RESEARCH COMMUNICATION

Establishment of a Fluorescent Implantation Metastasis Model of Bladder Cancer and Real-time Microscopic Detection in Nude Mice

Delin Yang, Haifeng Wang*, Jiansong Wang, Chao Zhang, Hongyi Xu

Abstract

Objective: To establish a fluorescent implantation metastasis model of bladder carcinoma with high metastatic potential in nude mice and observe development and metastasis. Methods: Human bladder cancer EJ cells with high invasive ability were screened and transfected with GFP plasmid to screen stable enhanced GFP-expressing clones instilled into the bladders of nude mice. Subsequent growth, invasion, and metastasis of the implanted tumors were observed and evaluated with a whole-body fluorescence optical imaging system. Results: The transfected bladder cancer EJ cells stably and efficiently expressed EGFP. The growth, invasion and metastasis of the implant bladder tumor were readily observed and accurately evaluated by fluorescent microscopy. In the bladders of nude mice, the rates of EGFP expression detected by flow cytometry at weeks 1-4 were 22.6%, 46.7%, 62.3% and 72.7%, respectively, with clear increase over time. Conclusion: GFP-labeled bladder cancer EJ cells display green fluorescence under fluorescent microscopy and show stable GFP expression. The model will provide a simple and reliable means for studying the mechanism of implantation metastasis of human bladder cancers in vivo.

Keywords: Urinary bladder - implantation metastasis - animal model - green fluorescent protein (GFP)

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Introduction

Metastasis and recurrence are distinctive features of malignant tumors, and metastasis is the main reason for failure of cancer treatment. Studies have shown that bladder cancer recurrence was 15% -50% or even up to 70% in one side of the upper urinary tract cancer (Hisataki et al., 2000). The recurrence rate of superficial bladder cancer patients who were performed by transurethral resection was nearly 50% (Dalbagni and Herr, 2000). Cavity implantation metastasis is the important reason for the postoperative recurrence of bladder cancer and multiple tumor formation (Fadl-Elmula et al., 1999). Consequently, in order to study the mechanics of implantation metastasis and evaluate the effective of drug treatment, including a single gene therapy and combined treatment, we established an efficient, in vivo, dynamic, and visual implantation metastasis animal model of human bladder cancer.

Materials and Methods

Materials

Human bladder invasive EJ cell line is from Institute of Urology, Peking University as a gift; NIH3T3 cell line is purchased from Kunming Institute of Zoology, Chinese Academy of Sciences; Petri dish is purchased from millipore company, USA.; Matrigel and ECM extracts of ESH rat sarcoma are purchased from BD company, USA.; pEGFP-C1 is a plasmid that contains enhanced green fluorescent protein (EGFP), and purchased from Clontech company, USA.; Extraction kit of the endotoxin removed plasmid DNA is purchased from Clontech company, USA.; Transfection reagent LipofectamineTM2000 is purchased from Invitrogen company, USA.; Balb/c, nu/nu nude mice are purchased from Beijing Vital River Co., Ltd. Mice aged 6-8W, weighing 18-20g.

Methods

Screening of high-invasive human bladder cancer cells. Reference to the method of Girnita et al (2006), we put chemokine into the 24-well plates, and put the plug-in culture dishes that coated with 100μg Matrigel into the 24-well plate, added the EJ cells on the membrane. After 7-hours cultures, we removed the invasion chamber, and culture the cells in the 24-well plates. The medium was changed after 4 to 6 days. Until reached lamellar fusion, the cells were digested and passaged. We repeated screening 3 times, and the amount of Matrigel increased 50μg/membrane compared with the previous one. The screened high invasive cells were named as EJ-m3.

*Department of Urology, The Second Affiliated Hospital of Kunming Medical University, Yunnan Institute of Urology, Kunming, China *For correspondence: wanghaifeng0871@gmail.com*

Cell transfection and selection. Reference to the operating manual of LipofectamineTM2000, we transfected pEGFP-C1 to EJ-m3 cell line. 48h after transfection, we screened the cells by the G418(200 ug / ml) culture medium for 2 weeks, and gained cell lines that stably expressing EGFP. We used the method of limited dilution of 96-well plates to screen monoclonal cells, and obtained six monoclonal cell lines that expressed EGFP. We selected the highest EGFP expression cells as the experimental monoclonal cell line, and named it EJ-m3-GFP.

Identification of the cell basic biological characteristics. a) Detection of the cell cycle and EGFP expression. We used the method of flow cytometry to detect the cell cycle and EGFP expression. b.) Growth curve. We made EJ, EJ-m3 and EJ-m3-GFP cells to be cell suspension, seeded them in seven 24-well plates after the cell count. Each cell line was set 3 repeat hole, and each hole was seeded 1×10^4 cells. Begin with the next day, we took one 24-well plate for a cell count, lasting for 7d. This experiment was repeated three times. Then, we drew the growth curve. c.) Detection of the invasion in vitro. EJ, EJ-m3 and EJ-m3-GFP cell lines are added into the invasion chamber, and cultured for 12h. We discarded the liquid on the upper chamber, wiped tumor cells on the membrane and Matrigel with a cotton swab, and made a formalin fixation for 30 min and conventional HE staining. Under the light microscope(×200), we divided the membrane into four quadrants by horizontal and vertical lines, and counted the cells. Then, we discarded the liquid of 24-well plate and made a gentian violet stain on them, and counted the cells attached to the 24 well-plate. Three numbers of Invasion chamber were used for each cell line, and calculated the avera.

Screening of bladder cancer cell line with cavity implantation metastasis potential. Balb/c, nu/nu female nude mice aged from 6 to 8 weeks, and weight ranged from 18 to 20 g. Reference to the method of Crook et al. (2000), we conducted experiments. Mice were sacrificed by dislocation after 4 weeks, and we took the bladder under sterile conditions, and isolated cell with mechanical separation. Then, the cells were cultured in the culture medium containing the neomycin analogue C20H40N4O10.2H2SO4 (G418). After 3 passages, gained cells were confirmed that the EGFP expression loss does not appear, we perfused the bladders of nude mice. This experiment was repeated three times. After 10 passages of the gained cells, we detected the EGFP expression by flow cytometry. We named the cell line with cavity implantation metastasis potential and stable EGFP expression as EJ-im3-GFP.

Establishment of intravesical implant model

We perfused the EJ-im3-GFP cells into the urinary bladder cavity of nude mice, and detected tumor cases by whole-body fluorescence optical imaging system in the 1, 2, 3, 4 W. Each time, after the detection, we sacrificed 1 mouse, and took the bladder under sterile conditions, washed it with PBS, cut it into pieces, resuspended it with trypsase, and put it on the swing bed(37°C). The next day, we detected the EGFP expression by flow cytometry.

Data processing

SPSS 11.5 statistical software was used to analyze the variance, and the results of quantitative data were shown as $\bar{x}$ ±s.

Results

Screening of high-invasive EJ cells

When the 1st screening, there were only more or less 10 cells that can permeate Matrigel in each invasion chamber. The polycarbonate membrane (12μm) attached to the bottom of 24-well culture plates, and part of the EJ cells died during the process of culture. There were only a few clones survive and proliferation. 20 days later, cells show a proliferation into a sheet. And 14 days after that, we made a 2nd screening. The permeated cells increased when making the 2nd and 3rd screening. And the number of the dying cells decreased during the process of culture. Cell proliferation was significantly faster. We named the high-invasive human bladder cancer EJ cells after the 3rd screening as EJ-m3.

2. Monoclonal cell line EJ-m3-GFP that expresses EGFP stable obtained

The adherent EJ-m3-GFP cells showed strong green fluorescence under the fluorescence microscopy, and the fluorescence distributed throughout the cell evenly. And the cells can highly express EGFP after 3 months without the pressure of G418.

3. EGFP expression and cell cycle of the three cell lines

The fluorescent positive rate of EJ-m3-GFP cells was 99.9% under the detection of flow cytometry, and we did not detect the expression of fluorescent cells in EJ-m3 cells. Cell proliferation index (PI) of EJ, EJ-m3 and EJ-m3-GFP cells were listed in Table 1. Using the statistical analysis of variance, we found that there were significant different between the EJ and EJ-m3, EJ-m3-GFP cell lines. The PI was significantly different ($P<0.03$). However, the PI of EJ-m3 and EJ-m3-GFP was no significant difference ($P<0.166$).

Growth curve

The population doubling time of EJ, EJ-m3 and EJ-m3-GFP cell lines were 31.7±0.1h, 27.6±0.2h and 28.5h±0.1 respectively. The cell growth was significant differences between EJ-m3 and its parent cell line EJ ($P<0.05$). And there was no significant difference between cells transfected with EGFP(EJ-m3-GFP) and EJ-m3. ($P>0.05$, Table 2). The growth curve is shown as Figure 1.

In vitro invasion

The number of EJ, EJ-m3 and EJ-m3-GFP invasive cells were 24.9±1.8, 123.0±5.3 and 124.8±7.2 respectively. Using the method of analysis of variance, we found that there was a significant difference between them ($P<0.001$). Q test confirmed there was no statistic

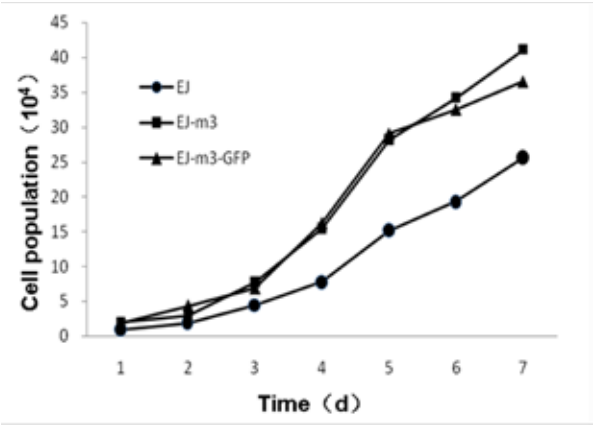

Figure 1. Growth Curves of the Three Cell Lines

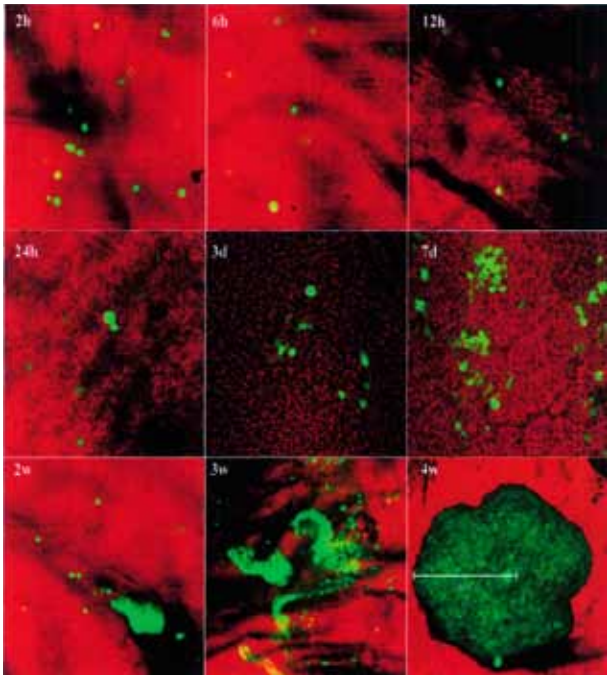

Figure 2. Results for Growth and Metastasis of Bladder Tumor observed under the Whole-body Fuorescence Optical Imaging System

difference of invasiveness between EJ-m3 and EJ-m3-GFP cells(P=0.96), which meant transfection had no effect on the cell invasion. However, the invasiveness between EJ and EJ-m3-GFP cells, EJ-m3 and EJ-m3-GFP cells were significantly different (P<0.001), which meant that the invasiveness of the cells after screening did not increase obviously.

6. Screening cells with cavity implantation metastasis potential

In the first screening, we perfused EJ-m3-GFP into the bladders of nude mice. The bladder of one mouse was found harden, and blood urine was also observed 15 d later, and another in the 19d. The other two were not found such a change. 4W later, the 4 nude mice were executed. We found that two mice had visible tumor formation. In the second screening, using bladder tumor cells that screened from the first screening, the 4 nude mice were found harden bladder and blood urine in the 14d, 15d, 19d and 22d. When the 4 nude mice were executed, all the mice were found visible tumor formation. In the third

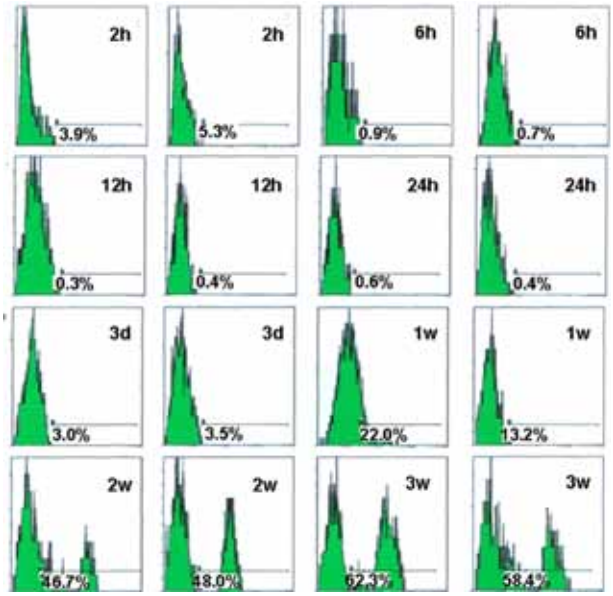

Figure 3. EGFP Expression Detected by Flow Cytometry

screening, using cells that screened from the second screening, the 4 nude mice were found harden bladder and blood urine all in about 14d.

After 24h culture, using the cell suspension obtained from the improved mechanical separation method, we could see the spindle and round-like adherent cells. When the first passage in 7d, the appearance of the cells was quasi-circular. We cultured the quasi-circular cells from each nude mouse bladder when the second and third screening. After 4d culture, using cell suspension gained from the third screening, we made a passage of the cells.

The screened cells shown strong green fluorescence under the fluorescent microscope, and the fluorescence distributed throughout the cell evenly. The cells obtained from the third screening 10 times, and detected the EGFP expression by flow cytometry. The rate of EGFP expression was 99.9%. We named this cell line as EJ-im3-GFP.

Establishment of intravesical planting model

We perfused EJ-m3-GFP into the bladders of 5 nude mice. There was no naked-eye change in the 1W, but we can see tumor formation under the whole-body fluorescence optical imaging system. In the 2W, 5 nude mice were found harden bladder and blood urine. And we can see multiple tumor formation. 3 weeks, 4 weeks later, tumor volume and number are significantly increased (Figure 2).

Using the method of flow cytometry, we found that the EGFP expressions were 22.0%, 46.7%, 62.3% and 72.7% in the 1W, 2W, 3W and 4 W. Moreover, it can be found the expression increased with the time going. And it was consistent with the results of the whole-body fluorescence optical imaging system (Figure 3).

Discussion

Invasion is the first step of metastasis, and the basic

characteristic of metastatic tumors. Adhesion, proteolysis and cell migration are the three main factors of invasion. In this research, we isolated high-invasive human bladder cancer subline using the invasion model in vitro, and took the following principles: (1) The NIH3T3 cell conditioned medium was as a chemokine, which made tumor cells moving to the 24-well plates; (2) Matrigel was used to simulate the basement membrane, because it was rich in type IV collagen and its components is very similar to the basement membrane. (3) The diameter of Millicell Chamber's carbon membrane pore was 12nm, which was smaller than the diameter of tumor cells. Tumor cells should make a deformation to move through the membrane hole. The tumor cells isolated from this model had a strong capacity of proteolysis and movement, which has a strong invasive potential.

Green fluorescent protein as a reporter gene is currently used in many fields, and widely applied in cancer research, especially in vivo and in vitro studies of tumor metastasis(Klemk et al.,2007; Boissonnaset al., 2007), tumor neovascularity(Amoh et al., 2007), and Cancer Gene Therapy (Goding et al., 2007). Studies show that tumor cells that express the green fluorescent protein stably could be used in the research of tumor growth and metastasis (Deng et al., 2007; Yanget al., 2007). Green fluorescent protein allows us to observe the location of the tumor cells in vivo. In this study, we used the method of liposome transfection to obtain the monoclonal EJ-m3-GFP cell line with high EGFP expression. And we used this cell line to perfuse the bladders of nude mice. After tumor formation, we took the bladder of the mice and recultivate it in order to obtain EJ-im3-GFP with cavity implantation metastasis potential. Moreover, we used EGFP as a marker and G418 as a screening tool to gain cells that glow under fluorescence microscopy. This method can be used for in vivo fluorescent cell screening and provides a new reliable way to screen cells in vivo.

The process of metastasis includes cells shed from the primary tumor, adhesion and invasion to the planting site, proliferation to form metastases. In this study, we use the technique of EGFP transfection and molecular imaging to establish a model of visible bladder cavity metastasis. This model has the following advantages: (1) Cells used to build the model are obtained by the two screening in vitro and in vivo, and had characteristics of high invasion and cavity implantation metastasis stable. Consequently, it can ensure the tumor formation rate of the model was nearly 100%; (2) Cells used to build mode was bladder cancer cells with high expression of EGFP, and we can use whole-body fluorescence optical imaging system to observe tumor cells through the body without mice were killed and dynamically observe the growth of tumor cells.(3) Bladder wall of the mouse is thin and EGFP expression in bladder tumor cells are relatively strong, thus even small tumors can be seen. Comparing to the traditional method of biopsy, this model can make us dynamically, intuitively and three-dimensional evaluate the size and number of the tumor. (4) The model can make a quantitative analysis of the amount of bladder tumor cells by flow cytometry and better reflect the growth of tumors.

In conclusion, this model can provide a way to study the mechanism of cavity implantation metastasis of bladder cancer, and can be used as an animal model to evaluate drugs on the prevention and treatment of bladder cancer.

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