RESEARCH ARTICLE

Momordica cochinchinensis Seed Extracts Suppress Migration and Invasion of Human Breast Cancer ZR-75-30 Cells Via Down-regulating MMP-2 and MMP-9

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Abstract

Objective: Metastases and invasion are the main reasons for oncotherapy failure. *Momordica cochinchinensis* (Mu Bie Zi in Chinese) had been used for a variety of purposes, and shown anti-cancer action. In this article, we focused on effects on regulation of breast cancer cell ZR-75-30 metastases and invasion by extracts of *Momordica cochinchinensis* seeds (ESMCs). Methods: Effect of ESMCs on ZR-75-30 human breast cancer cells proliferation were evaluated by MTT assay and on invasion and migration by wound-healing and matrigel invasion chamber assays. Expression and protease activity of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, were analyzed by Western blotting and gelatin zymography, respectively. Results: ESMC revealed strong growth inhibitory effects on ZR-75-30 cells, and effectively inhibited ZR-75-30 cell invasion in a dose-dependent manner. Western blot and gelatin zymography analysis showed that ESMC significantly inhibited the expression and secretion of MMP-2 and MMP-9 in ZR-75-30 cells. Conclusions: ESMC has the potential to suppress the migration and invasion of ZR-75-30 cancer cells, and it might prove to of interest in the development of novel inhibitors for breast cancer.

Keywords: *Momordica cochinchinensis* - breast cancer - invasion - MMP-2 - MMP-9

Asian Pac J Cancer Prev, **15 (3)**, 1105-1110

Introduction

Breast cancer is one of the leading causes of cancer death in women worldwide. More than 90% of lethality in cancer patients is caused by metastasis (Mueller et al., 2001; Weigelt et al., 2005; Fang et al., 2013). Lungs, bone and brain are the most common sites for breast cancer metastasis and the occurrence of distant metastases severely limits the prognosis of breast cancer patients. Factors, such as neoplastic cell molecular, genetic characteristics and biological environment, are thought to be determinant in the metastatic process (Chambers et al., 2002; Mendes et al., 2005).

To form metastases, neoplastic cells must invade through the basement membrane, enter lymphatic and blood vessels for dissemination into the circulation, and establish a new tumor in distant organs (Friedl and Wolf, 2003). To migrate, the cell body must modify its shape and stiffness to interact with the surrounding tissue structures. Hereby, the extracellular matrix (ECM) provides the substrate, as well as a barrier towards the advancing cell body (Yilmaz et al., 2007). Matrix metalloproteinases (MMPs), a family of highly homologous, zinc- and calcium-dependent extracellular enzymes , were capable of degrading essentially all of the components of the extracellular matrix and classified into 5 groups (collagenases, gelatinases, stromelysin, matrilysin and the membrane-type MMP) based on substrate specificity, protein domain structure, sequence homology and ability/inability to be secreted. MMP2 and MMP9 (gelatinases A and B or 72- and 92-kD type IV collagenases) are of particular interest because of their role in early cancer development and progression. They are implicated in tumor invasion and metastasis. Therefore, inhibition of the function of MMPs, especially MMP-2 and MMP-9, in the ECM is being most actively pursued for anticancer therapy (Hidalgo and Eckhardt, 2001; Somiari et al., 2006).

Now, cancer chemotherapy drugs are research hotspot. And nature plant is one of important sources to discover new therapeutic drugs (Gordon M. Cragg and Newman, 2005). *Momordica cochinchinensis* Spreng., a member of the Cucurbitaceae family, has been highly valued for its nutritional and medicinal qualities and wide range of adaptability. It has been used as an indigenous food and traditional medicine throughout East and Southeast Asia for a long time (Ishida et al., 2004; Tsoi et al., 2006; Sanwal et al., 2011; Parks et al., 2012). As a medicinal plant, the seeds of the fruit, used as traditional Chinese medicine

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(Mubiezi in Chinese, Figure 1 A), have anti-cancer actions (Wong et al., 2004; Kubola and Siriamornpun, 2011; Lin et al., 2012).

In this study, we focus on the tumor invasive cascade in the metastatic process and investigate the effect of the seeds of *Momordica cochinchinensis* on the migration and invasion of human breast cancer cells ZR-75-30. Furthermore, we investigate the effect of the seeds of *Momordica cochinchinensis* on enzymatic degradation of extracellular matrices in order to understand the mechanisms of its anti-metastasis effect.

Materials and Methods

Reagents

RPMI 1640, trypsin, MTT, DMSO were purchased from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Roche (CA, USA). FBS was purchased from Lanzhou National Hyclone Bio-engineering Co., Ltd (Lanzhou, China). MMP2 and MMP9 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). HRP-conjugated GAPDH monoclonal antibody was purchased from Proteintech Group (Chicago, IL, USA). Anti-rabbit antibody was purchased from Santa Cruz Biotechnology (CA, USA).

Cell culture

Human breast cancer cell line ZR-75-30 was obtained from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences. ZR-75-30 cells were cultured in RPMI 1640 medium (GIBCO, Invitrogen Corp., USA) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% carbon dioxide at 37 °C. All cells were used in experiments during the linear phase of growth.

Plant material

Momordica cochinchinensis (Lour.) Spreng. seed (Figure 1 B) were collected in China, June 2011, and identified by Dr. Xiaofeng Niu, Xi'an Jiaotong University. A voucher specimen (No. 11119) was deposited at the Institute of Materia Medica, School of Medicine, Xi'an Jiaotong University, China.

The seeds of *Momordica cochinchinensis* were shattered into fine powder. Then we decocted 50 g *Momordica cochinchinensis* powder in water for 1 h, and repeated the above steps twice. Merge all the filtrate collection, filtered and got the each filtrate collection. The extract (ESMC) was concentrated under rotary evaporator.

Cell proliferation assay

The effect of ESMC on ZR-75-30 cell proliferation was evaluated by the MTT assay. Exponentially growing cells were seeded into 96-well plates at a density of 2×10^4 cells per well in medium. After 24 h incubation at 37 °C, cells were treated with ESMC at various concentrations for 48 h. Then, 20 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. After the removal of medium, 150 μL of DMSO was added to each well, and the optical density of cells was determined with a microplate reader (Bio-RAD instruments, USA) at 490 nm and expressed as absorbance values (Zheng et al., 2013).

Wound-healing assay

ZR-75-30 cells were planted into a 6-well plate and allowed to grow to 70 % confluency in complete medium. Cells were then serum starved for 24 h, and cell monolayers were carefully scratched an artificial "wound" at 0 h with a pipette tip. Wounded monolayers were then washed to remove floating cells and photographed under an inverted microscope. Cells were incubated in medium with or without concentrations of ESMC. Cell migration into the wound surface and the average distance of migrating cells were observed in different times using a phase contrast microscope.

Migration and invasion assays

In vitro cell migration assays were performed using transwell chambers (8 μM pore size; Millipore, USA). Cells were allowed to grow to sub-confluency (75–80%) and were serum-starved for 24 h. After detachment with trypsin, cells were washed with PBS, and resuspended in serum-free medium. Cell suspension (2×10^5 cells/ml) was added to the upper chamber with ESMC (0, 30, 60 and 120 μg/ml) in 400 μl of 1 % BSA RPMI 1640 medium. The bottom chamber contained medium with 10 % FBS RPMI 1640 medium to serve as a chemoattractant to induce invasion. For the screen, after 24 h the cells that had not migrated were removed from the upper face of the filters using cotton swabs. To determine the number of migratory cells, the invaded cells were fixed with 100 % methanol and then stained with 0.2 % crystal violet (Beijing Chemical Works, China). Images of three different fields were captured from each membrane and the number of migratory cells was counted using a phase contrast microscope. The mean of triplicate assays for each experimental condition was used. Similar inserts coated with 100 μl (1 mg/ml) matrigel (Becton–Dickinson, USA) were used to determine invasive potential in the invasion assay.

Zymography

Secreted metalloproteinase were detected and characterized by zymography. Conditioned media were obtained by a 24 h incubation of ZR-75-30, which were treated overnight with ESMC (0, 30, 60 and 120 μg/ml) in serum-free media. The media were collected, centrifuged for 10 min at 4 °C at 2000 rpm. Conditioned media (20 μl) were loaded on 8 % SDS-PAGE gels that had been copolymerized with 1 mg/ml gelatin. The samples were not activated before running which allowed the latent and active forms of each enzyme to be visualized. Electrophoresis was performed under nonreducing conditions at 100 V for 2 h at 4°C. The gels were immersed in Triton X-100 (2.5% in water) for 3 h to deactivate the enzymes by removing the SDS, and were incubated in collagenase buffer (50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$) for 40 h at 37°C. Gels were stained with 0.5% Coomassie blue for 30 min at room temperature and destained until revealing clear bands containing proteolytic activity on a dark blue background. The presence of metalloproteinases was indicated by an

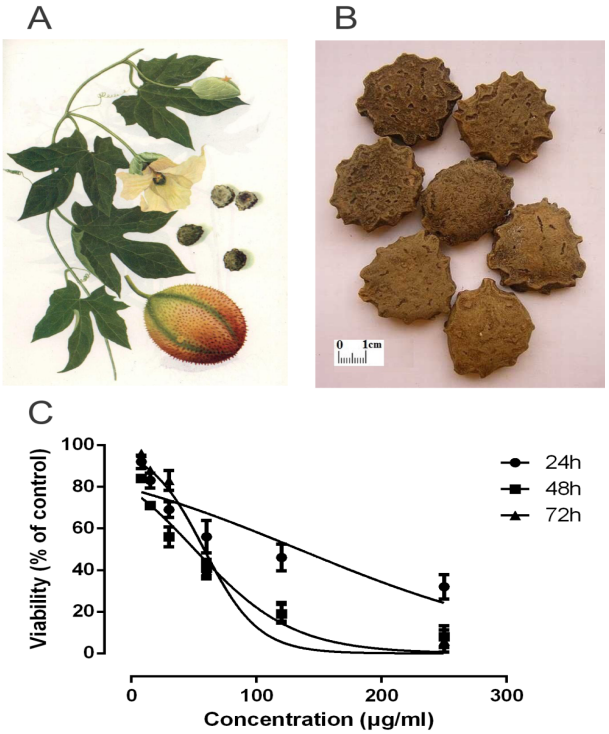

Figure 1. The Effects of ESMC on Viability of ZR-75-30 Cells. a The plant about *Momordica cochinchinensis* b The seed of *Momordica cochinchinensis* c The effects of ESMC on viability, ZR-75-30 cells were treated with various concentrations of ESMC for 24, 48 and 72 h. Data were represented as means ± SEM at least three independent experiments. Statistically significant changes at $*p < 0.05$, $**p < 0.01$ vs. the control group

unstained proteolytic zone in the substrate in terms of their molecular weights: 92 kDa and 72 kDa, corresponding to MMP-9 and -2 respectively. Both active forms and pro-enzymes were revealed by this technique as the exposure of pro-MMPs to SDS during SDS-PAGE leading to activation without proteolytic cleavage. Bands were quantified using an image quantitative analysis system (Image-Pro Plus, USA) (Rooprai et al., 2000).

Western blotting

ZR-75-30 cells treated with ESMC for 48 h were lysed with RIPA lysis buffer containing protease inhibitor cocktail and phosphates inhibitor cocktail on ice for 30 min, then lysis buffer was collected, and centrifuged at 12, 000 g, 4 °C for 10 min. The protein lysates were resolved by SDS-PAGE, and separated proteins were transferred to PVDF membranes and blocked with 5 % skimmed milk for 2 h. Then, the membranes were incubated with specific primary antibodies overnight at 4 °C, incubated with the relevant secondary antibodies at room temperature for 2 h. Finally, the blots were detected by Immobilon® Western (Milipore Corporation, MA, USA)

Statistics

All data were obtained from at least three independent experiments and expressed as mean ± SEM. Comparisons of the different groups were performed with Student's t-test. $p < 0.05$ was considered the minimal level of significance.

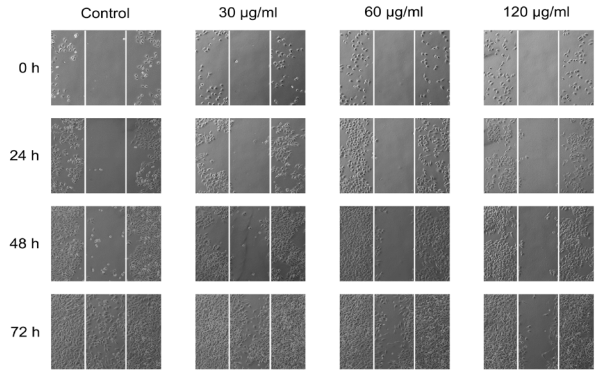

Figure 2. Effect of ESMC on ZR-75-30 Cell Migration in Vitro. Photographs of wound of cells treated with 0, 30, 60 and 120 μg/ml of ESMC for 24, 48 and 72 h

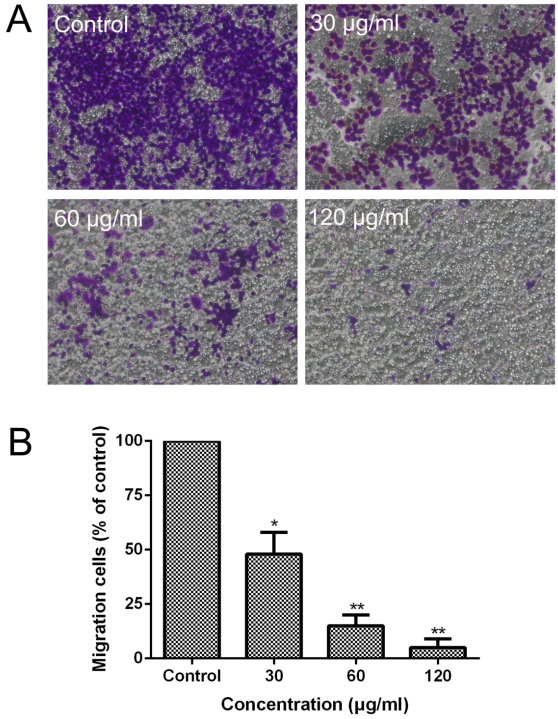

Figure 3. Effect of ESMC on ZR-75-30 Cell Migration in Vitro. a Photographs showed the cell migration through the polycarbonate membrane treated with concentrations of ESMC stained by 0.2 % crystal violet. The inhibitory effect of ESMC on the migration of the cells was in a concentration-dependent manner. b Quantification of the number of cells migrating through the polycarbonate membrane. Data were represented as means ± SEM at least three independent experiments. Statistically significant changes at $*p < 0.05$, $**p < 0.01$ vs. the control group

Results

ESMC supressed ZR-75-30 cell growth

We investigated the effect of ESMC on cell viability. ESMC showed significant anti-proliferative effect on human breast cancer ZR-75-30 cells in a dose- and time-dependent manner, as shown in Figure 1 C, and the 50 %-growth inhibitory concentrations (IC50) of ESMC at 24, 48 and 72 h were 93.24, 34.04 and 53.43 μg/ml. A significant inhibitory effect was noted, compared with the control group.

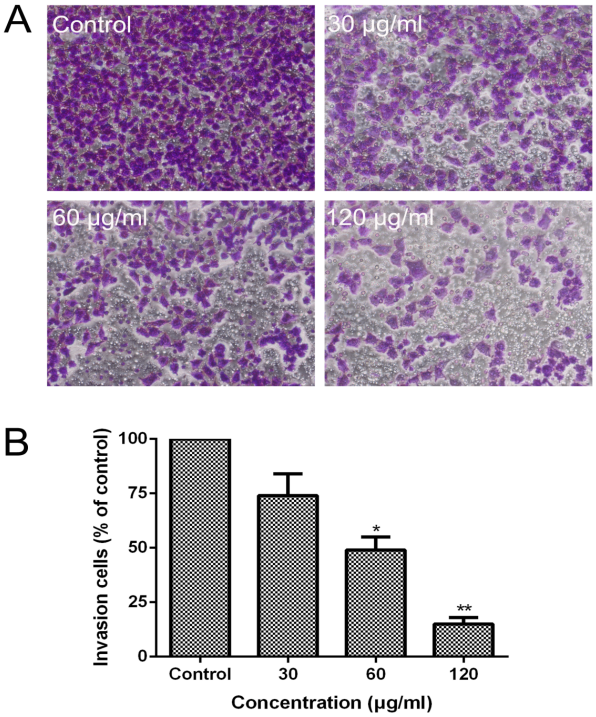

Figure 4. Effect of ESMC on ZR-75-30 Cell Invasion in Vitro. a Photographs of the cell invasion through the matrigel-coated polycarbonate membrane stained by 0.2 % crystal violet. The inhibitory effect of ESMC on the invasion of the cells was in a concentration-dependent manner. b Quantification of the cell invasion assay. Data were represented as means ± SEM at least three independent experiments. Statistically significant changes at $*p < 0.05$, $**p < 0.01$ vs. the control group

ESMC inhibited the migration of ZR-75-30 cells

To investigate the effect of ESMC on cell migration, wound-healing (scratch motility) and transwell invasion assays were used. Confluent monolayers of cells were scratched to form wounds, then cultured in the absence or presence of various concentrations of ESMC (30, 60, 120 μg/ml), and observed at a different time after cell monolayers had been wounded. As shown in Figure 2, ESMC-induced cells moved slowly compared with the control group in a dose-dependent manner. Similar results were obtained from the transwell invasion assays. Millicell was also used to determine the inhibitory effect of ESMC on ZR-75-30 cell migration. Results showed that, after 24 h of treatment with various concentrations of ESMC (30, 60, 120 μg/ml), the cell number on the lower surface of the membrane decreased in a dose-dependent manner (Figure 3). Taken together, our data suggested that ESMC could impair breast cancer cell ZR-75-30 migration.

ESMC inhibited the invasion of ZR-75-30 cells

The possible effect of ESMC on cell invasion was examined using matrigel-coated chambers. Cells were treated with various concentrations of ESMC (30, 60, 120 μg/ml) or vehicle for 24 h in the upper side, and then allowed to migrate through a membrane coated with matrigel. As shown in Figure 4, ESMC inhibited the invasion ability of ZR-75-30 cells in a dose-dependent manner. These data were consistent with results we found above.

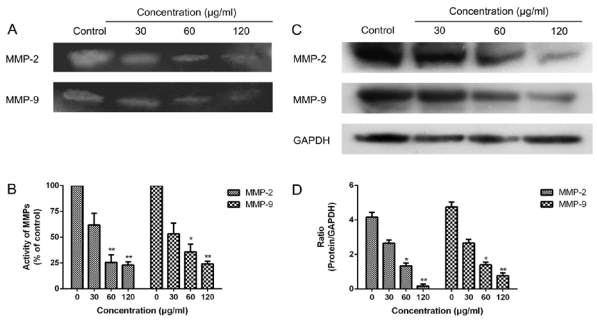

Figure 5. Effect of ESMC on Activities and Proteins Expressions of MMP-2 and MMP-9 in ZR-75-30 Cells. a Gelatin zymogragpy analysis of serum-free media conditioned by ZR-75-30 cells treated with concentrations of ESMC. It indicated that ESMC could exert an inhibitive effect on the secretion of MMP-2 and MMP-9 in a dose-dependent manner compared with the control group. b Quantification of the gelatin zymography assay. c Western blot analysis of ESMC in the downregulation of MMP-2 and MMP-9 expression in ZR-75-30 cells. It shows that ESMC could inhibit the MMP-2 and MMP-9 expression in a dose-dependent manner. d Quantification of the protein expression assay. Data were represented as means ± SEM at least three independent experiments. Statistically significant changes at $*p < 0.05$, $**p < 0.01$ vs. the control group

ESMC suppressed the activity of MMP-2 and MMP-9 in ZR-75-30 cells

The potential effects of ESMC pretreatment on MMP-2 and MMP-9 production by ZR-75-30 cells were detected by gelatin zymography. Pretreatment of ZR-75-30 cells with ESMC had significant inhibitory effects on the levels of MMP-2 and MMP-9 activity in a dose-dependent manner (Figure 5 A-B). These suggested that inhibition of invasion by ESMC was caused by changes in gelatinase secretion or activation.

ESMC down-regulated the protein expression levels of MMP-2 and MMP-9 in ZR-75-30 cells

We tested the protein expression of MMP-2 and MMP-9 in ZR-75-30 cells treated with ESMC (30, 60 and 120 μg/ml) for 48 h by western blot analysis. As shown in Figure 5 C-D, ESMC exerted an inhibitive effect on the expression of MMP-2 and MMP-9 in a dose-dependent manner compared with the control group. The results suggested that protein expression levels of MMP-2 and MMP-9 could be down regulated in ZR-75-30 cells treated with ESMC.

Discussion

Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver (Mueller et al., 2001). Tumor cell migration and metastasis are very important factors playing key roles in the generation, progress and prognosis of tumor, and these usually display a remarkably diverse set of clinical features. Therefore, researchers around the world continue to search for a cancer cure that is even more effective, and place extra emphasis on the metastasis.

Nature plants extracts are prime source of highly effective conventional anti-cancer drugs. In 2013's Asian Pacific Journal of Cancer Prevention, Munkhzaya, B. et al

found that ethanol extract of Saussurea involucrata have anti-metastatic potential against hepatic cancer, Waraporn, Y. found that Phyllanthus emblica extract suppressed metastasis on human fibrosarcoma cell, and Song, F.Q. et al focued on the anticancer mechanisms of medicinal mushroom. Traditional medicine has its boundedness that was due to active ingredients being unclear in cancer treatment. However, with the development of analysis technology, nature extracts provide leads for the potential novel agents (Munkhzaya et al., 2013; Waraporn et al., 2013; Song et al., 2013).

In this study, the authors demonstrated, for the first time, that *Momordica cochinchinensis* seed played a remarkable role in inhibiting metastasis via down regulation of MMP-2 and MMP-9 in breast cancer treatment.

In the pre-experiment, we have found that extracts of *Momordica cochinchinensis* seed indicated anti-cancer effect. The mechanism of this effect was not expounded yet. In this article, we explore the therapeutic mechanism of this plant by relevant experiment research.

The effect of ESMC on ZR-75-30 breast cancer cell invasion was investigated in this study by a matrigel chamber invasion assay. ESMC displayed obvious inhibition of invasion in a dose-dependent manner.

We examined whether the effect of ESMC on ZR-75-30 cells influenced cell motility. The scratch motility (wound healing) and millicell assays indicated that ESMC significantly reduced the migration of ZR-75-30 cells in a dose- and time-dependent. The ability of cells to migrate through uncoated porous filters in response to a chemotactic stimulus was examined in a Transwell migration assay. Treatment of ESMC displayed obvious inhibition of invasion in a dose-dependent.

Then, we examined whether the mechanism was linked to elevated levels of MMPs, which are well documented ECM-degrading enzymes and whose activity is associated with tumor invasiveness. MMPs activities were measured by a zymography assay of conditioned media from ESMC and control ZR-75-30 cells.

The expression and activity of MMPs against matrix macromolecules have been associated with the development of malignant phenotypes and the promotion of cell invasiveness and metastasis. ESMC's anti-invasive action is also reflected by its suppressive effects on the expression of MMP-2 and MMP-9, two major MMPs mediating the degradation of the ECM. In our study, ESMC treatment not only reduced the protein expression but also repressed the enzymatic activity of MMP-2 and MMP-9. These results suggest that ESMC's anti-invasive action is mediated, at least in part, by diminishing the ability of breast cancer cells to degrade the components of ECM by modulating MMP-2 and MMP-9 expression and activity.

Taken together, our results demonstrated that ESMC was able to inhibit breast cancer cell adhesion, migration and invasion. The mechanism underlying the above effects was attributed to attenuation of the activity and expression of MMP-2 and MMP-9. This study suggests ESMC is a potential candidate for interventions against breast cancer metastases.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant 81370088 and 81227802), the ministry of national science and technology during the significant new drugs creation special project (011ZX09401-308-031), the project of shaanxi star of science and technology (2012KJXX-06), and supporting plan of education ministry's new century excellent talents (NCET-13-0467).

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