Effect of metformin on the improvement of prostate cancer in diabetic rats

Kaijian Hou,1* Wansheng Ke2* and Jianping Xiong3

Abstract
This study was designed to investigate the effect of metformin on the improvement of prostate cancer in diabetic rats. A total of 20 Sprague Dawley (SD) rats were equally divided into control and intervention groups. The intervention group received intragastric metformin 200 mg/kg, while the control group was given intragastric drinking water for 4 weeks. Tumor volumes were compared, all tumor specimens underwent routine pathological examination, immunohistochemical detection of E-cadherin and N-cadherin, and western blot assay. The tumor volume of control and intervention group was 462.15 ± 45.67 and 23.46 ± 5.32 mm3, respectively. Hematoxylin and eosin (HE) staining showed partial visible glandular structure with deepened nuclear staining in the intervention group. Immunohistochemistry showed high expression (6.5 ± 0.28 vs 3.8 ± 0.26, P < 0.05) of E-cadherin and low expression (3.4 ± 0.12 vs 7.8 ± 0.34, P < 0.05) of N-cadherin in the intervention group. Western blot assay showed higher expression of E-cadherin, while low N-cadherin in the intervention group. Metformin can effectively alleviate lesion extent of prostate cancer and mechanism may be related to upregulation of E-cadherin and downregulation of N-cadherin expression.

Keywords
diabetes, E-cadherin, metformin, N-cadherin, prostate cancer

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Introduction
Prostate cancer is a relatively common tumor among the male, and in recent years, its incidence has been increasingly rising in developed areas like Beijing and Guangdong, especially in the elderly population. The development of prostate cancer is closely related to genetic factors, sexual life, and diet habit.1 Its early clinical symptoms are not obvious, which affects diagnosis and treatment of the disease and increases the risk of death in patients while exacerbating the difficulties of prostate cancer treatment.2 At present, laparoscopic radical prostatectomy has become the gold standard for the treatment of localized prostate cancer. However, the patients with advanced prostate cancer suffer from local metastasis of lymph node, bone, and lung; unable to receive surgery; and during drug treatment, most patients will develop into androgen-independent prostate cancer within 1–2 years with drug resistance and poor prognosis.3 Therefore, it is necessary to explore effective drugs for prostate cancer treatment.

Metformin is currently the first-line drug for treatment of type 2 diabetes mellitus. Related studies have found that4,5 it can effectively reduce blood sugar, lower insulin level, and increase insulin sensitivity with effects of anti-inflammatory, antioxidant stress, and anti-tumor. Relevant study
has shown that in type 2 diabetes patients treated with statins and metformin at the same time, the incidence of prostate cancer is significantly lower. But the mechanism of metformin against prostate cancer is unclear. A recent study has demonstrated that the mechanism of epithelial mesenchymal transition (EMT) is involved in prostate cancer invasion and metastasis. EMT refers to the process where epithelial cells experience the change of protein expression under the influence of external factors and transform into the cells with mesenchymal cells feature. Existing study shows that in the course of cell EMT, epithelial adhesion proteins such as E-cadherin are decreased and the expression of N-cadherin increased. It is suggested in literature that nuclear factor (NF)-κB is a downstream molecule of N-cadherin, and metformin regulates NF-κB signaling via suppressing N-cadherin. In this study, we constructed a diabetic rat model of prostate cancer to analyze the effect of metformin on prostate cancer and explore related mechanisms.

Materials and methods

Main experimental materials

A total of 20 healthy male Sprague Dawley (SD) rats, age of 40 days, were provided by the animal experimental center of certain university. Streptozocin (STZ; Sigma), rat PCa-RM1 cell line (reserved by the Department), RPMI1640 culture medium (Guangzhou Anjie Biotechnology Co., Ltd.), Dulbecco’s Modified Eagle Medium (DMEM) culture medium (Shanghai Yiyan Biological Technology Co., Ltd.), E-cadherin, N-cadherin mouse anti-human monoclonal antibody (Beijing Huaxia Ocean Technology Co., Ltd.), and SP Kit (Beijing noble Ryder Technology Co. Ltd.) were used as main experimental materials.

Experimental methods

Cell culture. RM-1 cells were placed in incubator with 5% CO₂ at 37°C and cultured in RPMI1640 medium containing 10% inactivated fetal bovine serum. The cells used in the experiments stayed in the logarithmic growth phase with the good growing state.

Establishment of animal models. STZ was dissolved with 0.1 mol/L citric acid buffer to make into 2% solution with the pH value of about 4.2, which was injected into the rats to establish diabetic rat model. The diabetic rats were injected with 0.1 mL phosphate-buffered saline (PBS) suspension of 1 × 10⁷ m⁻¹ RM-1 cell under abdominal anesthesia and the size of tumor was observed by vernier caliper, and the next step was taken when the tumor size reached about 80 mm³. A total of 20 rats with diabetes and prostate cancer were randomly divided into control group and intervention group, 10 rats in each group in which the intervention group received intragastric administration of metformin 200 mg/kg for 4 weeks, while the control group was given intragastric administration of the same amount of drinking water for 4 weeks; at the end of the intervention, the tumor size was measured and the tumor of each group was taken out for laboratory tests like pathological examination.

Routine serum analysis

Blood glucose concentration was determined with the help of automatic glucose analyzer, while serum insulin concentration was determined by enzyme immunoassay kit (EIA kit) while applying an enzyme-linked immunosorbent assay (ELISA) reader.

Routine pathological examination

The tumor tissue was fixed with 10% formaldehyde, and after paraffin embedding, it was conducted with routine histopathologic section and common hematoxylin and eosin (HE) staining followed by observation of pathological changes of tumor tissues under light microscope.

Immunohistochemical detection of E-cadherin and N-cadherin

Immunohistochemical detection of E-cadherin and N-cadherin was performed by ultra-sensitive SP immunohistochemistry method. The sections were dewaxed followed by gradient dehydration; the activity of endogenous peroxidase was eliminated with hydrogen peroxide followed by antigen heat repair and closure of tissue protein; one drop of diluted first antibody of E-cadherin mouse anti-human monoclonal antibody and N-cadherin anti-human monoclonal antibody were, respectively, added (with the dilution concentration of 1:100);
then it was incubated at 37°C for 15 min and washed by PBS buffer three times, 3 min/time, at 4°C for the overnight; one drop of biotin-labeled second antibody was added (with the dilution concentration of 1:100), and then, it was incubated at 37°C for 15 min and washed by PBS buffer three times, 3 min/time. 3,3′-Diaminobenzidine (DAB) stain was maintained at room temperature for 5–10 min and flushed by distilled water three times, 3 min/time, which was followed by dehydration, transparency, and sealing. Then, it was observed under microscope and the picture was taken.

**Standard for judging E-cadherin and N-cadherin staining degree**

The staining result was analyzed with scoring method, the positive count of tumor cell \(<10\%\) was scored 0; the positive cell count reaching 11% to 20% was scored 1; 21% to 50% 2; and the positive cell count \(>50\%\) was scored 3. The intensity of staining was divided as follows: pale yellow was scored 1; yellow or dark yellow 2; and brown or tan 3. The multiplication of both scores was defined as protein expression level, when the product \(\geq 4\) suggested positive result and the product \(<4\) negative result. The positive expression rate and expression level (optical density) of E-cadherin and N-cadherin were, respectively, calculated in the two groups. The results of the experiment were concluded by two experimental personnel and judged with double-blind method.

**Western blot assay of E-cadherin and N-cadherin**

The tumor tissue was washed twice with precooled PBS and proper amount of cell lysate was added followed by being placed on ice for 30 min, centrifuged at 12,000 r/min for 20 min at 4°C, and protein content was adjusted, and then, the same amount of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer was added followed by being placed in boiling water at 95°C–100°C for degeneration for 5 min, next SDS-PAGE vertical electrophoresis was conducted with electrorotation of polyvinylidene difluoride (PVDF) film, 50 g/L skim milk powder was added and then it was placed in cold storage at 4°C for the overnight; after phosphate-buffered saline supplemented with Tween 20 (PBST) washing of membrane, it was respectively added with first antibody of E-cadherin mouse anti-human monoclonal antibody, N-cadherin anti-human monoclonal antibody, \(\beta\)-actin goat anti-mouse monoclonal antibody (with the dilution concentration of 1:1000) followed by incubation at 37°C for 1 h and PBST washing of membrane; the mixture of same amount of electrogenerated chemiluminescence (ECL) reaction liquid A and B was added to the membrane for 1 min and finally X-ray showed color.

**Statistical processing**

SPSS 21 statistical software was used for statistical analysis. The difference between the two groups of measurement data was calculated by t-test, the rate of comparison was measured by \(\chi^2\) test, the table was \(P < 0.05\), and the difference was statistically significant.

**Ethical approval**

The ethical approval for this study was obtained from the institutional ethical committee of Shantou Traditional Chinese Medical Hospital, P.R. China. All the experiments were carried out as per National Institutes of Health (NIH) guideline for laboratory animals. The reference number is 1356/ERB/STCM/2017.

**Results**

**Comparison of tumor volume and HE staining after intervention**

After 4 weeks of intervention, the volume of the control group reached \((462.15 \pm 45.67)\ mm^3\); and that of the intervention group was significantly reduced with the volume of \((23.46 \pm 5.32)\ mm^3\), with significant difference between two groups \((P < 0.05)\) as shown in Figure 1(a). After 4 weeks of intervention, HE staining showed cell dispersing with no prostate gland structure in the control group, while in the intervention group there was partial visible glandular structure with deepened nuclear staining as shown in Figure 1(b).

**Immunohistochemistry results**

Compared with the control group, the expression level of E-cadherin in the intervention group was higher \((6.5 \pm 0.28 \text{ vs } 3.8 \pm 0.26, P < 0.05)\) and its
positive expression was also significantly higher in the intervention (70% (7/10) vs 20% (2/10), \( P < 0.05 \)) as shown in Figure 2(a) and (b).

N-cadherin had expression both in nucleus and membrane of prostate cancer cells as shown in Figure 2(c) and (d); the expression level of

**Figure 1.** Comparison of (a) tumor volume and (b) HE staining of prostate cancer tissue after intervention (400×). Intervention group versus control group, **\( **P < 0.05; \) (A) control group and (B) intervention group.

**Figure 2.** The expression of (a and b) E-cadherin and (c and d) N-cadherin in prostate cancer tissues (immunohistochemistry 400×): (a and c) control group and (b and d) intervention group.
N-cadherin in the intervention group was lower (3.4 ± 0.12 vs 7.8 ± 0.34, \( P < 0.05 \)), and its positive expression was also lower in the intervention group ((10% (1/10) vs 60% (6/10), \( P < 0.05 \)).

**Western blot assay result**

The results of Western blot assay showed that compared with the control group, the expression level of E-cadherin in the intervention group was higher (\( P < 0.05 \)) and that of N-cadherin was lower (\( P < 0.05 \)), as shown in Figure 3.

**Serum analysis**

Insulin levels in serum were significantly lower (\( P < 0.05 \)) in the intervention group than in controls. However, there were no significant differences between the rats of both groups for the serum insulin concentrations. The level of glucose in the serum of the intervention was lower than in the control group as shown in Table 1. The serum glucose lowering effect of metformin without a significant change in serum insulin concentration suggest that the metformin may involve an insulin-independent mechanism.

**Discussion**

Prostate cancer has become the most frequent type of cancer in men. Recent data suggest that diabetic patients taking metformin have a lower incidence of certain cancer, including prostate cancer.\(^8\) After 4 weeks of intervention, the volume of the control group reached and that of the intervention group was significantly reduced with the volume of significant difference between two groups; in the control group, HE staining showed cell dispersing with no prostate gland structure, while in the intervention group there was partial visible glandular structure with deepened nuclear staining. These results suggest that metformin can effectively inhibit the growth of prostate cancer in diabetic rats with anti-tumor effect.

In this study, the results of immunohistochemistry showed that compared with the control group, the expression level of E-cadherin in the intervention group was higher and its positive expression was also significantly higher, and N-cadherin expression level in the intervention group was lower with its positive expression also being lower. Our findings are in agreement to a study conducted by Ye et al.,\(^9\) who concluded that in tumor cells, E-cadherin can significantly prevent cell invasion and metastasis by stabilizing cell–cell junction. The results of western blot assay showed that compared with the control group, the expression level of E-cadherin in the intervention group was higher and that of N-cadherin was lower, suggesting that metformin can upregulate the expression of E-cadherin and downregulate the expression of N-cadherin in prostate cancer tissues of diabetic rats. A previous study suggested that the PC3 cells that knocked off the E-cadherin gene had an increased capability for invasion and metastasis and that the number of tumor cells with stem cell properties was also increasing. Similarly, our results are supported by Liu’s group who concluded that the expression of N-cadherin was significantly higher in prostate cancer tissues compared to that in prostatic hyperplasia, and the correlation analysis revealed that the expression of N-cadherin was significantly related to differentiation degree of prostate cancer, clinical staging, lymph node metastasis, and bone metastasis, proving that N-cadherin plays an important role in the occurrence and development of prostate cancer.\(^{10,11}\) To sum up, metformin can effectively alleviate lesion extent of prostate cancer in diabetic rats and its therapeutic mechanism may be related to upregulation of E-cadherin expression and downregulation of N-cadherin expression (Supplemental material).
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ORCID iD
Kaijian Hou https://orcid.org/0000-0003-1733-0068

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