Establishment of a visualized nude mouse model of cervical carcinoma with high potential of lymph node metastasis via total orthotopic transplantation

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Summary

Objective: To screen cervical carcinoma (CC) SiHa subgroups with high potential of lymphatic metastasis and establish a visualized nude mouse model of cervical carcinoma with a total orthotopic transplantation approach. *Methods:* A cervical carcinoma SiHa subgroup with high potential of lymphatic metastasis was isolated by in vitro and in vivo primary culture and continuous passage screening of cervical carcinoma SiHa cells that stably expressed enhanced green fluorescent protein (EGFP). Forty male nude mice aged 6-8 weeks were equally randomized to group A receiving unscreened SiHa/EGFP cells, and group B received in vitro and in vivo screened SiHa/EGFP cells with high potential of lymph node metastasis. *Results:* In the 20 animals of group A receiving orthotopic transplantation of unscreened SiHa/EGFP cells, the primary tumors were small; local lymph node metastasis was observed in five animals; local organ invasion and distal lymph node metastasis were observed in two animals; and no lung metastasis was observed. In the 20 animals of group B receiving screened SiHa/EGFP cells, local lymph node metastasis occurred in all animals; distal lymph node metastasis was observed in 18 animals; and lung metastasis was observed in seven animals. *Conclusion:* A cervical carcinoma SiHa subgroup with high potential of lymphatic metastasis was isolated and a visualized nude mouse model of cervical carcinoma with high potential of lymph node metastasis was established through total orthotopic transplantation successfully. This provided a good platform for the further study of cervical carcinoma-related mechanisms, especially mechanisms of lymphatic metastasis in cervical carcinoma.

Key words: Cervical carcinoma; Orthotopic transplantation; Lymphatic metastasis; Visualized animal model; Enhanced green fluorescent protein (EGFP).

Introduction

Cervical carcinoma (CC) is a common malignant tumor in gynecology, affecting about 500,000 women in the world yearly. One-third of the yearly total incidence occurs in China, and is an important cause of cancer-related deaths in women next to breast cancer. Lymphatic metastasis is the main metastatic route of CC, and the presence or absence of metastasis in pelvic lymph nodes is an independent prognostic factor in CC patients. It is therefore necessary and important to establish an ideal CC animal model to further explore the pathogenesis of CC and mechanisms of lymphatic metastasis, develop new contrast agents, and evaluate potential therapeutic methods for the treatment of CC.

Materials and Methods

Materials used in this study included enhanced green fluorescent protein (EGFP)-labeled CC SiHa cell strain prepared and stored in our laboratory; RPMl1640 solution (Genome Biomedical Technologies Co., Ltd., Hangzhou, China); fetal bovine serum (FBS) (sijiqing, Hangzhou); healthy specific pathogen-free (SPF) grade male nude mice aged 6-8 weeks and weighing 20-25 g and male nude mice aged 3-4 weeks and weighing 15-20 g (Experimental Animal Center of Southern Medical University, Guangzhou, China).

Inoculation of CC SiHa/EGFP cells via the tail root

CC SiHa/EGFP cells were cultivated routinely in 10% FBS-RPMI1640 at 37°C, 5% CO_2 and saturated humidity. Non-contaminated cells growing to 80-90% confluence (exponential phase) were harvested from the medium and washed twice with PBS, added with 0.25% trypsin containing 0.02% EDTA (just submerging the cell surface), digested for 1-2 min, and observed under an inverted microscope. When most cells became round or oval, the medium containing 10% FBS-RPMI1640 (more than two-fold volume of the trypsin) was added to terminate the digestion. Confluent cells were dispersed gently to form cell suspension, which was pipetted into a centrifuge tube and centrifuged at 600 rpm for 3 min. After removing the supernatant, cells were washed with serum-free RPMI1640 twice, added with an appropriate amount of RPMI 1640 for re-suspension with the cell concentration adjusted to 1 x 10^7 ↑·m1 $^{-1}$ to prepare single-cell suspension, which was then placed on ice for use.

Three male nude mice aged 3-4 weeks were injected with 0.2 ml single-cell suspension (2 x 10^6 cells/each) via the tail root, and raised in the SPF environment. The growth status of the animals and tumors was observed weekly.

Screening of highly metastatic CC cells

When the above tumor-bearing mice presented with obvious signs of cachexia failure, they were anesthetized by intraperi-

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toneal (IP) injection of 20 mg/kg 1% sodium pentobarbital. The distal metastatic lesion was removed under strict aseptic conditions under the guidance of total internal reflection fluorescence (TIRF) imaging, from which tissue that was active in the periphery were picked out, submerged in ice PBS containing 500 IU/ml penicillin and 500 IU/ml streptomycin, and transferred as soon as possible to the laboratory superclean bench for primary culture. The tumor tissue was removed from the superclean bench, and washed with PBS containing penicillin and streptomycin three times. Red blood cells and surface contaminants were removed. The tissue was washed more times if it was heavily contaminated. The washed tissue was then transferred to a clean sterile penicillin vial and sheared to 0.5-1 mm^3 chips with ophthalmologic scissors, which were spread evenly over the bottom of the flask with a dripper, to which 5 ml PRIM1640 containing 500 IU/ml penicillin, streptomycin and 20% FBS was added. The flask was placed upside down and cultured in a 37.5% CO_2 incubator until cells grew to confluence. One hour after culture, the flask was turned over gently to moisten the tissue chip, and at 2 h the flask was turned to the normal position gently. By 30 h, individual cells were seen creeping out of the periphery of the tissue chip, and increased in number daily. By day 4, more cells were seen creeping out of the tissue chip, and generation amplification was performed by day 10 when even more cells were seen creeping out of the tissue chip. Fluorescence-labeled cells were screened out by flow cytometry and amplified. The amplified cell suspension was injected to the tail root of the mice. This procedure was repeated in triplicate to screen out a CC SiHa subgroup with high potential of metastasis.

Establishment of the visualized nude mouse model through orthotopic transplantation

Ten healthy SPF male nude mice aged 3-4 weeks were equally randomized to two groups: animals in group A receiving unscreened CC SiHa/EGFP cells, and animals in group B receiving CC SiHa/EGFP cells that were screened continuously in vitro via subcutaneous neck injection of 0.2 ml single-cell suspension of cervix carcinoma (2 x 10^6 cells/each). The animals were then sent back and raised in the SPF environment, and the growth status of the mice and subcutaneous tumor was observed closely.

To raise the adaptive ability and survival of tumor cells in the body of the mice, they were passaged continuously in vivo. When the first passage of subcutaneous cervix carcinoma cells grew to a 0.5 cm^3 tumor, the animal was anesthetized IP with 20 mg/kg 1% sodium pentobarbital. The peripheral tissue with active tumor cell growth was removed, washed with normal saline (NS) containing l00 IU/ml penicillin and streptomycin three times, placed in a clean sterile container, sheared into 1-2 mm^3 tissue chips, placed on ice and moistened with NS. An additional five male nude mice aged 3-4 weeks were anesthetized and tumorigenized by the subcutaneous pocket method. This inter-mouse passage procedure was repeated three times, and each passage consisted of at least five mice. The subcutaneous tumor of the last passage was used as the tumor source for surgical orthotopic implantation (SOI) of CC.

Forty male nude mice aged 6-8 weeks were equally randomized to two groups: animals in group A received passage 3 subcutaneous tumor tissue from inoculation of unscreened SiHa/EGFP cells (control group), and animals in group B received passage 3 subcutaneous tumor tissue from continuous inoculation of screened SiHa/EGFP in vitro (study group). The animals were anesthetized by IP with 20 mg/kg 1% sodium pentobarbital, fixed and draped aseptically. A median incision was made in the lower abdomen to expose the abdominal cavity. The omentum and intestine were pushed upward and moistened with normal saline (NS)-soaked gauze. The urinary bladder was exposed, behind which was seen the "Y"-shaped tubular uterus. A small incision was made at the cervical level, through which the fresh tumor chip was fixed in the cervical muscle with a 9/0 non-traumatic suture. The abdominal organs were then restored in place, and the abdomen was closed with 4/0 absorbable catgut. The skin at the suture site was sterilized with alcohol. The animals were sent back to the SPF environment. Caution should be taken lest the uterus-related vessels and lymph node should be damaged during the surgical procedure.

Table 1. — *Correlation between tumor volume and metastasis in group B.*

Nude mice	Volume (mm^3)	Local lymph node	Adjacent organ (rectum and bladder)	Distal lymph node	Lung
1	327	+	+	+	–
2	456	+	+	+	+
3	163	+	+	+	–
4	406	+	+	+	+
5	179	+	+	+	–
6	189	+	+	+	–
7	252	+	+	+	–
8	308	+	+	+	–
9	489	+	+	+	+
10	512	+	+	+	+
11	297	+	+	+	-
12	634	+	+	+	+
13	128	+	–	–	–
14	381	+	+	+	+
15	208	+	+	+	–
16	189	+	+	–	–
17	316	+	+	+	–
18	484	+	+	+	+
19	298	+	+	+	–
20	186	+	+	+	–

Results

1) Subcutaneous and tail root injection of CC SiHa and SiHa/EGFP single-cell suspension (2 x 10^6 cells/each) and orthotopic transplantation of the tumor tissue chip were 100% successful.

2) The overall fluorescence imaging system suggested extensive distal metastasis at week 7 of tail root injection of tumor cells other than the primary focus, indicating that spontaneous metastasis was likely to occur in animals receiving tail root injection of tumor cells.

Metastasis of orthotopic transplantation of CC tumor

The 20 mice in group A collapsed and died about two to three months after orthotopic transplantation of the CC tumor, when autopsy showed that the tumors grew slowly; their size was relatively small; local lymph node metastasis was seen in five animals; local organ invasion and distal lymph node metastasis were observed in five animals; and no lung metastasis was observed. The 20 mice in group B collapsed and died about two

Fig. 1

Fig. 2

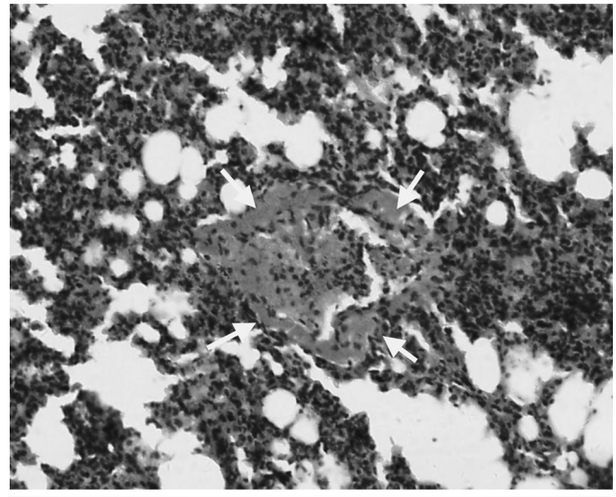


Fig. 3

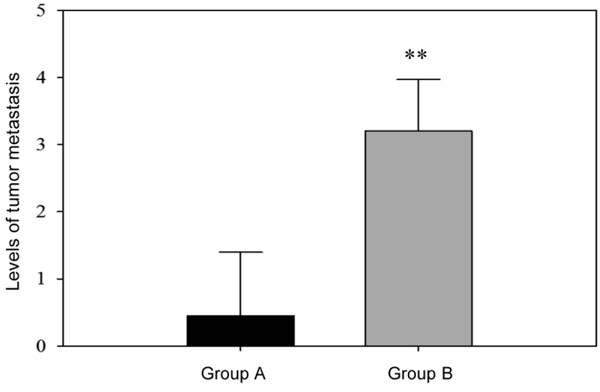


Fig. 4

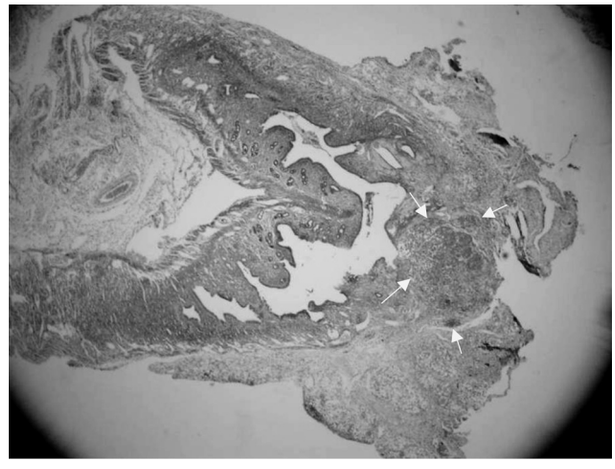


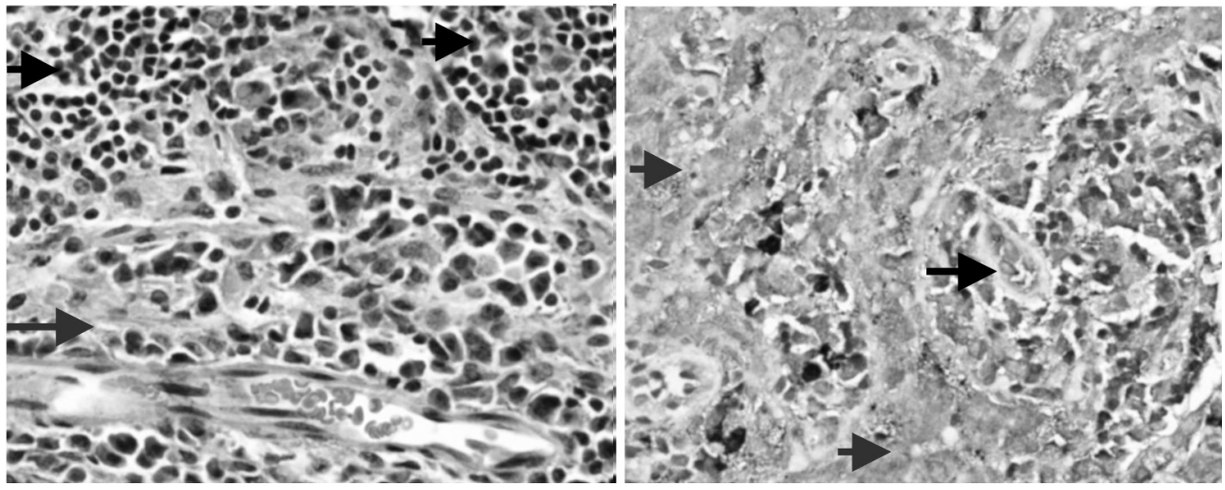
Figure 1. — Presence of local infiltration and distal metastasis after orthotopic transplantation of CC.
Figure 2. — Presence of lung metastasis after orthotopic transplantation of CC (arrow).
Figure 3. — Comparison of tumor metastasis in nude mice between group A and B, ** = $p < 0.01$.
Figure 4. — Pathological sections two weeks after orthotopic transplantation of CC (x 40) (arrow).

months after orthotopic transplantation of the CC tumor, when autopsy showed that other than the primary lesions, local lymph node metastasis was observed in all 20 animals; distal lymph node metastasis was observed in 18 animals (Figure 1), and distal lung metastasis was observed in seven animals. The metastatic lesions were removed and HE stained (Figure 2). The degree of metastasis was converted digitally as + = 1, ++ = 2, +++ = 3 and ++++ = 4, analyzed statistically, and mapped (Figure 3). The result showed that the difference between the two groups was statistically significant ($p < 0.01$).

A CC Siha subgroup with high potential of lymph node metastasis was screened out successfully, and a visualized nude mouse model through orthotopic transplantation was established. The overall fluorescence imaging system suggested that local lymph node metastasis was the main form of metastasis in the early phase of CC orthotopic transplantation, followed by invasion of the lung and other distal organs, and that the size of the primary tumor was positively correlated with invasion (Table 1). The volume (V) of the tumor chip was determined by measuring three mutually vertical diameter lines with a caliper, which were marked as L (length), W (width) and H (height). Calculation was made by using the equation: V = L x W x H/2.

Pathological results

Pathological specimens obtained at two weeks after orthotopic transplantation suggested that the location of orthotopic transplantation was correct. Microscopy showed that the tumor cells had a large volume, rich cytoplasm, deep-stained nuclei, and obvious atypia. The formation of cancer nests was seen (Figure 4). The cancer tissue inside the lymph node metastasis had an extremely rich blood supply; tumor cells were poorly differentiated; the nuclei were large and deeply stained, mostly showing pathological mitosis. Lymph node CK immunohistochemistry showed brown-yellow (Figure 5).



A) x 400 B) Lymph node CK immunohistochemistry (x 400)

Figure 5. — Pathological sections of orthotopic transplantation of CC (blue arrow indicating cancer cell, and black arrow indicating lymphocyte).

Discussion

The orthotopic transplantation strategy and tumor cell heterogeneity are two important ideas promoting the rapid development of tumor animal models in recent years. They are the important and generally accepted theoretical basis for human cancer invasion and metastasis [1]. Lymphatic metastasis is the main route of CC metastasis. Early detection of lymph node metastasis in the region of primary CC is the core problem in selecting an appropriate therapeutic program. Therefore, establishment of an ideal animal model of lymph node metastasis is an indispensable tool for such research, but there is no study reporting the establishment of a nude mouse model of CC through orthotopic transplantation.

Tumor metastasis is a complex and highly selective process, based on the theory of tumor cell heterogeneity advanced by Fidler *et al*. [2] in the 1970s. Fidler *et al*. [3] succeeded in isolating B16-FIO subgroups with high potential of lung metastasis by injecting B16 melanoma cells through the C57BL/6 mouse tail vein. Subsequently, Nicolson *et al*. [4] isolated subgroups with high potential of adrenal and ovarian metastasis from B16. Later, many researchers successfully isolated tumor cells with high potential of metastasis by posing selection pressure intentionally. They even isolated metastasis-associated tag genes in breast cancer [5-7], digestive tract tumors [8], and respiratory tract tumors [9]. Compared with their parent cell lines, these highly metastatic subgroups presented with high abilities of invasion and metastasis, and high tumorigenesis as well.

Based on the above findings, we postulated that lymph node metastasis in CC patients may be due to subgroups of cells with high potential of metastasis in the primary tumor. In light of previous experience of other studies, we obtained a Siha subline with high potential of lymph node metastasis by primary culture and continuous passage screening via tail root injection in nude mice, and established a visualized nude mouse model through orthotopic transplantation to analyze the status of lymph node metastasis. The result confirmed the existence of a subgroup with high potential of lymph node metastasis, thus laying a foundation for exploring the molecular mechanism of lymph node metastasis in CC.

The development of bioluminescence technique provides a new approach to the study of tumor biology. In 1997, Chishima *et al*. [10] first used green fluorescent protein (GFP) in tumor research in vivo, thus opening up a visual precedent for cancer research. Later, Hayashi *et al*. [11] used GFP-bearing tumor cells and observed how cells moved in lymphatic ducts and how they entered lymph nodes. Cells used in the present study fully exhibited the necessary characteristics of the fluorescent tumor model system, whereby micrometastatic foci could be easily detected by the fluorescent label borne by tumor cells, and actual tumor growth could be reflected more accurately, thus reducing human experimental errors.

In summary, the development and progression of CC is complex. The establishment of the nude mouse model of cervical carcinoma with high potential of lymph node metastasis through orthotopic transplantation makes it possible to reproduce the biological characteristics of CC and reflect multiple clinical aspects of the disease, especially the patterns of CC tumor invasion and metastasis. It was found that metastasis in CC follows the pattern of local lymphatic metastasis in the early phase, followed by invasion of adjacent organs, distal lymphatic metastasis, and finally metastasis to the lungs and other organs. It was also found that the size of the primary tumor is positively correlated with tumor metastasis and invasion. These findings indicate that it is feasible to use the established model to explore mechanisms underlying the development and progression of CC, develop new therapeutic methods and drugs, and study mechanisms and

therapies of lymph node metastasis. In addition, the existence of a fluorescent reporter molecule makes quantitative analysis of the corresponding experimental results more flexible and effective.

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