Original Article

Intraductal administration of N-methyl-N-nitrosourea as a novel rodent mammary tumor model

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Background: Chemically induced animal models of breast cancer (BC) using N-methyl-N-nitrosourea (MNU) have been widely used in preclinical research. The conventional approach entails intraperitoneal (i.p) or intravenous injection of a carcinogen, leading to tumor induction at unpredictable locations. This study aimed to establish a modified MNU-induced rat mammary tumor model using intraductal (i.duc) administration and to evaluate its biological behavior, morphology, and response to chemotherapy drugs.

Methods: In a pilot experiment, female Sprague-Dawley (SD) rats were injected with either i.duc MNU or vehicle to test the feasibility of this approach. We explored the appropriate dosage for stable tumor formation in pubescent female SD rats by testing a single i.duc dose of MNU (0.5, 1.0 and 2.0 mg) or vehicle.

Results: An i.duc injection of 20 μL (1 mg/per duct) MNU in the fourth rat mammary gland induced stable carcinomas *in situ*. Immunohistochemical (IHC) analysis showed positive expression of estrogen receptor (ER), negative expression of human epidermal growth factor receptor 2 (Her-2), and low expression of Ki-67. Histopathology revealed atypical hyperplasia in the mammary gland 4 weeks after carcinogen injection, developing into carcinoma *in situ* 5–6 weeks after treatment, with loss of α-SMA and calponin expressions during tumor progression. Albumin-bound paclitaxel (nab-PTX) was injected i.duc and intravenously (i.v) 5 weeks after administration of MNU. The tumor growth rate of the nab-PTX i.duc-treated group was lower than in the i.v and control groups. The number of TUNEL-positive apoptotic cells was significantly higher in the nab-PTX i.duc-treated group.

Conclusions: Using i.duc MNU (20 μL, 1 mg) to establish a rat mammary tumor model resulted in a predictable location in the rat mammary gland and exhibited better consistency; i.duc administration of nab-PTX permitted a smaller drug dose, but produced a better drug response, than i.v injection.

Keywords: N-methyl-N-nitrosourea (MNU); mammary tumor; rat; albumin-bound paclitaxel (nab-PTX); intraductal administration

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Introduction

Breast cancer (BC) remains the most frequently diagnosed cancer in women worldwide (1). With accessible screening technologies, such as ultrasound, mammography, and magnetic resonance imaging (MRI), the detection rate of early-stage BC, including ductal carcinoma in situ (DCIS), is growing rapidly (2,3). Increasing our understanding of BC biopathology is very important to the development of new preventive and therapeutic approaches to BC, and animal models are frequently used to study its development and progression. These include rodent models of spontaneous, chemically induced, transgenic, knockout, syngeneic and human xenograft model systems (4). Of these, the chemically induced rat mammary tumor model can mimic human BC initiation and development and has particular advantages: a short latency period, high reproducibility, low-cost, and simple operation. N-methyl-N-nitrosourea (MNU) is a potent carcinogenic agent and the longest-known of the nitroso compounds that cause DNA alkylation that does not require metabolic activation (5).

Among the various rat strains, random-bred female Sprague-Dawley (SD) rats have been generally recommended for use in experimental protocols to study MNU-induced mammary tumors (6,7). The conventional approach entails 1–2 intraperitoneal (i.p) or intravenous injections of MNU to complete the model-building; mammary tumors then appear randomly in the six pairs of mammary glands (8,9). Very occasionally, other organs, such as ovaries, uterus, spleen, kidney, and liver, may also develop carcinoma (10), and these drawbacks limit the stability of the model for studying preventive therapies. Intraductal (i.duc) administration of MNU places the agent directly into the mammary ductal system, thus making it possible to induce tumors at predictable locations (11). Direct contact of rat luminal epithelial cells with MNU may also improve the efficiency of carcinogenesis and i.duc delivery can reduce the administration of MNU. Furthermore, the i.duc mode of injection may also increase the therapeutic efficiency of drug delivery (12). We hypothesized that MNU injected directly into the rat mammary duct system through the nipple would establish a stable mammary tumor model with no effect on untreated mammary glands. We conducted a series of experiments to optimize the dose to induce tumors in the treated mammary gland without affecting other glands or organs. We also used albumin-bound paclitaxel (nab-PTX), an antitumor agent, to demonstrate the suitability of this novel MNU-induced rat model for testing therapeutic agents.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-21-1540).

Methods

Animal studies

Female SD rats, 3–5 weeks of age, were obtained from Hunan Silaike Jingda Laboratory Animal Co., Ltd. (Changsha, China). All animals were housed in groups of three per cage under well-controlled conditions of temperature (22±2 ℃), humidity (55%±10%), and a 12-h light-dark cycle for at least 3 days before undergoing the experimental procedures. The rats had free access to a standard laboratory diet and distilled water. Experiments were performed under a project license (No.: 20180915) granted by Laboratory Animal Welfare & Ethics Committee, Renmin Hospital of Wuhan University, in compliance with Institutional Animal Care and Use Committee guidelines for the care and use of animals.

For the i.duc injection of the carcinogen or drug, anesthesia was induced with 4% isoflurane (1% oxygen) and maintained at 2% isoflurane (1% oxygen). The keratin plug was removed from the treated teat by rubbing it gently with a cotton swab infused with liquid paraffin oil, revealing the duct's orifice, which was cannulated with a 33-G, blunt-ended needle attached to a syringe. MNU or the drug was infused slowly into the mammary gland under visualization with a dissection microscope. The human dose equivalent was calculated with a body surface of 100 g weight of the rat, using the formula $(k\times w^{2/3})\times 10^{-4}$, where k is a constant ($9.5\ m^2/g^{2/3}$) and w is the weight of the rat in grams. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Carcinogen and antitumor drug

Mammary tumors were induced with MNU (Toronto Research Chemicals Co., Toronto, ON), which was dissolved in dimethyl sulfoxide (DMSO; Sigma) and 0.9% saline at a ratio of 1 (gram):10 (mL):10 (mL), respectively. Nab-PTX dissolved in 0.9% saline was provided by CSPC Pharmaceutical Group Limited (Shijiazhuang, China). The MNU working fluid and nab-PTX were freshly prepared

prior to each experiment.

Experimental design

In a pilot experiment, six rats were randomly divided into two groups. The control group of three rats received 2.5 mg i.duc of solvent (DMSO and 0.9% saline), and the experimental group of three rats received a 2.5 mg i.duc dose of MNU (2.5 mg/50 μL solvent) in the 4th mammary gland. Both groups received a second injection 1 week later to ensure tumorigenesis. Based on the results of this pilot, we reduced the dosage of MNU and used a continuous dose gradient design.

A total of 24 rats were randomly divided into four equal groups. The control group (n=6) was given 2 mg i.duc solvent into the mammary duct; different doses of MNU (0.5, 1.0 and 2.0 mg) were given to each of the other three experimental groups (n=6). An additional six rats received an i.p injection of MNU (50 mg/kg) to provide a control for immunohistochemical (IHC) staining for the expression of the estrogen receptor (ER), human epidermal growth factor receptor 2 (Her-2), and Ki-67, with these rats receiving 1 mg i.duc MNU. Before the drug intervention experiment, we also selected an optimal time point. A total of 15 rats were divided into five groups (n=3); after administration of 1 mg i.duc MNU, the rats' mammary glands were removed in the 3rd week for the first group, in the 4th week for the second group, and so on until the 7th week. Mammary glands were processed for hematoxylin and eosin (HE), α-SMA, and calponin staining.

During each experiment, the rats were palpated for tumors every 2 days, beginning 2 weeks after the administration of the MNU. The latency period for palpable tumors was then calculated from the time of the last administration of the carcinogen until the appearance of the first tumor. Visible tumor growth was measured using a dial caliper, and the tumor volume was calculated as follows: tumor volume = (length × width^2)/2. Tumor volume and body weight measurements were taken weekly.

Finally, a drug intervention experiment was conducted to test the efficiency of i.duc delivery. In this study, 18 rats were randomly divided into three groups and administered 20 μL of MNU i.duc. After 5 weeks, one group received i.v (260 mg/m^2, 8 mg) nab-PTX and one received i.duc nab-PTX (100 μL, 0.5 mg), while the third was a vehicle control. The tumor or mammary glands were removed for HE and TUNEL staining when the experiment was terminated.

The pilot experiment was terminated 10 weeks after MNU injection. The subsequent experiments were terminated 16 weeks after administration of MNU. All the animals were killed humanely by CO_2 asphyxiation, followed by physical confirmation of euthanasia and collection of the tumors and internal organs.

Histopathology

Formalin-fixed tissues were processed conventionally through xylene, graded ethanol, and paraffin embedding to obtain 4-μm-thick sections, which were HE stained for histopathological examination.

Immunohistochemistry

Duplicate paraffin sections were deparaffinized, and IHC staining for ER, Her-2, Ki-67, α-SMA, and calponin was performed using rabbit anti-ER polyclonal antibody (pAb) (1:100, Bioswamp, PAB30632), anti-ERBB2 pAb (1:100, Bioswamp, PAB33179), anti-SMA pAb (1:100, Bioswamp, PAB35136), and anti-calponin pAb (1:200, Proteintech, 13938-1-AP). IHC staining was performed as previously described (12). IHC quantification was performed using ImageJ software according to the ImageJ User Guide.

TUNEL staining

Apoptotic tumor cells in paraffin sections of the rat mammary gland tissues were detected by TUNEL assay using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Tissues and cells were prepared according to the manufacturer's instructions, then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme and stained with fluorescein. All nuclei were stained blue by DAPI, and apoptotic cell nuclei were stained green by the TUNEL assay.

Statistical analysis

All the data are shown as mean ± SD. The results were analyzed using GraphPad Prism version 7 (GraphPad Software, Inc.), and statistical differences between means were tested using one-way ANOVA followed by Tukey's test; a significance level of $P<0.05$ was used.

Results

Feasibility of i.duc MNU

The pilot study was conducted to verify that i.duc administration of MNU would induce mammary gland tumors. All three female SD rats in the experimental group to which a i.duc dose of MNU (50 mg/kg) was administered developed measurable tumors in the 4th mammary gland at latencies of 35, 38, and 39 days, respectively. One rat also developed tumors in the untreated 2nd mammary gland (left side), and two developed tumors in both the 3rd and 6th glands (right side). We thus confirmed that i.duc administration of MNU can induce mammary tumors and determined an appropriate dose range for provoking tumors only in the treated mammary glands.

Histopathological alterations in i.duc MNU-treated rat mammary glands

To find the precise dose of MNU that induced mammary tumors only in the treated glands, the main experiment used a dose gradient. All three groups on that gradient (0.5, 1.0 and 2.0 mg) developed mammary tumors, while the control group did not. The rats' tumor-formation latency was recorded every 2 days, beginning 2 weeks after administration of the MNU (*Figure 1A*), and the mean latencies of the three experimental groups (0.5, 1.0 and 2.0 mg) were 43.8, 45.2, and 38.5 days, respectively; according to analysis by one-way ANOVA, this was not a statistically significant difference between the three groups. However, the latencies within the 0.5 mg group showed larger individual differences, and the 2 mg group had the same problems as the pilot experiment, with tumors appearing in untreated mammary glands.

Body weight and tumor volume were measured weekly. The body weights in the control group were slightly higher than those of the experimental groups under the same feeding conditions, but no significant differences were found. The 1 and 2 mg i.duc MNU groups had larger tumor volumes, but there was no statistically significant difference between the three groups (*Figure 1B,C*).

The rats were killed 16 weeks after i.duc MNU administration, and mammary tumors and internal organs were removed, including lungs, liver, bones, spleen, and kidneys. Tumor tissues were HE-stained to identify pathological types; the histological classification of the mammary tumors is shown in *Table 1*. Histological evaluation of tumors revealed the proportion of malignant tumors was higher in the i.duc group. Tumor sections (including the i.p group mentioned below) showed that there were 43% cases of papillary carcinoma, 26.1% cribriform carcinoma, 8.7% ductal carcinoma, 4.3% tubular carcinoma, 8.7% atypical hyperplasia, 4.3% intraductal papilloma, and 4.3% fibroadenoma (*Figure 1D,E*).

Six rats received i.p injections of MNU according to the schematic diagram in *Figure 2A*. One died without tumor development before the end of the i.p group experiment. Tumors appeared in random glands in the i.p group (5 rats available, 60 glands exposed), whereas in the i.duc group tumors were observed only in the treated glands (*Figure 2B*). Development of the tumors was followed at weekly intervals for 16 weeks (*Figure 2C*). The total number of tumors that developed in the i.p group and in the three i.duc groups (0.5, 1.0 and 2.0 mg) were 14/60 (23.3%), 8/12 (66.7%), 10/12 (83.3%), and 9/12 (75%), respectively (*Figure 2D*). The paraffin-embedded tumor tissue in the i.p group was IHC stained for comparison of ER, Her-2, and Ki-67 with the 1 mg i.duc group. ER expression in the 1 mg i.duc group was higher than in the i.p group (49.5% *vs.* 25.2%), but there were no significant differences in Her-2 (0.65% *vs.* 0.52%) or Ki-67 (10.2% *vs.* 11.1%) expression (*Figure 2E,F,G,H*).

Treatment of mammary tumors in novel rat model

Based on these results, it was reasonable to choose the 1 mg i.duc MNU protocol for the rat mammary tumor model, because the i.duc 0.5 mg group had long latency and the 2 mg group had tumor development in unpredictable locations.

To investigate the appropriate time point for early drug intervention, 15 rats were administered 1 mg i.duc MNU, randomly divided into five groups, and then one group was humanely killed and had the mammary glands removed each week from the 3rd to the 7th week thereafter (*Figure 3A*). The paraffin-embedded mammary gland tissues were processed for HE staining and IHC staining for α-SMA and calponin (*Figure 3B*). HE staining showed that mammary intraductal epithelial cells developed from the usual ductal hyperplasia to atypical hyperplasia and then to DCIS. HE and IHC staining showed that the time point of development from atypical hyperplasia to DCIS was between the 5th and 6th weeks after i.duc MNU. We therefore selected the 5th week for nab-PTX intervention.

In the drug intervention experiment, the body weights in the control group were significantly higher than in the

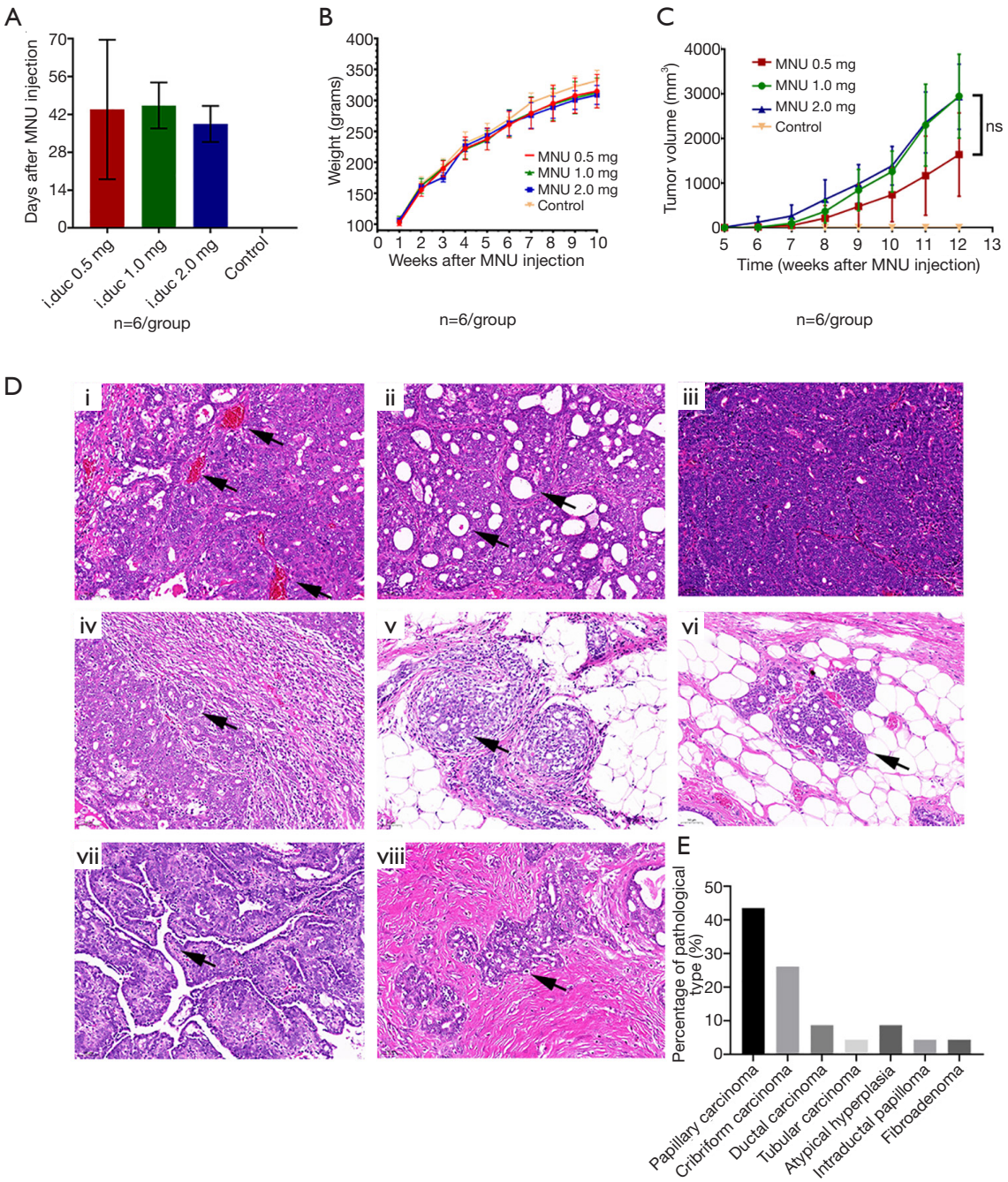

Figure 1 Mammary tumors induced by intraductal (i.duc) dose gradient (0.5, 1.0 and 2.0 mg) N-methyl-N-nitrosourea (MNU). (A) Tumorigenic latency in each group. (B) Curves of body weight recorded weekly. (C) Tumor volume curves recorded after the administration of MNU. (D) Hematoxylin-eosin staining (×200) of different histological patterns in the intraperitoneal (i.p) and i.duc groups [(i) papillary carcinoma (arrows: fibrovascular axis), (ii) cribriform carcinoma (arrows: sieve-like structure), (iii) ductal carcinoma, (iv) tubular carcinoma (arrow: tubule-like structure), (v) ductal carcinoma *in situ* (arrow: lack of myoepithelial cells), (vi) atypical hyperplasia (arrow), (vii) intraductal papilloma (arrow: papillary structure), and (viii) fibroadenoma (arrow: hyperplasia)]. (E) Percentage of each histological pattern observed in the i.p and i.duc groups.

Table 1 Histological classification of mammary tumors developed by MNU in i.duc and i.p groups

Lesions	Number of lesions	
	i.duc MNU	i.p MNU
Benign lesions		
Intraductal papilloma	0	1
Fibroadenoma	0	1
Preneoplastic lesions		
Atypical hyperplasia	2	0
Malignant lesions		
Papillary carcinoma	8	2
Cribriform carcinoma	5	1
Ductal carcinoma	2	0
Tubular carcinoma	1	0
Total	18	5

i.duc, intraductal; i.p, intraperitoneal; MNU, N-methyl-N-nitrosourea.

experimental groups (*Figure 4A*). The i.duc and i.v nab-PTX groups had smaller tumor volumes than the control group, but the difference between the i.v group and the control group was not significant (*Figure 4B*). Setting 4,000 mm^3 as the tumor volume overall survival endpoint, the i.duc nab-PTX group survival rate was 83.3% versus 33.3% for the i.v nab-PTX group and 16.7% for the control group (*Figure 4C*). HE staining showed apoptotic areas in the tumor, and TUNEL-positive apoptotic cells were identified in larger numbers in the i.duc nab-PTX group (*Figure 4D,E*). Ki-67 expression in the i.duc nab-PTX group was also lower than in the other two groups (*Figure 4F*).

Internal organs, including lungs, liver, bones, spleen, and kidneys, were also collected from the drug experiment group. No metastases were found, with the exception of one case of lung inflammation (a large degree of lymphocyte infiltration was observed, but no tumor cells) and one case of regional liver necrosis (Figure S1A,B).

Discussion

BC is the most commonly diagnosed malignant tumor in women worldwide (1), and animal models are one of the most efficient ways to investigate carcinogenesis and the efficacy of therapies for BC (4,13). These include spontaneous, chemically induced, transgenic, knockout, syngeneic and human xenograft models. The carcinogen MNU is the oldest member of nitroso compounds with the ability to alkylate DNA. This carcinogen has been widely used to study the carcinogenesis of breast, ovary, uterus, prostate, liver, spleen, kidney, stomach, small intestine, colon, hematopoietic system, skin, retina and bladder. The MNU-induced rat mammary tumor model is a widely used animal model for mimicking the development of human BC. The conventional approach, using i.p or i.v injection of MNU, leads to unknown numbers of tumors at random locations in the six pairs of mammary glands (14). Because MNU can induce rat mammary tumors through i.p or i.v injection, we hypothesized that i.duc administration of MNU could establish a stable rat BC model with less metastasis in distant organs and with tumors that formed only in the MNU-injected mammary glands. A pilot experiment gave us confidence to continue with a larger study.

The human mammary gland has between 5 and 12 lactiferous ductal systems, while the rat has only one (15); i.duc MNU will thus spread to the whole mammary gland, which was the basis of our hypothesis. Prior to the present study, our team tested i.duc fulvestrant therapy for ER-positive DCIS and found that i.duc fulvestrant inhibits tumor growth better than delivery by intramuscular injection (4). Much of the research on i.duc injection has been related to therapeutic treatment; Stearns, Chun and Jacobs *et al.* (9,16,17) evaluated the effect of commonly used chemotherapy drugs, such as carboplatin, PTX, pegylated liposomal doxorubicin, 5-fluorouracil, and methotrexate, although using an i.p injection MNU rat model. However, when using i.p injections of MNU to establish a model, not all mammary glands develop tumors, yet drug therapy must still cover all six pairs of glands in the rat due to the unpredictable tumor locations. Consequently, i.duc injection of MNU could avoid extra work during research on BC prevention and treatment that uses the MNU-induced rat model.

The i.duc administration of MNU (20 μL, 1 mg) corresponds to a dose that is equivalent to nearly one-eighth that of i.p and tail vein injections. Control group in drug intervention experiment showed a tumor-formation rate of 83.3% using i.duc MNU, and was comparable to the previous studies (18,19). A lower dose of MNU in a localized mammary gland has the advantage of limiting tumor appearance to the treated mammary gland only, reducing the workload of follow-up treatment. For example,

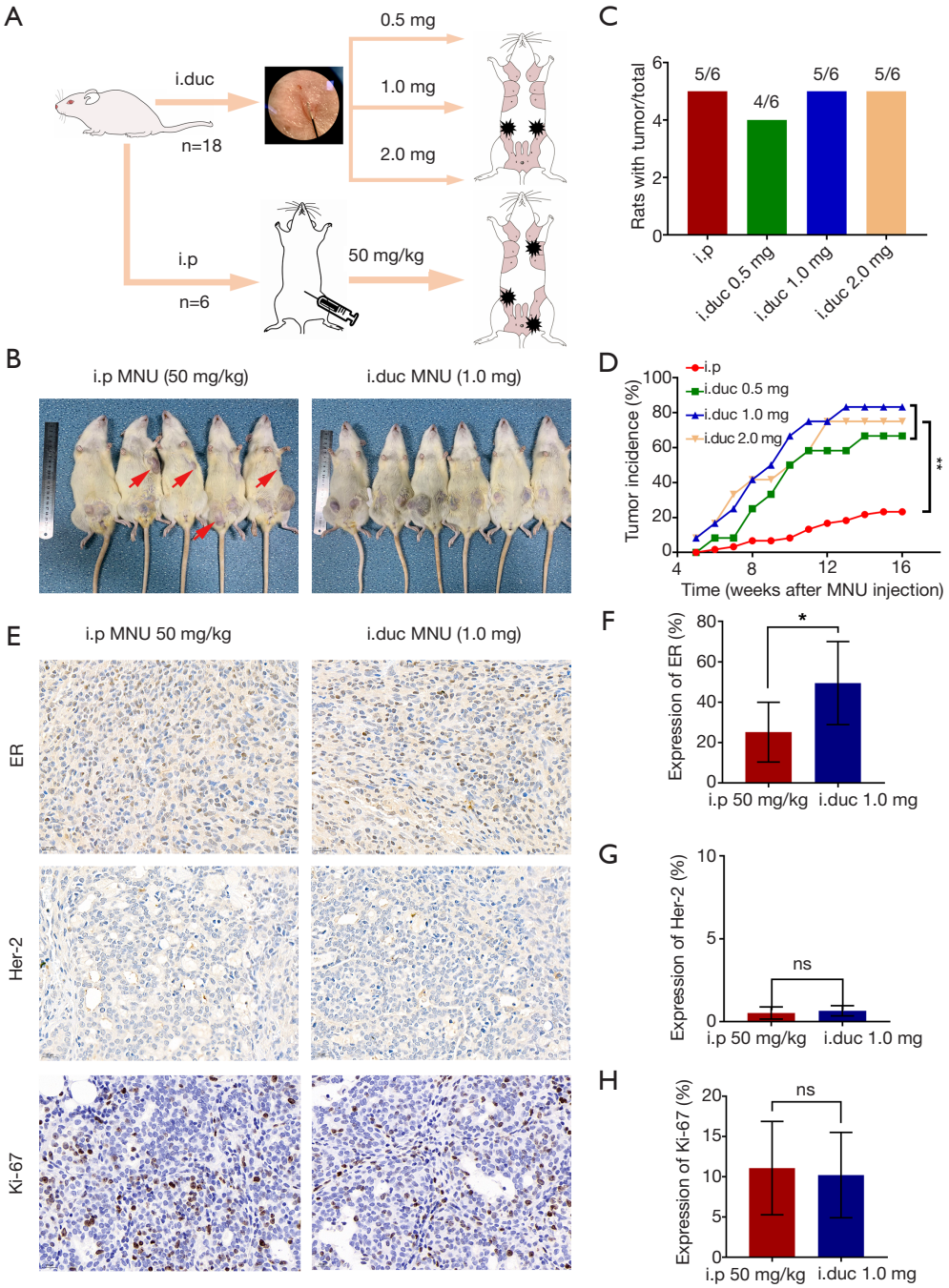

Figure 2 Intraductal (i.duc) N-methyl-N-nitrosourea (MNU) to induce rat mammary tumor has the advantages of predictable tumor location and incidence of tumorigenesis. (A) Schematic diagram of intraperitoneal (i.p) and i.duc tumor model establishment. (B) Rats with induced mammary tumor in the i.p and i.duc groups (red arrows show an arbitrary position in the i.p group compared with the i.duc group). (C) Tumor incidence in each group. (D) Rate of tumor development in i.p and i.duc groups. The total number of tumors induced was 23.3% in the i.p group (60 mammary glands exposed) and 8/12 (66.7%), 10/12 (83.3%), 9/12 (75%) in the i.duc group (12 mammary glands exposed) **, P<0.01. (E) Immunohistochemical expression of the estrogen receptor (ER, 400×), Her-2 (400×) and Ki-67 (400×) in the i.p and i.duc (1.0 mg) groups. (F) ER, (G) Her-2 and (H) Ki-67 are the quantitation of (E). *, P<0.05. ns, not statistically significant.

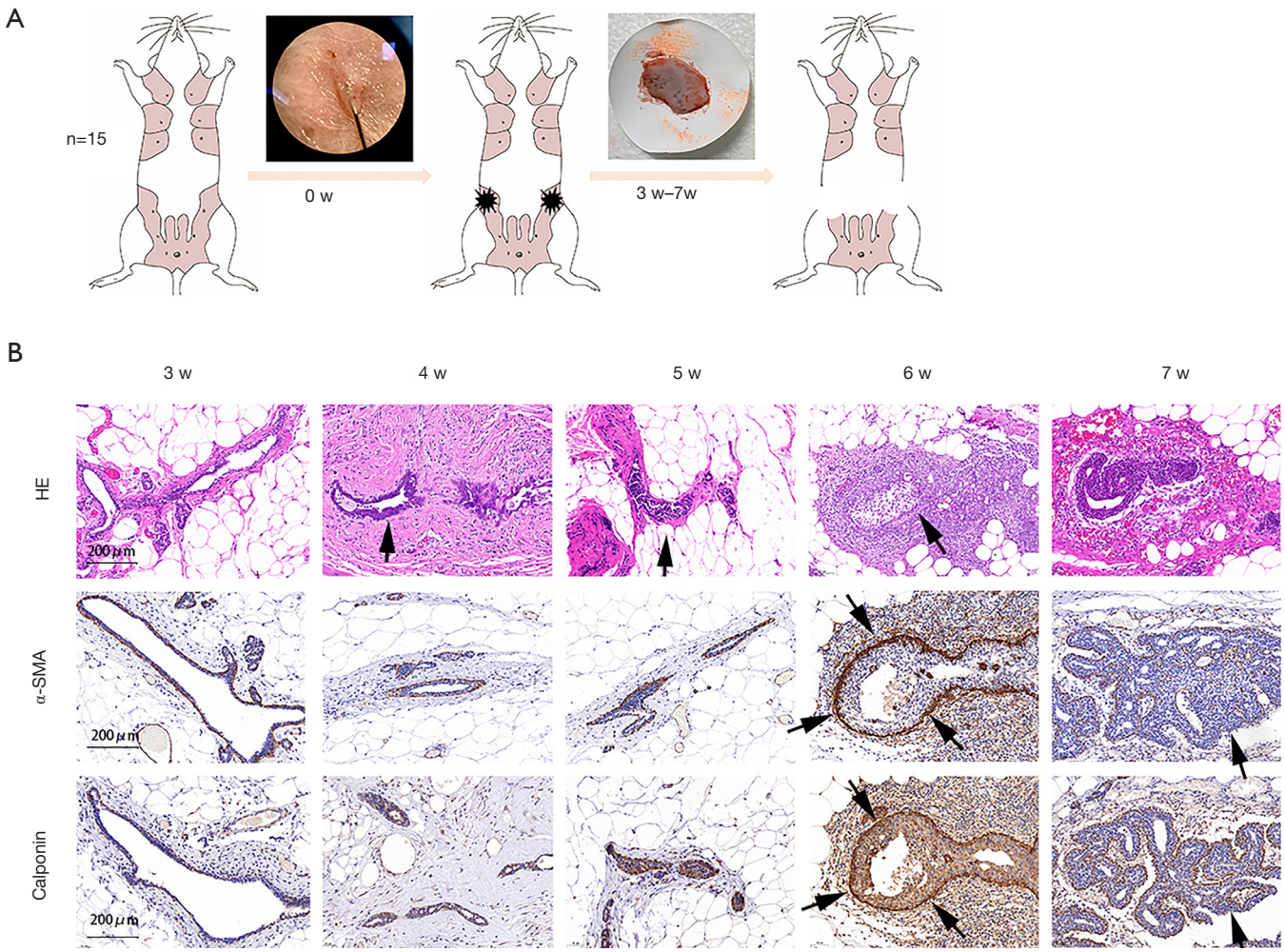

Figure 3 Tumorigenesis induced by intraductal (i.duc) N-methyl-N-nitrosourea (MNU) gradually developed from atypical hyperplasia to ductal carcinoma in situ (DCIS). (A) Schematic diagram of acquiring the rat mammary glands. (B) Histology and immunohistochemical staining of obtained mammary glands. Hematoxylin-eosin (HE) staining shows that i.duc MNU-treated glands developed atypical hyperplasia during the 4–5th week (black arrows) and then DCIS in the 7th week. α-SMA and calponin show myoepithelial cells stained but mammary ductal cells did not, suggesting cell mass was possibly DCIS in the 6–7th week.

with the drug intervention study, we are able to deliver the drug only to the MNU-treated glands, also using i.duc administration.

As a potent carcinogenic agent, MNU induces *Ha-ras-1* mutations in normal mammary ductal epithelial cells (20-22). Mutant ductal epithelial cells gradually develop abnormal hyperplasia, from the usual ductal hyperplasia to atypical hyperplasia to DCIS to invasive carcinoma (4). In this study, induced mammary tumors developed from the rat's mutant cells, and we observed various pathological types and different stages of tumor with individual differences. Papillary carcinoma and cribriform carcinoma accounted for almost 69.6% of tumors, but not all induced tumors were malignant. These results are in accordance with previous studies by Alvarado *et al.* (18) and Faustino-Rocha *et al.* (18,19).

Tumor incidence in the i.p group was lower than in the i.duc group, possibly due to systemic MNU dilution during i.p injection. IHC staining suggested that the i.duc MNU rat model was similar to luminal A type BC, with positive ER expression, negative Her-2 expression, and low Ki-67 expression. The tumor growth rate was slower than that of xenograft tumors, such as in nude mice with mammary fat pad injection of MCF-7 cell lines (23,24). The i.duc MNU

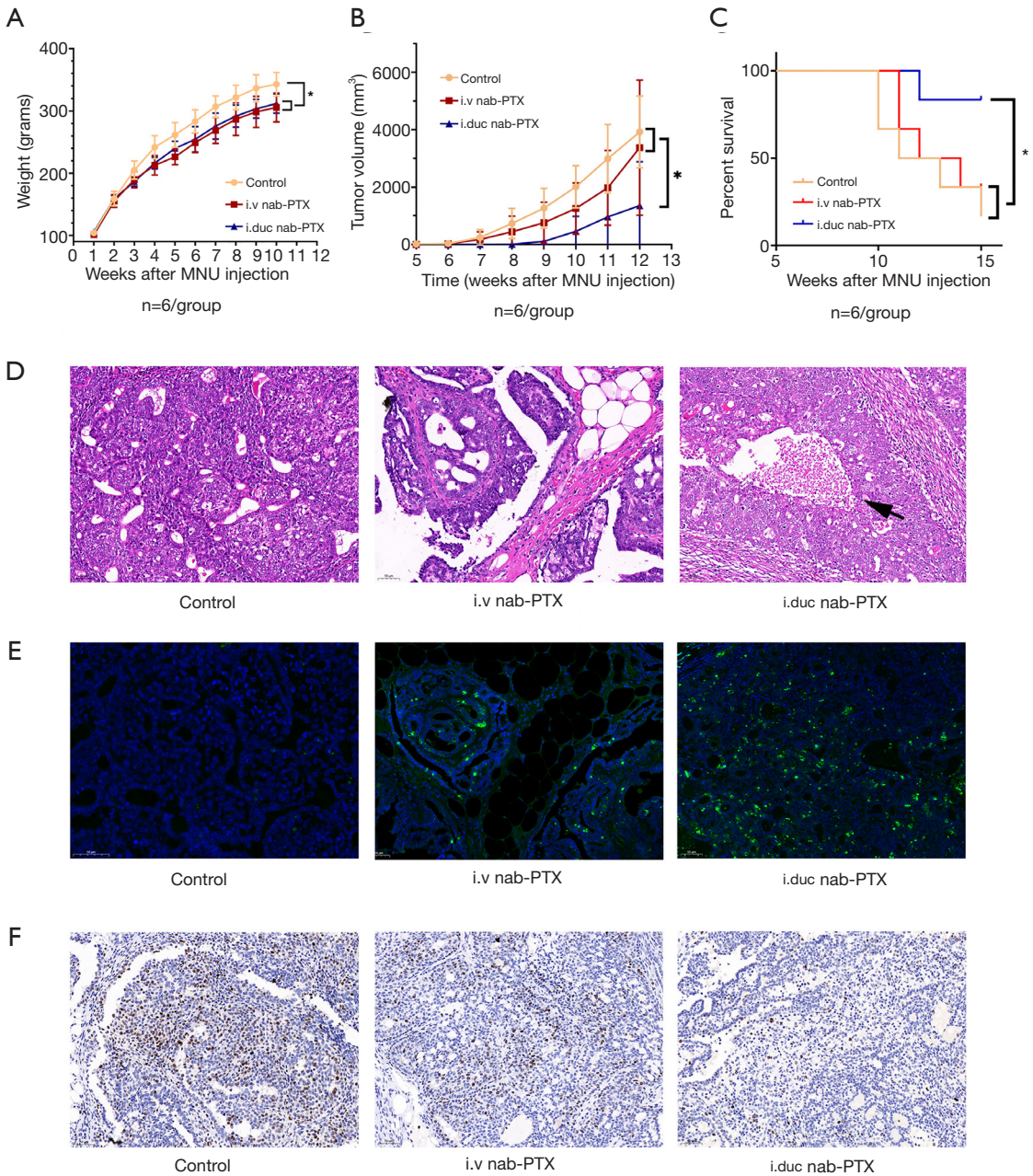

Figure 4 Sensitivity of mammary tumors induced by intraductal (i.duc) N-methyl-N-nitrosourea (MNU) to antitumor drug albumin-bound paclitaxel (nab-PTX). i.duc delivery of nab-PTX to i.duc 1-mg MNU-induced mammary tumor was more effective than i.v injection. (A) Curves of body weight recorded weekly. *, P<0.05. (B) Tumor volume curves recorded after i.duc administration of 1 mg MNU in each group. *, P<0.05. (C) Tumor burden survival (endpoint was set at 4,000 mm^3 for each rat) in i.duc nab-PTX group was significantly higher than for the i.v nab-PTX group and control group. *, P<0.05. (D) Hematoxylin-eosin (HE) staining in each group (black arrow shows necrotic region of tumor, ×200). (E) TUNEL staining in each group and positive cells appear green in color (×200). (F) Ki-67 expression in each group (×200).

rat model was not suitable for drug screening, but does mimic the onset and evolution of BC.

In the drug intervention experiment, rats in the i.v nab-PTX group showed a reduction in physical activity within 1 week of drug administration, and tumor growth in the i.duc nab-PTX group slowed after the intervention. These results suggested both effects and adverse reactions from the drug. The i.duc nab-PTX group received a smaller dose, revealing better efficiency, and HE and TUNEL fluorescence staining both showed apoptotic areas. Okugawa *et al.* (25) administered PTX by i.duc administration in an i.p MNU rat model and did not observe any toxic side effects. The tumor burden in the i.duc group was significantly reduced compared with the i.p. PTX-treated group. Interestingly, in our study one nodule in the nab-PTX-treated mammary gland was confirmed as lymphadenopathy with increased germinal centers. We conjecture that the immune system of that rat may have had a role in overcoming the malignant or premalignant cells (Figure S2).

In these experiments, we did not find any metastases in the internal organs, possibly because the lower dose of i.duc MNU was below the critical amount that could affect distant organs. The tumors induced by MNU may also have poor invasive capacity due to their luminal type with low Ki-67 expression.

Some limitations to the present study should also be noted. Although we could observe the whole process of development from hyperplasia to malignant tumor in the mammary gland, there were also benign tumors, such as intraductal papilloma and fibroadenoma. Benign tumors may not suitable for chemotherapy. In addition, further study needs to elucidate the pathogenesis caused by MNU.

In conclusion, i.duc administration of MNU to establish a rat BC model is feasible. Compared with conventional approaches, such as i.p or i.v injection, the tumor location was fully predictable when using an appropriate dose of MNU. Biological behavior, morphology, and molecular typing were similar to luminal type BC; there was no metastasis to distant organs during the experiment; and this model was sensitive to i.duc drug therapy delivery. The i.duc MNU (20 μL, 1 mg) model could be a novel rodent mammary tumor model for use in the field of BC prevention and treatment.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No.: 20180915) granted by Laboratory Animal Welfare & Ethics Committee, Renmin Hospital of Wuhan University, in compliance with Institutional Animal Care and Use Committee guidelines for the care and use of animals.

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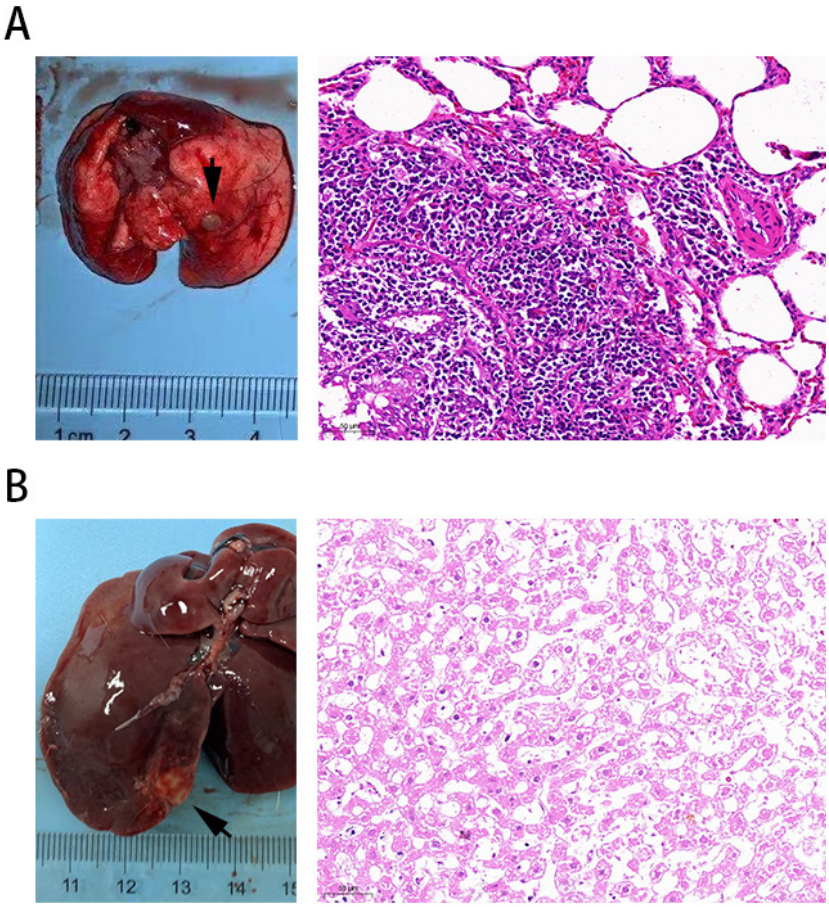

Figure S1 Metastasis was not found during the experiment, although one case of inflammation in the lung tissue and one regional necrosis in the liver were observed. Gross appearance (arrows: abnormal nodule) and hematoxylin-eosin staining (×200) of lung (A) and liver (B).

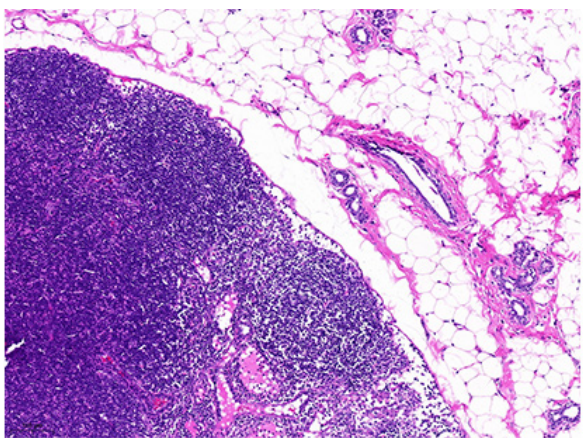

Figure S2 Lymph node without tumor observed in the intraductal (i.duc) group (HE staining, ×200).

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