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A Novel Animal Model for Locally Advanced Breast Cancer

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Abstract

Background—Locally advanced breast cancer (LABC) poses complex management issues due to failure of response to chemotherapy and progression to local complications such as skin erosion, superinfection and lymphedema. Most cell line and animal models are not adequate to study LABC.

Methods—A patient-derived xenograft (IOWA-1T) from a patient with LABC was characterized for expression profile, short tandem repeat (STR) profile, oncogenic mutations, xenograft growth and response to therapy.

Results—STR profile authenticated the cell line as derived from a human female. The primary tumor and derived xenografts were weakly ER α -positive (<5%), PgR-negative and HER2 non-amplified. Expression array profile compared to MCF-7 and BT-549 cell lines indicate that IOWA-1T was more closely related to basal breast cancer. IOWA-1T harbors a homozygous R248Q mutation of the *TP53* gene and *in vitro* invasion assay was comparable to BT-549, and greater than MCF-7. IOWA-1T xenografts developed palpable tumors in 9.6 $\pm$ 1.6 days, compared to 49 $\pm$ 13 days for parallel experiments with BT-20 cells (p <0.002). Tumor xenografts became locally advanced, growing to >2 cm in 21.6 $\pm$ 2 days characterized by skin erosion necessitating euthanasia. The SUMO inhibitor anacardic acid inhibited the outgrowth of IOWA-1T xenografts, while doxorubicin had no effect on tumorigenesis.

Conclusions—IOWA-1T is a novel cell line with an expression pattern consistent with basal breast cancer. Xenografts recapitulated LABC and provide a novel model for testing therapeutic drugs that may be effective in cases resistant to conventional chemotherapy.

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Keywords

Breast Cancer; Locally-advanced; Cell Line; Animal Model; Sumoylation

Introduction

Locally advanced breast cancer (LABC) is an advanced-stage of local disease defined as tumors larger than 5 cm, fixed, or with skin ulceration (T3 or T4 tumors), or with extensive nodal disease (N2 or N3) ¹. Prognosis of LABC depends on the size of the primary tumor, lymph node status, estrogen receptor- α (ER α), progesterone receptor (PgR), HER2 receptor and Ki-67 ²⁻⁵. Significantly longer disease-free intervals and overall survival were predicted for patients with ER α -positive LABC while ER α PgR negativity and mutated p53 were associated with shorter overall survival times ^{5,6}. One of the most significant factors predictive of recurrence and survival in LABC is response to chemotherapy ^{2,7}. The resistance to therapy and the associated worse outcomes of basal breast cancer have generated significant interest in developing approaches for pre-clinical screening of new chemotherapeutical compounds using xenograft transplantation models that mirror the clinical disease.

There are few basal xenograft models and many of them have long latency periods after inoculation. For example, tumorigenesis after injection of MDA-MB-231 cells is commonly 30-40 days ⁸ and for BT-20 approximately 55 days ⁹. We report the isolation of a patient-derived xenograft line (IOWA-1T) with basal molecular profile enriched for cells expressing cancer stem cell (CSC) markers. IOWA-1T rapidly forms xenografts that replicate the clinical features of LABC and provides an animal model that can be used for testing novel therapy and fundamental mechanisms of tumorigenesis.

Materials and Methods

Creation of IOWA-1 and IOWA-1T Cell Lines

The IOWA-1 cell line was derived from a primary breast cancer from a patient with LABC using established methods ¹⁰. Briefly, the tumor specimen, obtained at the time of surgery with IRB approval and informed consent, was immediately mechanically disaggregated. Enzymatic digestion was done with Gentle 10 $\times$ Collagenase/ Hyaluronidase (Stemcell Technologies) at 37°C overnight. Cell pellet was suspended in 0.25% Trypsin-EDTA solution (Life Technologies) and incubated for 2 min at 37°C. Cells were washed with DMEM/F-12 medium (Life Technologies), 5% fetal bovine serum (Life Technologies) and resuspended in serum-free DMEM/F-12, supplemented with 10 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 5 μ g/ml insulin, and bovine serum albumin (Sigma). After filtration through a 30 μ m filter, cells were plated on plastic 6-well dishes at 10⁶ cells/well. Cells were split 1:5 every 3 days by pipetting. The IOWA-1T cell line was derived by inoculating IOWA-1 cells into the flank of a nude mouse. 6 $\times$ 10⁶ IOWA-1 cells were mixed with BD Matrigel (BD Biosciences) and were injected into nude mice flanks. When the tumor reached 2 cm, the mouse was euthanized, and a cell line established as

described above. All animals were cared for in accordance with guidelines established by the University of Iowa Institutional Animal Care and Use Committee.

RNA Isolation, cDNA Synthesis, Real-Time-PCR and Gene Profiling by Microarray

RNA gene expression analysis was determined by quantitative real-time PCR using TaqMan primers and detection probes. RNA sample preparation, the subsequent hybridization and microarray analyses were done at the University of Iowa DNA Facility according the manufacturers' recommended protocols ¹¹.

Flow cytometry

IOWA-1T cells were detached by trypsin, washed by PBS and incubated with CD24 – PE (eBioscience) and CD44 – APC (RD Systems) antibodies on ice for 30 min. Hoechst Stains (Invitrogen) was used for living cells separation. Flow cytometry was performed on a FACS (fluorescence-activated cell sorting) DiVa (Becton Dickinson, Franklin Lakes, NJ, USA) machine.

Short Tandem Repeat Profile

STR profile for cell line authentication was done by the Fragment Analysis Facility at Johns Hopkins University.

Sequencing of 50 Common Oncogenic Genes

Ion AmpliSeq Cancer Hotspot panel v2 (Life Technologies) was used for sequencing of 50 cancer-related genes and was performed by the University of Iowa Department of Pathology.

Growth of Xenografts

Xenografts were generated by inoculating 6×10^6 BT-20 and IOWA-1T cells into nude mice as described above.

Treatment with SUMO Inhibitor and Doxorubicin

Anacardic acid was administered in 10 mg/kg dosage by gavage daily for three weeks. Doxorubicin was administered in 10 mg/kg intravenous injections once weekly for three weeks.

Invasion Assay

Cell Invasion Assay Kit (Chemicon International) was used for invasion assay according to instructions from the manufacturer.

Results

Creation of IOWA-1 and IOWA-1T Breast Cancer Cell Lines

The original tumor was obtained from a patient who presented with a left breast invasive ductal carcinoma, which rapidly progressed to locally advanced disease. The patient was treated with cyclophosphamide, doxorubicin and paclitaxel; however, the tumor was

resistant to chemotherapy and had rapid progression. The patient underwent surgical resection with an attempt to gain local control. The final pathologic diagnosis was a grade 3, triple-negative invasive ductal carcinoma, which was weakly ER α -positive (<10%), PgR-negative and HER2 non-amplified. The patient died from respiratory failure and sepsis with increasing pleural effusions and pneumonia. The patient had a CT scan of her head and chest/abdomen/pelvis. The breast tumor directly invaded into the pleural space and into the ribs. She had new multiple small bilateral pulmonary nodules in addition to the pleural effusions. There was no evidence of disease in the brain, liver, adrenals, or other abdominal organs. There was no evidence of bony metastasis during her last admission or on a previous bone scan six weeks previously.

After informed consent, primary tumor material obtained during mastectomy was used to establish a breast cancer cell line, called IOWA-1. Interestingly, *in vitro* IOWA-1 grew as a suspension cell culture with no attachment to plastic plates, forming spheroids and cell aggregates. Similar morphologic properties were described for newly established cancer cell lines rich in stem cell populations^{10,12,13}.

To create tumor xenografts, 6×10^6 cells from an early passage of the IOWA-1 cell line were injected into the flank of a nude mouse. Tumor growth was rapid with progression to 2 cm size within three weeks, necessitating euthanasia. The IOWA-1 xenograft was excised and a cell line established. The cell line derived from the IOWA-1 xenograft after a single passage in mice was called the IOWA-1T cell line. IOWA-1T cells demonstrated the same morphological and growth characteristics as the parental IOWA-1 cell line (Figure 1A). The cells do not adhere to plastic and grow in spheroids and cell aggregates.

Characterization of the IOWA-1T Cell Line

IOWA-1T cells were analyzed by short tandem repeat (STR) profile confirming the purity of the newly derived cell line. The profile did not match any existing commonly used breast cancer cell lines and was confirmed to be human female in origin. Expression array was used to examine global patterns of gene expression in comparison to the MCF-7 luminal cell line and the BT-549 basal cell line. The global pattern of expression showed that the IOWA-1T expression profile was more closely related to the basal cancer cell line BT-549 than the luminal cancer cell line MCF-7 (Figure 1B & C). The expression profile was examined in detail for genes associated with luminal and basal breast cancer lines¹⁴. Figure 1C shows detailed analysis of luminal (*ESR1*, *FOXA1*, *KRT8*, *MUC1*, *RET*, *GATA3*, *GREB1*, *MYB*) and basal gene markers (*MMP 14*, *SFRP1*, *GSTP1*) from the array results and confirms that the expression profile of IOWA-1T was more similar to the basal cell line.

RNA was further analyzed by RT-qPCR for select markers. The basal markers were expressed at a relatively high level in BT-549 and IOWA-1T cell lines while luminal targets were expressed at relatively lower levels (Figure 2). RT-qPCR data were confirmed by western blot and show that luminal markers were detected only in luminal MCF-7 cells but not in the IOWA-1T or BT-549 basal cells (Figure 3). Basal markers SFRP1, GSTP1 and MMP14 were expressed in IOWA-1T and BT-549 cells (Figure 3).

The IOWA-1T cells were analyzed for mutations of 50 commonly mutated oncogenes and tumor suppressor genes. A homozygous R248Q mutation of *TP53* was detected by AmpliSeq Cancer Hot Spot panel test. The *TP53* gene mutation identified in IOWA-1T is a well-described hot spot for p53 mutation found most frequently in colorectal and breast cancer¹⁵.

Flow cytometry (FACS) analysis using the markers CD44 and CD24 showed enrichment in the CD44^{+/hi}/CD24^{-/low} cancer stem cell (CSC) population, with 93% of cells expressing CSC markers in the IOWA-1T cell line (Figure 4A). The BT-549 cell line had 98.6% of the cells sorted to the CD44^{+/hi}/CD24^{-/low} population (Figure 4A). In contrast to the basal lines and consistent with other reports, the MCF-7 luminal cell line had <1% of the cells in the CD44^{+/hi}/CD24^{-/low} CSC population.

Immunohistochemistry (IHC) was performed on the IOWA-1T xenografts and compared to the original clinical cancer biopsy. IOWA-1T cells were weakly ER α -positive (<5%), PgR-negative and HER2 non-amplified (Figure 4B). The IHC profile of the IOWA-1T xenografts matched the expression profile of the original primary cancer (Figure 4B).

IOWA-1T Model for LABC

The invasive property of the IOWA-1T cell line was compared to MCF-7 and BT-549. The *in vitro* relative invasion of IOWA-1T was significantly greater than MCF-7 but was not statistically different than BT-549 (Figure 5A). Tumorigenesis of IOWA-1T was assessed after inoculation of cells into the flank of nude mice. The IOWA-1T cells rapidly form xenografts that develop skin erosions necessitating euthanasia of the animals (Figure 5B). The growth pattern of the IOWA-1T cell line was compared to the BT-20 basal cancer line. 6×10^6 IOWA-1T or BT-20 cells were injected into the flanks of nude mice and tumor growth was monitored. Palpable tumor growth was detected with IOWA-1T inoculations in a mean time of 9.6 ± 1.6 days, compared to 49 ± 13 days for parallel experiments performed with BT-20 cells ($p < 0.002$) (Figure 5C). The IOWA-1T xenografts grew to >2 cm in a mean time of 21.6 ± 2 days requiring euthanasia (Figure 5B & D). Similar to human LABC, the IOWA-1T xenografts formed large tumors with skin erosions. By comparison, tumors formed from inoculation of BT-20 cells had a mean diameter of 0.5 mm after 3 months (data not shown). Pulmonary metastases were assessed in several of the euthanized animals with IOWA-1T xenografts by serial sections of lung tissue; however, no metastases were identified (data not shown).

SUMO Inhibitors Delayed IOWA-1T Tumorigenesis

Recently SUMO inhibitors were reported as chemical compounds with potential anti-cancer activity¹⁶. The small molecule SUMO inhibitor anacardic acid (AA), an E1 activating enzyme inhibitor¹⁷, was used for *in vivo* studies using IOWA-1T xenografts. After inoculation of xenografts, animals were either gavaged with AA or treated with tail vein injection of doxorubicin. Whereas, administration of AA prevented the outgrowth of IOWA-1T xenografts, doxorubicin treatment failed to alter tumorigenesis compared to untreated animals (Figure 5E). These findings highlight the potential of testing novel

therapeutic agents using the IOWA-1T cell line, which is unresponsive to conventional doxorubicin chemotherapy.

Discussion

Clinical Focus on Locally Advanced Breast Cancer

The initial treatment of patients with LABC involves chemotherapy. Patients who achieve a pathologic complete response (pCR) have improved disease-free and overall survival ^{7,18,19}. In patients with LABC treated with neoadjuvant chemotherapy, the median five-year disease-free survival is approximately 60%, and the five-year overall survival is approximately 80% ²⁰. Patients who have an incomplete response or progress despite treatment have a particularly poor prognosis ²¹, as exemplified by the patient from whom the IOWA-1T cell line was derived. Patients with LABC who do not respond to neoadjuvant chemotherapy have limited treatment options, and developing models to investigate new treatment modalities is critically important.

IOWA-1T xenografts recapitulate clinical LABC in terms of the size of tumors formed and in local skin ulceration. Molecular profiling with ER α , PgR, and HER2 matched the pattern of the primary cancer, and is consistent with other studies showing that the molecular profile of patient-derived xenografts often match the original tumor isolate ²². The IOWA-1T cell line expressed a low level of ER α RNA and protein; although some investigators suggest that tumors with any ER α expression should be considered ER α -positive, there is compelling data that tumors with less than 5-10% of the cells staining positive for ER α indicate a response and prognostic profile most often characterized as ER α -negative ²³⁻²⁵.

The IOWA-1T cell line has several attractive features as a model for LABC. First, propagation in culture is relatively rapid and the fact that the cells do not adhere to plastic facilitates expanding the number of cells by cell culture. Second, when inoculated as xenografts, the IOWA-1T cells rapidly form large tumors, with tumors forming in 1-2 weeks after inoculation of 6×10^6 cells compared to 1-2 months for parallel experiments performed with BT-20 cells. Finally, the IOWA-1T xenografts were unresponsive to doxorubicin. Hence, the IOWA-1T cell line has many attributes that facilitate investigations of new treatment strategies for LABC. The cell line is particularly attractive for development of new treatment strategies for breast cancers that are unresponsive to conventional chemotherapy. For example, IOWA-1T cells have relative overexpression of GSTP1, which was reported to be responsible for doxorubicin resistance ^{26,27} and may contribute to doxorubicin resistance of IOWA-1T cells.

IOWA-1T Cells as a Model for Examining Cancer Stem Cells

The IOWA-1T cell line expresses a high percentage of cells characterized by the CD44^{+/hi}/CD24^{-/low} cancer stem cell (CSC) markers ²⁸. The cell population expressing CSC markers are specifically the cells possessing tumor-initiating ability ²⁸ and are likely responsible for tumor recurrence ²⁹. A high percentage of breast cancer cells expressing CSC markers is predictive of chemotherapy resistance, and a serial comparison of tumors before and after chemotherapy has demonstrated a preferential increase in the CSC population ^{30,31}. The

abundance of CSC in the IOWA-1T cell line is likely related to its resistance to chemotherapy and the rapid formation of xenografts. The sensitivity of the IOWA-1T cell line to SUMO inhibitors is due to transcriptional activation of SUMO unconjugated TFAP2A, which represses CD44 expression and clears the CSC population¹⁶. The IOWA-1T cell line has relatively high expression of TFAP2A (data not shown) and is consistent with the molecular mechanism of SUMO inhibition of basal cancer tumorigenesis. Hence, the IOWA-1T cell line is a valuable reagent for preclinical testing of novel SUMO inhibitors that might be developed for clinical application. Given that CSCs are most likely responsible for resistance to conventional chemotherapy and cancer recurrence²⁹, the ability of SUMO inhibitors to clear the CSC population makes this line of investigation particularly attractive. The IOWA-1T cell line will also be useful for testing other therapies directed at the CSC population.

Models of Invasion and Metastasis

An interesting feature of IOWA-1T expression profile is an enrichment of MMP14, which is involved in tumor cell invasion and metastasis^{32,33}. Thus, MMP14 may be involved in LABC invasion pathogenesis and potentially responsible for skin erosion of IOWA-1T xenografts. IOWA-1T cells can be used for pre-clinical trials of MMP14 specific inhibitors to inhibit the invasive properties and/or skin erosion. Since the IOWA-1T xenografts grow rapidly requiring euthanasia of the animals, there is insufficient data to know if the animal model develops metastases. Further experiments utilizing survival surgery after xenografts form to allow the animals to live for longer periods of time will be needed to determine the metastatic potential of the cell line.

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Synopsis

Locally advanced breast cancer (LABC) poses unique clinical challenges that require new models to study issues of rapid tumor growth, skin erosion and drug resistance. Herein, we report the development of a novel patient-derived xenograft that mimics LABC.

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Figure 1. IOWA-1T Cell Culture and Expression Array

A. Shown is a photomicrograph of the IOWA-1T cell line morphology by light microscopy. The cells do not adhere to plastic and grow in spheroids and cell aggregates. **B.** Expression array for the IOWA-1T cell line in comparison to MCF-7 and BT-549 with whole genome array with genes that differ by a factor of 2. **C.** Selected genes characteristic of luminal (*ESR1*, *FOXA1*, *KRT8*, *MUC1*, *RET*, *GATA3*, *GREB1*, *MYB*) and basal (*MMP14*, *GSTP1*, *SFRP1*) expression profiles.

Figure 2. RNA Expression of Luminal and Basal Gene Expression

RT-qPCR analysis of RNA expression is shown for selected luminal (*ESR1*, *FOXA1*, *KRT8*, *MUC1*, *RET*, *GATA3*, *GREB1*, *MYB*) and basal (*MMP14*, *GSTP1*, *SFRP1*) genes for MCF-7, IOWA-1T and BT-549 cell lines. Relative expression was normalized to MCF-7. The findings confirm the expression array data and indicate that the IOWA-1T cells have a basal-like expression pattern. Statistical significance of differences compared to MCF-7 are noted by an asterisk, * $p < 0.05$.

Figure 3. Western blots for Luminal and Basal Genes

Western blot data for protein expression of ESR1/ER α , FOXA1, KRT8, MUC1, RET, GATA3, GREB1, MYB, MMP14, GSTP1, SFRP1 for MCF-7, IOWA-1T and BT-549 cell lines demonstrate that the IOWA-1T cells have a basal-like phenotype by protein expression.

Figure 4. FACS Analysis with CD44 and CD24 and Immunohistochemistry

A. Flow cytometry is shown for CD44 and CD24 staining for MCF7, IOWA-1T and BT-549 cells. The data demonstrates that 93% of the cells in the IOWA-1T cell line are contained within the CD44^{+/hi}/CD24^{-/low} cancer stem cell population. By comparison the BT-549 cell line had 98.6% and MCF-7 had 0% cells in the CD44^{+/hi}/CD24^{-/low} population. **B.** Immunohistochemistry of the primary tumor from the original core biopsy (top) and from IOWA-1T xenografts (bottom) stained for ERα, PgR and HER2. Both the primary tumor and IOWA-1T xenografts were ERα weakly positive (<10%), PgR-negative and HER2-negative.

Figure 5. Characterization of IOWA-1T Invasion and Xenograft Growth

A. *In vitro* invasion assay comparing MCF-7, IOWA-1T and BT-549 cells. For comparisons, * $p < 0.05$ and NS: not significantly different. **B.** Example of IOWA-1T xenograft showing large tumor with skin erosion. **C.** Tumor free survival with time from tumor cell inoculation for IOWA-1T vs. BT-20 cells. **D.** Overall survival for IOWA-1T vs. BT-20 cells from time of tumor cell inoculation. **E.** Tumor-free survival for IOWA-1T xenografts with mice treated with vehicle (control), doxorubicin by tail vein injection (Doxorubicin) or anacardic acid via gavage (Anacardic) demonstrates increased TFS with anacardic acid and no significant effect of doxorubicin.