Effect of HMGN2 on proliferation and apoptosis of MCF-7 breast cancer cells

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Received June 20, 2017; Accepted October 16, 2018

DOI: 10.3892/ol.2018.9668

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Key words: high mobility group protein N2, breast cancer, proliferation, apoptosis

Abstract. We investigated the effect of high mobility group protein N2 (HMGN2) on the proliferation and apoptosis of the human MCF-7 breast cancer cell line, and its effect on tumor growth in a subcutaneous heterotopic transplantation tumor model of breast cancer. The cell viability assay was used to verify the effect of the recombinant human HMGN2 on MCF-7 cell proliferation. The Transwell chamber assay was used to verify the effect of HMGN2 on MCF-7 cell migration. Flow cytometry and Hoechst staining were used to detect the effect of HMGN2 on MCF-7 cell apoptosis. MCF-7 was injected to establish a subcutaneous heterotopic transplantation tumor model of breast cancer in nude mice. The size, weight and volume of tumor in each group were compared after the administration of different concentrations of HMGN2 solution around the tumor tissue at day 1, 3, 5 and 7. The tumor tissue was removed and cut into sections, and the apoptotic cells in tumors of nude mice were detected by a TUNEL kit. The CCK-8 assay showed that HMGN2 at different concentrations inhibited the proliferation of the MCF-7 breast cancer cells, and the proliferation of MCF-7 cells were significantly inhibited when the concentration of HMGN2 reached 3 µg/ml (P<0.01). The Transwell chamber assay showed that 3 µg/ml of HMGN2 significantly decreased the migration capacity of MCF-7 cells (P<0.01). Flow cytometry and Hoechst staining showed that 3 µg/ml of HMGN2 significantly increased apoptosis of MCF-7 cells (P<0.01).

After the nude mouse model of breast cancer was established, HMGN2 at different concentrations was injected around the tumor tissue at day 1, 3, 5 and 7. We demonstrated that the growth of breast cancer was significantly inhibited when the concentration of HMGN2 reached 15 µg/ml. TUNEL staining showed that the number of apoptotic cells in the 15 µg/ml dose group was significantly higher than that in the control group (P<0.01). Therefore, in vitro and in vivo experiments proved that recombinant human HMGN2 could significantly inhibit the proliferation and migration of breast cancer cells, which increased the apoptosis of breast cancer cells and exerted anti-breast cancer effects, which enriched our understanding of the biological roles of HMGN2.

Introduction

Breast cancer is one of the most common female tumors, and is a leading cause of female mortality worldwide. The incidence of breast cancer is on the increase annually with a trend towards the younger population (1,2). With the continuous development of medical technology, research on the pathogenesis of breast cancer has revealed abundant information that was previously unknown. The treatment of breast cancer includes a local operative treatment, radiotherapy and chemotherapy and endocrine therapy. Other treatments may be added at the discretion of the oncologist. These treatments have improved the quality of life and prolonged the survival period of breast cancer patients (3,4).

In recent years, it has been shown that, high mobility group protein N2 (HMGN2), which is a type of protein with antibacterial activity against gram-negative bacteria, exerts potential anti-viral and antitumor effects (5,6). Hu et al (7) and Dong et al (8) found that HMGN2 affects anti-human oral squamous cell carcinoma, which is expected to be developed as a potential treatment for oral squamous cell carcinoma. Previous findings have shown that HMGN2 can effectively reduce the proliferation and migration of lung cancer cells, thus affecting both the occurrence and development of lung cancer (9). Currently, chemotherapeutic drugs for breast cancer, which is a type of squamous cell carcinoma, are characterized
by unsatisfactory treatment effects and a high recurrence rate. In literature, to the best of our knowledge, there is no previous study on the effects of HMGN2 on breast cancer.

The aim of the present study was to investigate the effect of HMGN2 on the proliferation, migration and apoptosis of breast cancer MCF-7 cells via in vitro and in vivo experiments in order to enrich the understanding of biological function of HMGN2 and to find new ideas for the treatment of breast cancer.

Materials and methods

Animals. Thirty-two nude mice were obtained from the SLAC laboratory animal Co., Ltd. (Shanghai, China). The mice were housed in isolated and ventilated cages (≤5 mice per cage). The environment was kept between 16 and 26°C with relative humidity between 30 and 70%. Autoclaved laboratory rodent diet (Western Research Products, Orange, CA, USA) and water were provided ad libitum.

Ethics approval for the study was obtained from the West China College of Basic and Forensic Medicine, Sichuan University (Chengdu, China).

Materials and instruments. Recombinant human HMGN2 was prepared as previously described (7). The MCF-7 cell line (Kunming Cell Bank of Chinese Academy of Sciences), Cell Counting Kit-8 (CCK-8; Sigma, St. Louis, MO, USA), DMSO (Sigma), RIPA lystate (Wuhan Google Biotechnology, Ltd., Wuhan, China), Transwell chamber (Millipore Corp., Bedford, MA, USA), phosphorpylated protease inhibitor (Wuhan Google Biotechnology, Ltd.), Hoechst detection kit (Wuhan Google Biotechnology, Ltd.), TUNEL apoptosis detection kit (Cell Signaling Technology, Beverly, MA, USA), flow apoptosis detection kit (Cell Signaling Technology), hydrogen peroxide (Sigma), paraformaldehyde (Sigma), paraffin (Wuhan Google Biotechnology, Ltd.), ultraviolet spectrophotometer (Beckman Coulter, Miami, FL, USA), electronic balance (Thermo, Germany), electrophoresis apparatus (Corning Inc., Corning, NY, USA), and pipettor (Eppendorf, Hamburg, Germany) were all obtained commercially.

Effects of HMGN2 on proliferation, migration and apoptosis of breast cancer cells

Detection of effect of HMGN2 on the proliferation of MCF-7 breast cancer cells via CCK-8. The viability of MCF-7 cells was determined using the CCK-8 according to the manufacturer's protocol. Briefly, MCF-7 cells were plated at 5x10^4 cells per well in 96-well plates and incubated overnight in DMEM medium supplemented with 10% FBS. After treatment with various concentrations of HMGN2 protein (0, 1, 2, 3, 4 and 5 µg/ml) for 24 h, 10 µl CCK-8 liquid was added to the test well and incubated for 3 h. Then absorbance was measured at a wavelength of 450 nm.

Detection of effect of HMGN2 on the migration of MCF-7 breast cancer cells via Transwell assay. The migration assays of MCF-7 cells were carried out using Transwell insert chambers. MCF-7 cells (1x10^5) were plated into the upper chamber in serum-free medium in triplicate. The medium in the upper and lower chambers contained different concentrations of HMGN2 (0, 1, 2, 3 µg/ml) in different groups. After incubation of the cells for 24 h, the cell in the upper chambers were removed by wiping with a cotton swab. Cells that migrated to the lower surface of filter were fixed in 70% ethanol for 30 min and then stained with 0.2% crystal violet for 5 min. The cell migration was scored by counting five random fields per filter under a light microscope (Nikon Instech Co., Ltd., Tokyo, Japan).

Detection of effect of HMGN2 on the apoptosis of breast cancer MCF-7 cells via flow cytometer. MCF-7 cells were selected and the cell density was adjusted to 5x10^6 cells/ml. Then, the cells were incubated in a 6-well plate and divided into the blank control group and HMGN2 with different concentration groups (1, 2, 3 µg/ml). After 24 h, operations were performed in strict accordance with the instructions of the apoptotic kit. After digestion, the cells were washed using pre-cooled PBS twice, and the fluorescent solution was added after re-suspension and centrifugation at 450 x g for 5 min in order to incubate cells at room temperature in the dark for 15 min, which was followed by detection via a FACSCalibur flow cytometer (BD Biosciences, NJ, USA).

Detection of effect of HMGN2 on apoptosis of breast cancer MCF-7 cells via Hoechst staining. MCF-7 cells were selected and the cell density was adjusted to 5x10^6 cells/ml. Then, the cells were incubated in a 6-well plate, the medium containing different concentrations of HMGN2 (0, 1, 2, 3 µg/ml) in different groups. After 24 h, the culture solution was removed and the cells were washed using cold PBS twice. Operations were performed in strict accordance with the instructions of Hoechst-33258 kit. Paraformaldehyde (4%) was added to fix the cells for 5 min and then the prepared staining solution A was added to incubate cells in the dark for 15 min. Then, washing solution B was used to wash cells twice, which was followed by observation under a fluorescence microscope (Olympus Co., Tokyo, Japan).

Establishment of subcutaneous heterotopic transplantation tumor model in nude mice. Thirty-two nude mice were selected and randomly divided into four groups with eight mice in each group. After incubation of the MCF-7 breast cancer cell line, cells in the logarithmic growth phase were collected and the cell density was adjusted to 5x10^7 cells/ml. The cells were inoculated into the nude mice under sterile conditions and HMGN2 was injected into the nude mice at day 1, 3, 5 and 7 around the tumor tissue 3 weeks after the tumor cell transplantation. According to the different HMGN2 doses, the nude mice were divided into the control (normal saline), low-dose (5 µg/ml HMGN2), medium-dose (10 µg/ml HMGN2) and high-dose (15 µg/ml HMGN2) groups. Each nude mouse received 10 ml/kg of the HMGN2 according to their body weight. After inoculation, changes in body weight, tumor size and tumor growth status of the nude mice were observed and recorded. Data at day 1, 2, 3, 4, 5, 6 and 7 were recorded and analyzed. Then the nude mice were sacrificed by cervical dislocation, and the tumor tissue was removed and sectioned, followed by paraffin sealing for subsequent experiments. After the nude mice were sacrificed, the ascites of each group were taken out and weighed, and the cachexia of nude mice was evaluated by (ascites / body weight) x 100%. 
Detection of apoptotic cells in breast cancer tissues. The breast cancer tissue section (1.5x1.5 cm) in each group was dewaxed and placed into the water. It was treated in 3% hydrogen peroxide for 10 min and washed using PBS 3 times. The experiment was conducted in strict accordance with the TUNEL kit. The Proteinase K solution was added onto the slice, and the slice was placed in the wet box for digestion at 37˚C for 10 min, and then washed 3 times using PBS. TdT and DIG-d-UTP mixed solution (40 µl) was added onto the slice, and the slice was placed in the wet box for labeling at 4˚C for 2 h, and then washed 3 times using PBS. Sealing fluid (40 µl) was added onto the slice, and the slice was sealed at room temperature for 30 min. Rabbit anti-human HMGN2 monoclonal primary antibody (1:100; cat. no. 9437; Cell Signaling Technology, Inc., Boston, MA, USA) was added and the slice was placed in a wet box for reaction at 37˚C for 40 min. Then, it was washed 3 times using PBS. Anti-fluorescent quenching sealing liquid was dropped for sealing, followed by observation and images being captured under the fluorescence microscope. Cells with yellow-green fluorescence were the positive cells, namely the apoptotic cells.

Statistical analysis. The data in this study are presented as mean ± standard deviation. SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. A t-test was used for measurement data and the Chi-square test was used for enumeration data. One-way ANOVA was performed for other data. The Bonferroni method was used for pairwise comparison under homogeneity of variance, while the Welch method was used under heterogeneity of variance. Dunnett’s T3 method was used for multiple comparisons. P<0.05 was considered statistically significant.

Results

Detection of effect of HMGN2 on the proliferation of breast cancer MCF-7 cells via CCK-8 assay. The effect of HMGN2 on the proliferation of MCF-7 cells was detected using the CCK-8 assay (Fig. 1). The results show that the proliferation of the MCF-7 breast cancer cell line was inhibited and the proliferation capability was decreased after HMGN2 at different concentrations was added. When the concentration of HMGN2 reached 3 µg/ml, the inhibition rate of MCF-7 cells was as high as 59.6% (P<0.01). In this study, to explore the antitumor effect of HMGN2 on breast cancer MCF-7 cells, we chose 1, 2, 3 µg/ml for the subsequent experiments and the action time was set as 24 h.

Detection of effect of HMGN2 on migration of breast cancer MCF-7 cells via Transwell assay. The effect of HMGN2 on the migration of MCF-7 cells was detected via the Transwell assay (Fig. 2). These results show that the migration of MCF-7 cells was inhibited and the migration capability was decreased after HMGN2 at different concentrations. When the concentration of HMGN2 reached 3 µg/ml, the migration of MCF-7 was significantly inhibited (P<0.01). This result demonstrated that HMGN2 was able to suppress the progression of breast cancer.
Detection of the effect of HMGN2 on apoptosis of MCF-7 cells via flow cytometer. The effect of HMGN2 on the apoptosis of the MCF-7 breast cancer cells was detected via flow cytometry (Fig. 3). These results show that the number of apoptotic breast cancer MCF-7 cells was increased after HMGN2 at different concentrations was added. When the concentration of HMGN2 reached 3 µg/ml, the number of apoptotic MCF-7 cells was significantly increased compared to that in the blank control group (P<0.01).

Detection of the effect of HMGN2 on apoptosis of breast cancer MCF-7 cells via Hoechst staining. The effect of HMGN2 on apoptosis of the MCF-7 breast cancer cell line was detected via Hoechst staining, as shown in Fig. 4. Fluorescence intensity of normal cells in the control group was weak (Fig. 4A-a). The characteristics of apoptotic cells were chromosome enriched and combined with more Hoechst-33258, showing strong blue fluorescence (Fig. 4A-b, c and d). The results showed that the number of apoptotic MCF-7 cells was increased with the higher concentrations of HMGN2 in different groups. The apoptosis induced by HMGN2 occurred in a dose-dependent manner, as shown in Fig. 4B. When the concentration of HMGN2 reached 3 µg/ml, the number of apoptotic MCF-7 cells was significantly increased compared to that in the control group (P<0.01).

Effect of HMGN2 on subcutaneous heterotopic transplantation tumor in nude mice. The subcutaneous heterotopic transplantation tumor model of nude mice was established 3 weeks after MCF-7 cells were injected. The different concentrations of HMGN2 were injected at day 1, 3, 5 and 7 around the tumor tissue during the modeling. The body weight, tumor volume and tumor growth of nude mice were recorded at day 1, 2, 3, 4, 5 6 and 7. There was no statistically significant difference in the body weight among groups within 7 days after injecting HMGN2 (P>0.05). The body weight of the experimental animals was slightly increased because of the tumor growth (Fig. 5). The tumor growth speed and status of nude mice in the control group were significantly higher than those in the HMGN2 groups (P<0.01) (Fig. 6). When the HMGN2 dose was at 15 µg/ml, the inhibitory effect on the tumor growth of nude mice was the most significant (P<0.05). After modeling and administration, the tumor tissue of nude mice in each group was taken out and weighed. The tumor volume was calculated as follows:

\[ \text{Volume} = \text{Width} \times \text{Length} \times \text{Height} \]
The longest diameter exhibited by a single tumor was 1.4 cm in the animals of the blank control group. Multiple tumors were not observed in the experimental animals. The tumor volume in the high-dose group (15 µg/ml HMGN2) was significantly smaller than that in the model group, and the difference was statistically significant ($P<0.01$) (Fig. 7). On the 7th day, the nude mice in each group were sacrificed to take out and weigh ascites. The ascites of each group were 0.7-3.2 ml, and the cachexia of each group was evaluated. The results showed cachexia in the high, middle and low dose groups was significantly lower than that in the model group ($P<0.01$), and that in the high dose group was the lowest (Fig. 7).

Detection of apoptotic cells in tumor tissue via TUNEL staining. The tumor tissue of nude mice in each group was removed and the cell apoptosis of tumor tissue was detected via TUNEL staining (Fig. 8). The cells with yellow-green fluorescence were TUNEL-positive cells, namely the apoptotic cells. We found more positive cells in the HMGN2-treated group than that in the control group, and that in high-dose group was significantly larger than that in control group, and the difference was statistically significant ($P<0.01$).

Figure 7. Tumor growth status of nude mice in each group during controlling. Through comparing the growth status of breast tumor in each group at 7 days after HMGN2 injected, it was found that the growth status of nude mice in HMGN2 group was significantly lower than that of control group, and the growth status of nude mice in high-dose group was significantly lower than that in control group. The difference was statistically significant. $^*P<0.01$, compared with control group.

Figure 8. Detection of TUNEL-positive cells in tumor tissue via TUNEL staining. (A) Image under microscope, bar=50 µm; (B) statistical diagram. The results showed that the number of TUNEL-positive cells in tumor tissue in HMGN2 group was significantly increased, and that in high-dose group was significantly more than that in control group, and the difference was statistically significant. $^*P<0.01$, compared with blank control group.

Discussion

The malignant transformation of tumor mainly manifests as an accelerated proliferation of tumor cells, easy invasion and metastasis. The optimal treatment or operation opportunity of breast cancer, one of the frequent malignant tumors in females, is often lost due to tumor cell invasion, reduction in quality of life and shortening of the survival time of patients (10,11). Currently, the development of antitumor drugs mainly focuses on killing tumor cells and inhibiting tumor cell migration (12). HMGN2 is an important member of the HMG family and consists of 90 amino acid residues with a molecular weight of approximately 9.2 kDa, which is closely related to DNA transcription, replication and repair (13,14). HMGN2 was discovered and purified early due to its potent antimicrobial activity, which has proven to be potent in anti-gram-negative bacterium (15). Currently, it is reported that HMGN2 is closely related to the basic vital phenomena, such as tumorigenesis and embryonic development. HMGN2 may also be involved in the whole process of embryonic development, tumorigenesis and cell apoptosis (16,17). Due to the regulatory effects of HMGN2 in the occurrence and development of breast cancer, its mechanism remains unclear.

In this study, the effects of HMGN2 on the proliferation and apoptosis of the MCF-7 breast cancer cell line was
studied by in vitro experiments, and the effect of HMGN2 on the growth of subcutaneous heterotopic transplantation tumor of nude mice was investigated by in vivo experiments. The results showed that HMGN2 affected the proliferation, migration and apoptosis of breast cancer cells in a dose-dependent manner. When HMGN2 concentration reached 3 μg/ml, the proliferation and migration of MCF-7 cells were significantly inhibited, and cell apoptosis occurred. Musselman and Kutateladze (18) found that HMGN2 exerted an effect of activating chemokines. Activation of chemokines can gather leukocyte and other immune cells by killing tumor cells, and inhibiting tumor cell proliferation, which may also be the main reason for the inhibitory effect of HMGN2 on breast cancer cell proliferation. HMGN2 activates chemokines in a concentration-dependent manner, and therefore, its inhibitory effect of tumor cell proliferation was also dose-dependent. There were no significant differences in the body weight of nude mice between the HMGN2 and blank control groups when the subcutaneous heterotopic transplantation tumor model was established. The main reason was animals developing ascites with tumor growth during the animal experiments. In addition, the activity of the experimental animals was limited and the body weight of the experimental animals declined. The results showed that the toxicity of HMGN2 within the effective dose range was low and did not affect the physiological function of model animals.

Flow cytometry and Hoechst staining were used to clarify the effect of HMGN2 on tumor cell apoptosis with regard to survival and death rate. Results from both of these assays indicated that HMGN2 could promote apoptosis of breast cancer MCF-7 cells. Dal Cin et al (19) demonstrated that HMGN2 can significantly increase cell apoptosis in thyroid tumors, effectively inhibiting the growth of thyroid tumors. Subramanian et al (20) also found that HMGN2 can inhibit angiogenesis of tumor tissue and reduce the nutritional supply of tumor cells, thereby promoting cancer cell death and indicating its potential use as a cancer therapy. In this study, subcutaneous heterotopic transplantation tumor model of nude mice was established to study the effect of HMGN2. The results showed that the tumor grew slowly and the tumor volume became smaller. Animal experiments showed more intuitively that HMGN2 could significantly inhibit breast cancer cell proliferation, and TUNEL staining also showed that the number of apoptotic cells in tumor tissue was significantly increased. Its effect on tumor growth and apoptosis was also demonstrated in a concentration-dependent manner. The results were consistent with the cell results. The limitations of this study include that the molecular mechanism of HMGN2 of inhibiting cell proliferation was not investigated more deeply, and this will be the focus of our research group in the future.

In conclusion, this study demonstrated that HMGN2 could be used to treat breast cancer through the inhibition of the proliferation and increasing the apoptosis of breast cancer cells. The results enriched the understanding of the biological role of HMGN2 which may represent a candidate effector molecule for antitumor activity.

Acknowledgements

Not applicable.

Funding

This study was supported by the Natural Science Foundation of Jiangsu Province (no. BK20140220) and the National Natural Science Foundation of China (nos. 81401817, 0040105401132).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

BF and SS worked on CCK-8 assay and Transwell assay. XS and XY constructed subcutaneous heterotopic transplantation tumor model. NL and GW were responsible for Hoechst staining and flow cytometer. XG and NH helped with TUNEL assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval for the study was obtained from West China College of Basic and Forensic Medicine, Sichuan University (Chengdu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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