RESEARCH ARTICLE

Unique Pattern of Intraperitoneal Inoculation of Murine Breast Cancer Cell Line 4T1 in Female BALB/c Mice: Invasion of Skeletal Muscle by Breast Cancer

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ABSTRACT

Breast cancer is one of the most commonly diagnosed cancers in women that generally metastasizes to local and distal organs. However, invasion of skeletal muscle by breast cancer is an unusual and rare phenomenon. We report here a murine breast cancer model of invading skeletal muscles by breast cancer. The model consists of an intraperitoneal inoculation of 4T1 cells, a murine breast cancer cell line in female BALB/c mice. After 4 weeks of injections, all the animals developed a butterfly-like sheath structure on the dorsal side of the liver. Histological evidence suggests invasion of the skeletal muscles by breast cancer cells. Metastasis to the liver and lung was observed in some animals. To our knowledge, this is the first observed occurrence of its kind in an animal model. This model could be beneficial in the development of anti-metastatic and anti-cancer drugs.

1 Introduction: Primary or secondary cancers are the leading cause of death world-wide.[1,2] Breast cancer is the second most common cause of death in women in the United States. Most breast cancer deaths are due to metastasis to distal sites or recurrence at the primary site.[3] Breast cancer typically metastasizes to the lung, liver, brain, lymph nodes and bone. However, invasion of skeletal muscle by breast cancer is rare and uncommon.

Since one model will not fit all studies, depending on the nature of the study, a number of murine models are used to mimic the natural process of breast cancer.[4,5,6] These models include tissue or cell inoculation, via ectopic, xenograft, subcutaneous, orthotopic, intra-cardiac, and

tail vein or spleen administration.

Various human and murine breast cancer cell lines are used in clinical settings and experimental breast cancer studies. Among several murine cell lines, the 4T1 breast cancer cell line is the most popular and extensively used in in vitro and in vivo studies in female BALB/c mice. The 4T1 cell line has several characteristics that make it suitable for experiments in animal models for human cancer. It has several advantages over other cancer cell lines because it closely resembles tumor growth and metastasis as seen in human breast cancers. Depending upon the route of administration, the inoculated 4T1 cells metastasize to the lung, liver, heart, spleen, lymph nodes, spine, brain and bone. These include[7-10]:

i) Subcutaneous: Lung

ii) Subcutaneous (Mammary Pad): Lung, liver, heart, brain, lymph nodes and bone

iii) Orthotopic: Lung and bone

iv) Tail Vein: Lung and spine

v) Spleen: Liver

vi) Cardiac: Spine

In the present study we report the invasion of skeletal muscles by 4T1 breast cancer cells when administered intraperitoneally into female BALB/c mice. Skeletal muscle breast cancer metastasis is a rare phenomenon; however, we believe this study will further the understanding and various pathways of metastasis. This is a part of a larger project of experiments where we focus on different aspects of metastasis, to investigate the triggering factors for cancer cells to metastasize to different organs. In the following study we focused on skeletal muscle aspect of metastasis.

2 Materials and Methods:

2.1 Cells and culture: The murine breast carcinoma cell line 4T1 was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). The 4T1 cells were maintained in DMEM supplemented with 10 percent fetal bovine serum, and 100 U/ml penicillin (antibiotic) and 100 µg/ml streptomycin (antibiotic). The media and sera used were obtained from ATCC, and penicillin and streptomycin (antibiotics) were from Gibco BRL (Long Island, NY, USA). The 4T1 breast cancer cells were cultured in T-175 flasks in complete media. At near confluence the cells were trypsinized, centrifuged at 4^0 C at 1500 rpm for 10 minutes. They were washed twice with sterile PBS. The pellet was suspended in PBS and the number of cells counted.

2.2 Animals: The Female BALB/c mice (approximately 5 to 6 weeks old on arrival) were purchased from Simonsen Laboratories (Gilroy, CA, USA). They were maintained in microisolator cages under pathogen-free conditions on a 12hr light/12hr dark schedule for one week. All procedures were performed according to humane, customary care and use of experimental animals and followed the protocol approved by the Internal Animal Care and Use Committee (IACUC).

2.3 Experimental Design: After housing for one week, the female BALB/c mice (n = 5) were inoculated intraperitoneally (IP)

with $1x10^6$ 4T1 cells in sterilized PBS. After inoculation, the mice were returned to their cages and they had free access to a diet consisting of Purina rodent feed and water. Four weeks later the mice were sacrificed and the abdominal cavity of each animal was opened. A colorless butterfly-like sheath attached to the dorsal side of the liver of each animal was dissected out and washed with saline. The liver, lung, kidney, heart and spleen were excised and inspected for metastasis.

2.4 Histopathology: The tissue and liver samples were fixed in 10% buffered formalin, embedded in paraffin and cut into 4- to 5-micron sections. The sections were deparafinized through xylene and graded alcohol series of water and stained with H&E for microscopic evaluation by IDEXX Reference Laboratory (Sacramento, California).

3 Results:

3.1 Mean initial and final weight of the animals: The weight of the animals before IP inoculation was 19.5 +/- 1.1 grams and at the time of sacrifice 21.2 +/- 1.6 grams.

3.2 Tumor Morphology: The abdominal cavity of each animal was opened and all the mice had a colorless butterfly-like sheath attached to the dorsal side of the liver (Figure 1). Each tissue was excised and washed. A single excised tissue is shown in Figure 2. No metastasis was evident in the kidney, spleen and heart. However, metastasis in the liver (Figure 3) and lung (Figure 4) was observed in 3 of 5 mice.

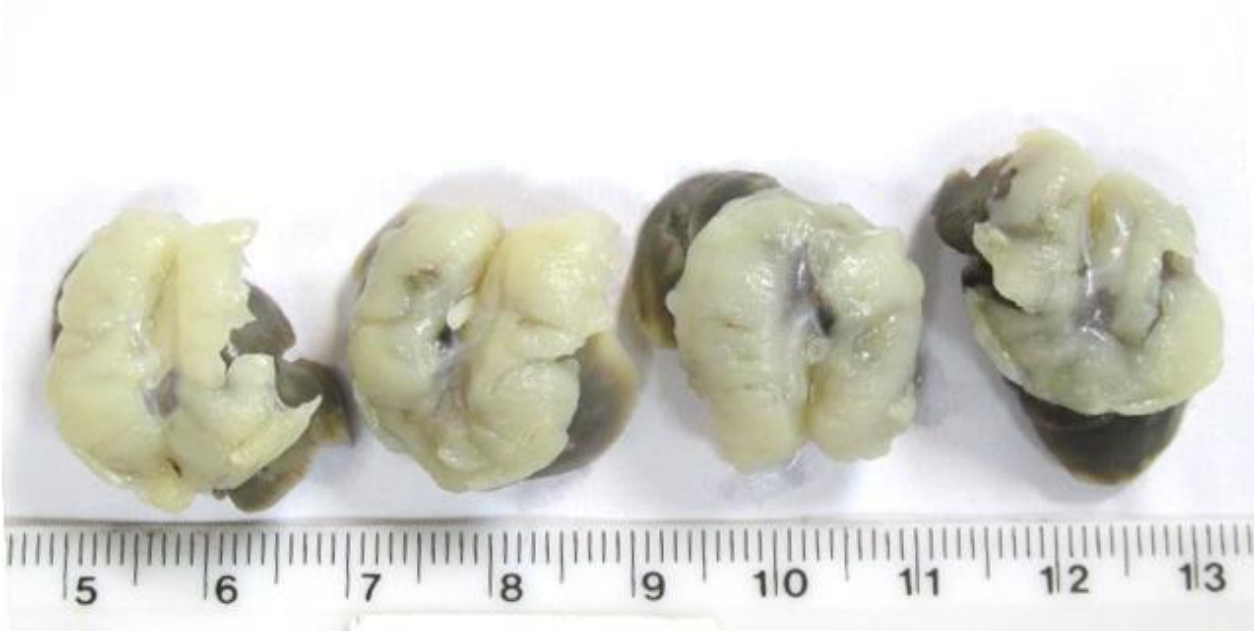

Figure 1- *White "butterfly like" sheath seen on the dorsal side of the liver after inoculation of 4T1 cells*

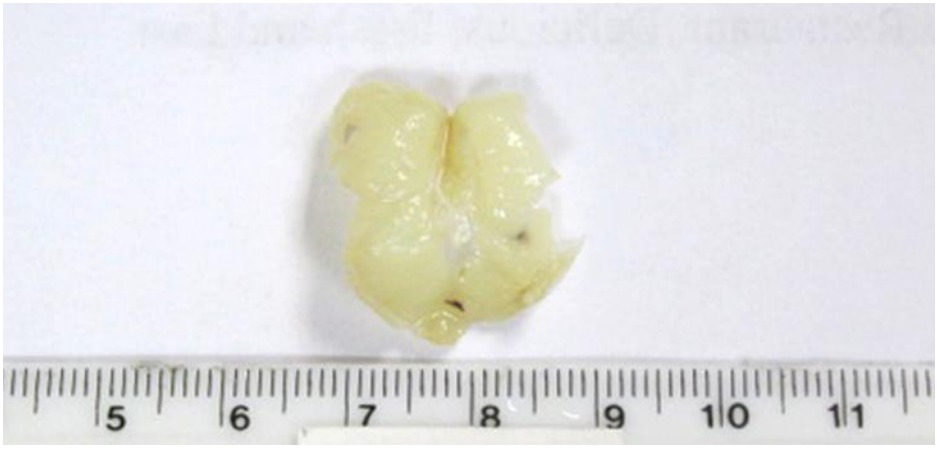

Figure 2- *Single "butterfly like" sheath tissue excised from the liver*

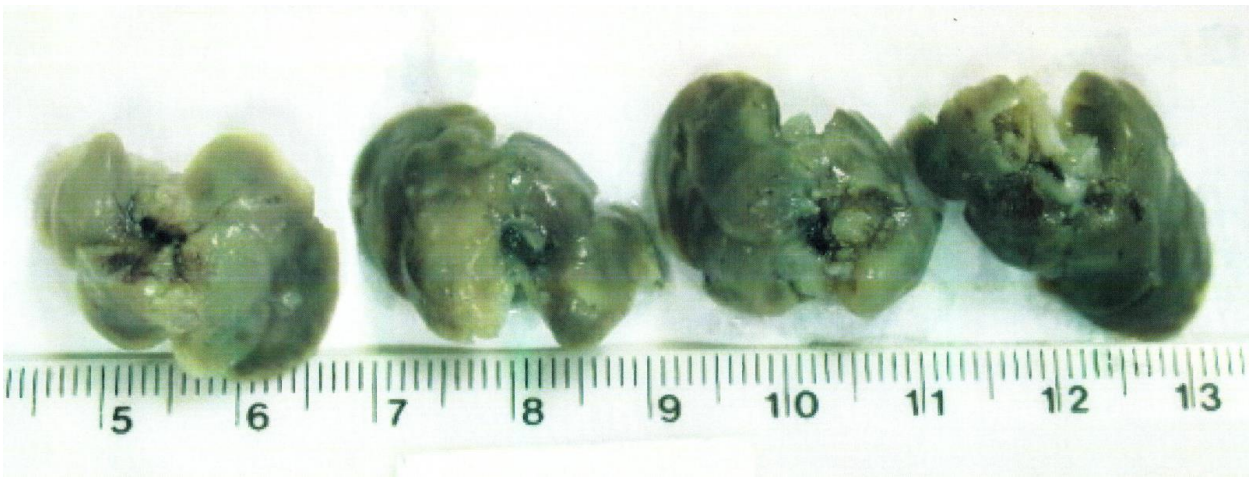

Figure 3- *Metastasis seen on the liver after inoculation of 4T1 mammary carcinoma cells*

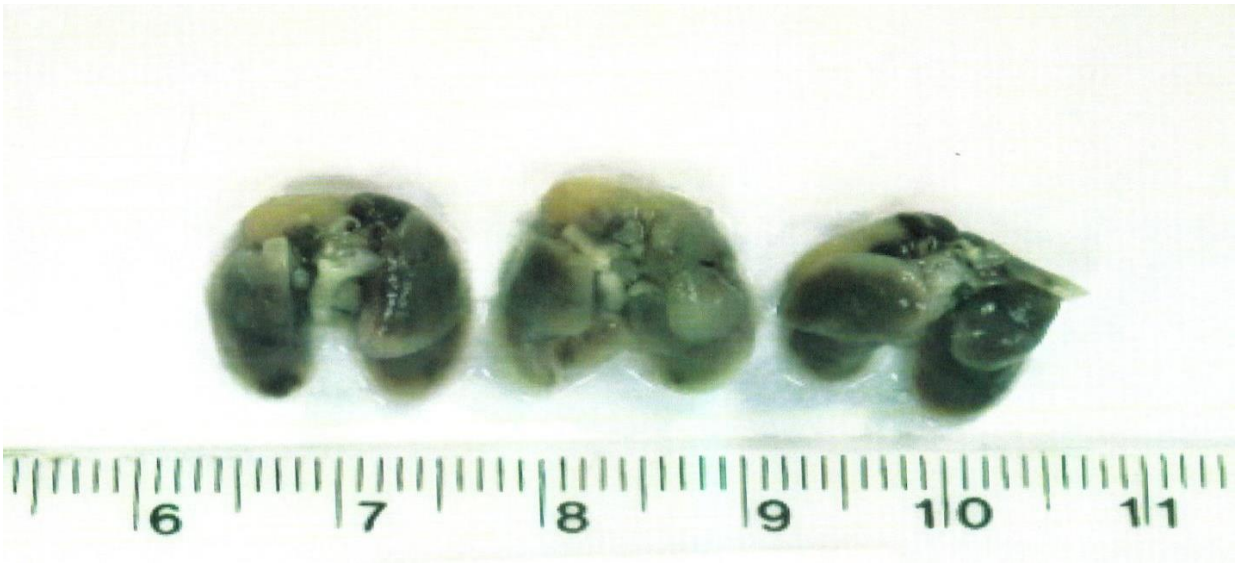

Figure 4- *Metastasis seen on the lungs after inoculation of 4T1 mammary carcinoma cells*

3.3 Tumor Histology: The H&E staining of the butterfly tissue (Figure 5) was evaluated by a pathologist at the IDEXX Reference Laboratory (Sacramento, California). Irregular, long sections of tumor tissue were observed with evidence of skeletal muscle invasion. It was seen that tumor cells are irregularly round, many with vacuolated cytoplasm and large irregularly-round vesiculated nuclei. Tumor cell necrosis was minimal to none. Mitotic figures ranged from 0-1 per high-power field.

Histological evidence strongly suggests the invasion of skeletal muscles by 4T1 breast cancer cell line.

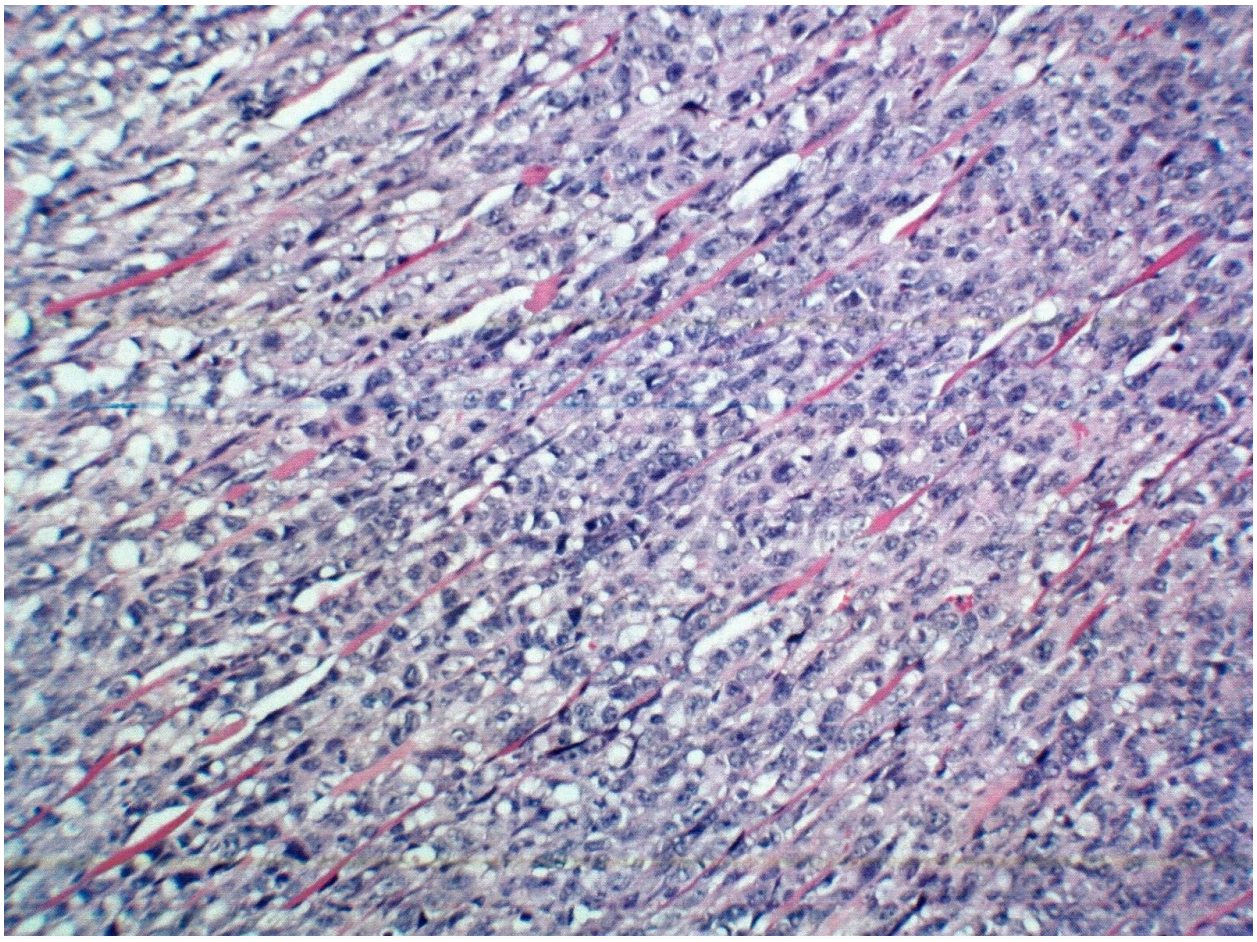

Figure 5- *Histopathology of the butterfly-like tissue after inoculation of 4T1 cells in BALB/c mice*

The H&E staining of the liver shows metastatic foci of neoplastic cells. All sections are congressed and have varying number of foci of extramedullary hematopoietic activity. Three sections of multiple, small foci of liver necrosis were shown in (Figure 6).

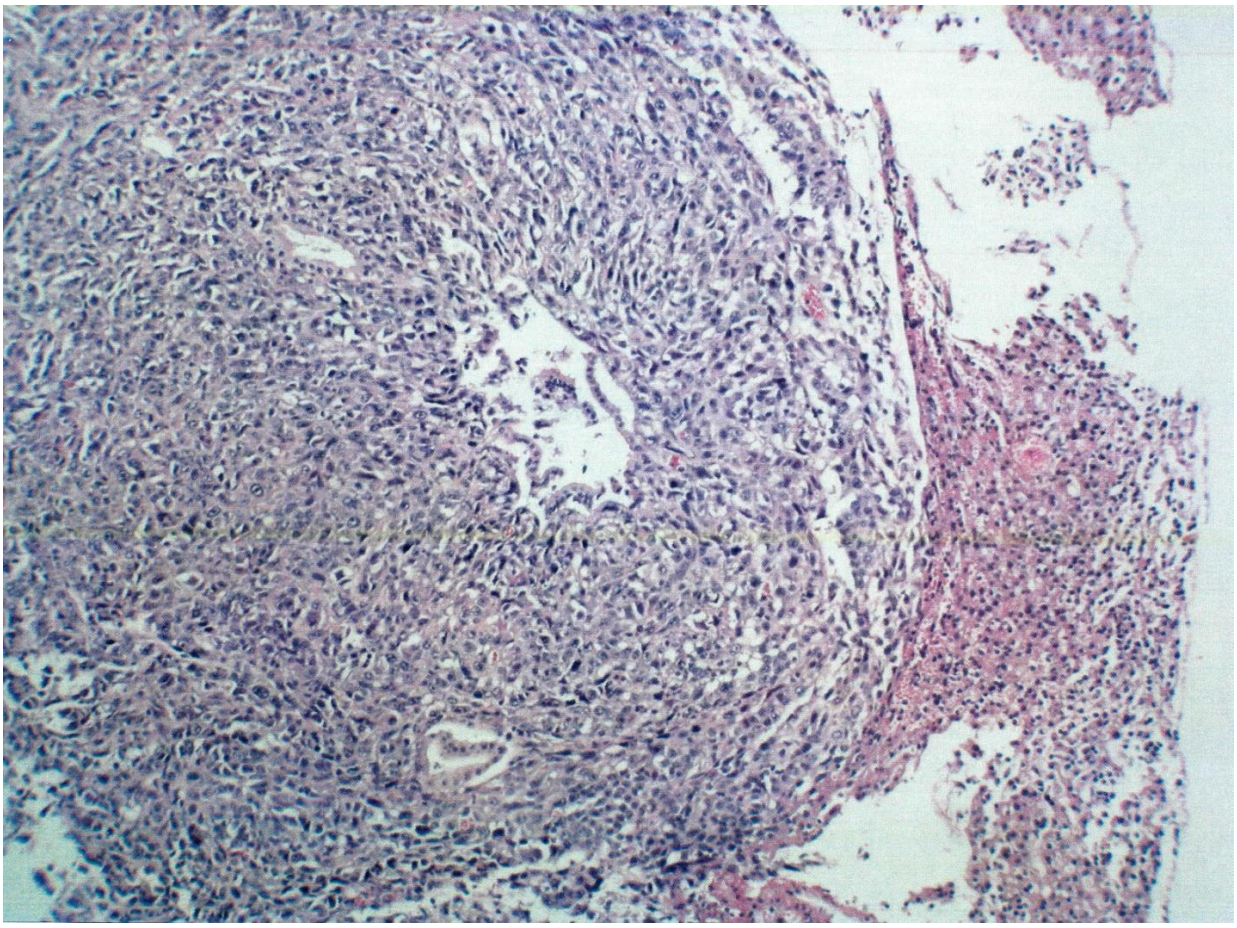

Figure 6- *Tumor histopathology in liver after inoculation of BALB/c female mice with 4T1 mouse mammary carcinoma cells*

Since 4T1 breast cancer cell lines have been characterized as triple negative; immunohistochemistry for the expression for receptors for estrogen, progesterone and HER-2 was not carried out.

4 Discussion: Breast cancer is one of the most dreadful and the second most common cancer in women. It generally metastasizes to several organs in the body (liver, lung, bone, lymph nodes, spine and brain). However, metastasis to the skeletal muscle is rare and seldom seen. Diagnosis of the skeletal muscle is very difficult, it could easily be mistaken for soft tissue metastasis - as signs and symptoms are similar. It is encountered in advanced stages of breast cancer and has a poor prognosis. Since this is an uncommon event, there have been few literature reports of breast cancer metastasis to the skeletal muscle.[11-17] Radiology, CT, MRI and PET/CT are tools used to diagnose skeletal muscle metastasis.[13-15]

Currently there is no definite treatment for skeletal muscle metastasis, mostly it is individualized depending on the patient's condition and mainly palliative. Therapeutic options include chemotherapy and surgery.

Clinically, invasion of skeletal muscle by breast cancer is a rare entity. Skeletal

muscle is resistant to most cancer metastasis because its harsh environment makes it difficult for any cancer cell to survive.[11,12,18] In addition, other factors include muscle motion contributing to tumor destruction, harsh muscle pH and the muscle's ability to remove lactic acid generated during the process, which induces angiogenesis. Besides, natural killer cells and lymphocytes which play an important role in the inhibition of muscle metastasis may be activated in the skeletal muscle.

There are several models for 4T1 cancer cells to metastasize to different organs, but no model to study the metastasis of breast cancer to the skeletal muscle was reported. While it is a rare occurrence of skeletal muscle breast cancer metastasis, this was a part of a larger project of experiments with a focus on metastasis of breast cancer cells in different organs. In our previous studies we demonstrated that inoculation of 4T1 in the mammary pad metastasized to the lung, liver and heart[19]; and kidney inoculation to the lung and spleen.[20] In a more recent study we showed that 4T1 inoculation via the tail vein metastasized to the lung and injection into the spleen metastasizes exclusively to the liver.[21] In the present study, we demonstrate that skeletal muscle was invaded by the breast cancer cells. However, it was a pilot study, although we think it is metastasis of the breast cancer cells to the skeletal muscles, we were not able to differentiate with a certainty between metastasis to the skeletal muscle or infiltration at this point. Nevertheless, we believe that this gives an understanding of another aspect of metastasis of breast cancer, as we continue to explore more pathways of cancer metastasis.

A good model for invasion of skeletal muscles to breast cancer is needed. We believe that this is a good model to study the invasion of skeletal muscle to the breast. The microenvironment of the peritoneal cavity as compared to the mammary pad or intravasculation site is certainly very different, and perhaps the cell line was successfully able to establish adhesion and invasion of the skeletal muscle of the diaphragm because the microenvironment of the skeletal facilitated this response of the malignant cell.

This model is simple, doable, novel and histological evidence strongly suggests invasion of skeletal muscles by breast cancer cells. Our model should prove to be useful for the development of drugs to target and test anti-metastatic and anti-cancer drugs especially to the skeletal muscle.

Acknowledgments: The research study was funded by Dr. Rath Health Foundation (Santa Clara, CA, USA), a non-profit organization. Ms. Cathy Flowers provided the proofreading assistance.

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