Comparison of Tumor Recurrence After Resection of Highly- and Poorly-Metastatic Triple-negative Breast Cancer in Orthotopic Nude-Mouse Models

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Abstract. *Background:* Triple-negative breast cancer (TNBC), defined by the absence of receptors for estrogen, progesterone and human epithelial receptor 2, is a recalcitrant disease in need of effective therapy. We previously isolated highly-metastatic variants of the human TNBC cell line MDA-MB-231 using serial orthotopic implantation in nude mice. *Materials and Methods:* In the present report, we compared local and metastatic recurrence in lymph nodes in orthotopic nude-mouse models after bright-light surgery (BLS) of tumors from highly-metastatic variants or poorly-metastatic parental MDA-MB-231-RFP cells. Orthotopic tumors from parental MDA-MB-231 or highly-metastatic MDA-MB-231 were resected under bright light similar to an operating room. *Results:* After resection of primary tumors, local recurrence from highly-metastatic MDA-MB-231 cells grew more rapidly than did parental MDA-MB-231 cells. Lymph-node metastasis from highly-

metastatic MDA-MB-231 cells occurred after primary tumor resection much more extensively than after parental MDA-MB-231 tumors were resected. *Conclusion:* The results of the present report suggest that conventional surgery under bright light was unable to control highly-metastatic compared with poorly-metastatic MDA-MB-231 TNBC.

Triple-negative breast cancer (TNBC) is defined by a lack of expression of estrogen and progesterone receptors, and human epithelial receptor 2, and are the most aggressive and metastatic type of breast cancer. The absence of effective treatment for this recalcitrant disease has led to a higher rate of local and systemic relapse after primary tumor resection (1, 2).

We previously developed and characterized a highly-invasive and metastatic TNBC variant by serial orthotopic implantation of MDA-MB-231 human breast cancer cells in nude mice. The variant thereby isolated is highly-invasive in the mammary gland and metastasized to lymph nodes in 10 of 12 mice compared to tumor from the parental cell line in two out of 12 mice. Extensive lymphatic trafficking by highly-metastatic, but not parental, MDA-MB-231 cells was also observed (3).

Surgical resection of the MDA-MB-231 highly metastatic variant primary tumor also resulted in rapid and enhanced lymphatic trafficking of residual cancer cells and extensive lymph-node and lung metastasis that did not occur in the non-surgical mice (4).

Materials and Methods

Cell culture. Parental and highly-metastatic red fluorescent protein (RFP)-expressing TNBC breast cancer, MDA-MB-231P-RFP, were maintained and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 5% penicillin/streptomycin (5).

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Figure 1. Representative time-course images after surgical resection of a parental MDA-MB-231-RFP tumor.

Figure 2. Representative time-course images after surgical resection of a highly-metastatic MDA-MB-231-RFP tumor.

Mice. Athymic nude mice (AntiCancer, Inc.) were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485; Envigo, Indianapolis, IN, USA). All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under

assurance A3873-01. All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by intramuscular injection of a 0.02 ml solution of 20 mg/kg

Figure 3. Bar graph shows the comparison of fluorescence intensity of recurrent lymph-node metastasis from parental and highly-metastatic MDA-MB-231-RFP. Data are shown as average $\pm$ SD. N=5.

ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO₂ inhalation if they met the following humane endpoint criteria: prostration, skin lesions, significant body weight loss, difficulty breathing, epistaxis, rotational motion and body temperature drop. Animals were housed with no more than five per cage. Animals were housed in a barrier facility on a high-efficiency particulate arrestance-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet (5).

Isolation of highly-metastatic TNBC MDA-MB-231-RFP breast cancer variant. Initially, MDA-MB-231-RFP cells (1×10^7 cells/site) were injected subcutaneously in the flank of nude mice. For orthotopic transplantation, fragments of the harvested subcutaneous tumor were grafted into a mammary gland. After growth, the tumor was resected. Residual cancer cells grew into tumors which were harvested and divided into fragments and re-implanted into a mammary gland. After the tumor metastasized to lymph nodes, it was harvested and divided into fragments and re-implanted into mammary gland. After growth, the orthotopic tumor was resected. The residual cancer cells formed primary and metastatic tumors. A lymph-node metastasis was harvested, divided into fragments and re-implanted into the mammary gland. Highly metastatic MDA-MB-231 tumors were developed after seven orthotopic transplantations described above (3-8).

In vivo whole-body/whole-tumor imaging. For whole-body or whole-tumor imaging, the OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), was used. The OV100 contains an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), for whole body, as well as

subcellular imaging in live mice. The optics of the OV100 have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. Four individually optimized objective lenses, parcentered and parfocal, provide a 105-fold magnification range. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and CellR (9-16). Bright-light surgery was carried out under white light of intensity similar to that of an operating room.

Statistical analysis. Five mice were used per group. Fluorescence intensity of recurring tumors was compared after surgical resection in each group. Data are shown as means $\pm$ standard deviation (SD). For comparison between two groups, significant differences were determined.

Results and Discussion

Bright-light surgery can control local recurrence and distant metastasis of poorly-but not highly-metastatic MDA-MB-231 tumors. Local recurrence of primary tumors and metastatic recurrence in lymph nodes was compared after bright-light surgery of orthotopic highly-metastatic and parental poorly-metastatic MDA-MB-231-RFP. Orthotopic tumors from parental MDA-MB-231 and highly-metastatic MDA-MB-231 cells were resected under bright light (Figure 1). After resection of primary tumors, local recurrence from highly metastatic MDA-MB-231 was much greater than parental MDA-MB-231 cells (Figures 1 and 2).

Lymph-node metastasis from highly metastatic MDA-MB-231 cells occurred after primary tumor resection much more extensively than with parental MDA-MB-231. Fluorescence intensity of recurrent lymph-node metastasis was more than 40-fold higher with the highly-metastatic variant compared to the poorly-metastatic parental MDA-MB-231-RFP (Figure 3). These results suggest that conventional surgery under bright light cannot control highly-metastatic compared with poorly-metastatic MDA-MB-231 tumors, which may explain the high rate of recurrence after resection of highly-metastatic TNBC in the clinic.

The major problem for recurrence with the highly-metastatic variant is that this variant is highly invasive (Figure 2) (16) and bright-light surgery cannot detect all the cancer cells. The parental cells are not as invasive, and the cancer cells can therefore be fully resected.

Disclosure of Potential Conflicts of Interest

Y. Urata is President & CEO of Oncolys BioPharma, Inc. H. Tazawa and T. Fujiwara are consultants of Oncolys BioPharma, Inc.

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