metastasis. The change of adhesion is one of key elements of malignant invasion and metastasis, cell migration is central to the process of tumor metastasis, the decrease of cell adhesion function can result in enhancement of migration, which is basic mechanism of tumor metastasis, the metastases take place more easily in the tumor cells with low cells adhesion and high migration[23,24]. Cell morphological observation showed that B16 cells and B16 pLNCX cells insufficiently spread, overlapped and had more division phases, that B16 pLNCX-RI cells become flatter, less nucleoli, less division phases and weaker alkalophilic quality of cytoplasm, which should imply cell proliferation viability was decreased and malignant phenotype was improved on the cell transfected RI. The data showed that the RI transfected led to decrease of cells proliferation and migration, increase of adhesion *in vitro*. Doubling time was (24.98 ± 0.16) h, (25.62 ± 0.28) h, (32.64 ± 1.11) h in B16 cells, B16 pLNCX and B16 pLNCX-RI cells respectively. Cell adhesion rate was significantly increased by 19.5% and 17.8%; cell migration was reduced by 60% and 61.4% in B16 pLNCX-RI cells compared with pLNCX B16 cells and B16 cells. Our study is accordance with the opinion that RI would have relation with the cell growth and differentiation[8]. It was also found that the transfected RI resulted in a significant inhibition of tumor metastasis with lighter lung weight, fewer metastasis nodules, a lower incidence rate, a lower density of blood vessels and

longer survival in mice bearing tumor with respect to control group *in vivo*. These results indicated that RI could exert a significant suppressive effect on melanoma metastasis as well as invasion and suggest that this effect was partially responsible for antiangiogenesis, inhibiting cell proliferation, decreasing cell migration, increasing cell adhesion and changing cell malignant phenotype. Our previous study demonstrated that the down-regulation of RI expression in some cancer cell lines as well as the partial demethylation and restoration of expression after treatment with methylation inhibitor. These finding may hint that RI is involved in a candidate tumor suppressor gene function in some tissues. All data showed that RI could become a target gene for gene therapy, a promising new therapeutic protein medicine and an inhibitor of angiogenin for antitumor. The further study will be required to identify the biological function of RI and its exact molecular mechanism of suppressive metastasis.

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转染人核糖核酸酶抑制因子基因对 B16 黑色瘤细胞及肿瘤转移的影响*

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摘要　人核糖核酸酶抑制因子（human ribonuclease inhibitor，RI）是一种细胞质中分子质量为 50 ku 的酸性糖蛋白. RI 能抑制核糖核酸酶 A（RNase A）的活性，RNase A 与血管生成因子（angiogenin，Ang）的氨基酸有着高度保守的同源序列. Ang 是 RNase A 超家族的一员，RI 通过与 RNase A 和 Ang 的紧密结合而抑制其活性. 血管生成及新血管的形成，是肿瘤发生和转移的必要条件. 所以抗血管生成将是一种很有希望的对抑制肿瘤生长和转移的有效方法. 实验显示 RI 能有效地抑制肿瘤诱导血管的生成. RI 由含有许多亮氨酸重复序列的多肽组成. 含有这样重复序列的 100 多种蛋白质显示了广泛的功能，包括细胞周期调节，DNA 修复，对细胞外基质相互作用以及抑制酶活性等. RI 被认为是胚胎发育，创伤愈合及肿瘤发生中新血管形成的一种调节因子. RI 定位于染色体的 11p15.5，与 *ras* 基因邻近，在肿瘤病人中经常存在染色体 11p15.5 部位的变异和异常. RI 可能与细胞的生长和分化有关，因此，RI 可能还具有尚未知的生物学作用. 为了进一步了解 RI 的潜在功能以及探讨 RI 与肿瘤侵润、转移的关系，将人的核糖核酸酶抑制因子基因的 cDNA 通过逆转录包装细胞 PA317，并转染到 B16 小鼠黑色瘤细胞中，用转染空载体和未转染的 B16 细胞作为对照. 通过 PCR，RT-PCR，蛋白质免疫印迹，免疫荧光分析鉴定，获得稳定表达人核糖核酸酶抑制因子的细胞株. 结果显示，转染的 RI 基因在体外能显著地抑制细胞增殖和细胞迁移，增加了细胞的粘附以及改善细胞的恶性形态，B16，B16 pLNCX，B16 pLNCX－RI 3 种细胞的倍增时间分别为（24.98±0.16）h，（25.62±0.28）h，（32.64±1.11）h. 与对照组相比，转 RI 的细胞粘附率增加 17.8% 和 19.5% 而迁移降低了 61.4% 和 60%. 转 RI 的细胞比对照组细胞较平展，核仁和分裂相较少，胞质嗜碱性减弱，提示细胞增殖活性降低和恶性表型的改善. 将 3 种 B16 细胞静脉注射到 C57BL/6 小鼠中，结果表明，转染 RI 基因的实验组显著地抑制了肿瘤的转移，与两个对照组相比，荷瘤小鼠有更长的存活时间，少得多的转移节结，更低的肿瘤血管密度和肺重量. 结果显示，RI 的表达可能与黑色瘤的转移有关，提示 RI 能显著地抑制肿瘤的转移，可能由于其与抑制血管作用，增加细胞粘附，降低细胞迁移及增殖有关.

关键词　B16 细胞，核糖核酸酶抑制因子，血管生成，肿瘤转移
学科分类号　R34

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