

Long-term progression and therapeutic response of visceral metastatic disease non-invasively monitored in mouse urine using β -human choriogonadotropin secreting tumor cell lines

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Abstract

Historically, the use of mouse models of metastatic disease to evaluate anticancer therapies has been hampered because of difficulties in detection and quantification of such lesions without sacrificing the mice, which in turn may also be dictated by institutional or ethical guidelines. Advancements in imaging technologies have begun to change this situation. A new method to non-invasively measure tumor burden, as yet untested to monitor spontaneous metastases, is the use of transplanted tumors expressing secretable human β -chorionic gonadotropin (β -hCG) that can be measured in urine. We describe examples of β -hCG-transfected tumor cell lines for evaluating the effect of different therapies on metastatic disease, which in some cases involved monitoring tumor growth for >100 days. We used β -hCG-tagged mouse B16 melanoma and erbB-2/Her-2-expressing human breast cancer MDA-MB-231 models, and drug treatments included metronomic low-dose cyclophosphamide chemotherapy with or without a vascular endothelial growth factor receptor 2-targeting antibody (DC101) or trastu-

zumab, the erbB-2/Her-2-targeting antibody. Both experimental and spontaneous metastasis models were studied; in the latter case, an increase in urine β -hCG always foreshadowed the development of lung, liver, brain, and kidney metastases. Metastatic disease was unresponsive to DC101 or trastuzumab monotherapy treatment, as assessed by β -hCG levels. Our results also suggest that β -hCG levels may be set as an end point for metastasis studies, circumventing guidelines, which have often hampered the use of advanced disease models. Collectively, our data indicates that β -hCG is an effective noninvasive preclinical marker for the long term monitoring of untreated or treated metastatic disease. [Mol Cancer Ther 2008;7(10):3452–9]

Introduction

The decision to evaluate a new anticancer drug or treatment in phase I and II clinical trials is usually preceded by preclinical therapy studies using tumor-bearing mice. The models used often involve transplanted “primary” tumors grown s.c. or in orthotopic organ sites (1, 2) but also spontaneously arising primary tumors arising in genetically engineered mouse models of cancer (3). In contrast, patients in phase I and II trials typically have advanced high-volume metastatic disease. This difference is likely one significant factor for the frequent failure to reproduce encouraging therapeutic results in preclinical models when the same therapies are evaluated in the clinical setting, as bulky metastatic disease is usually more difficult to treat than microscopic disease, in addition to the fact that the organ environment in which a metastatic lesion resides, e.g., the brain, can also limit therapeutic efficacy (4, 5). Given the dominance of (advanced) metastatic disease as the target for most cancer therapies, it is surprising to note the rarity of preclinical studies, which involve initiation of treatment at this stage of tumor progression (4). Instead, studies of metastatic cancer therapy often involve treatment of low-volume microscopic disease, e.g., initiating treatment within a few days after i.v. injection of tumor cells (4). In part, this has been due to the inherent difficulties of detecting metastases, monitoring their response to therapies over time, and quantifying overall tumor burden. However, recent developments in practical noninvasive imaging methods such as whole body optical (bioilluminiscent) imaging of luciferase-tagged tumor cells (6), or analysis of (tumor derived) DNA in plasma (7), has stimulated increased interest in preclinical therapy studies of metastatic disease.

A complementary non-invasive molecular method of detecting tumor burden involves the use of secreted

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tumor-associated cell marker protein. The use of prostate-specific antigen levels in the blood of men with recurring prostate cancer and reductions of prostate-specific antigen levels after therapy are examples of this approach. In this regard, Shih et al. (8) developed a method that permits a marker to be used in a similar fashion for any transplanted tumor. The cDNA for the β -subunit of human chorionic gonadotropin (β -hCG) is transfected into tumor cell lines, which are then injected into recipient mice. Secreted β -hCG (9) is then measured in the urine as a surrogate marker of tumor growth, tumor burden, and response to therapy (8). Others and we successfully used this approach to detect relative primary or ascites tumor burden (8, 10, 11) but not for metastatic disease. Here, we report that monitoring of β -hCG is also a reliable and robust surrogate marker for monitoring the progression and response of metastatic disease, even over extended periods of observation.

Materials and Methods

Cell Lines

The human breast cancer cell line MDA-MB-231, the derived erbB2 transfectant 231-H2N (12), the human cancer cell line MDA-MB-435, and murine B16 melanoma cells were grown in DMEM. Murine EMT-6 breast cancer cells were grown in Waymouth's medium. All medium was supplemented with 10% FCS and 2 mmol/L L-glutamine. Human prostate cancer cells PC3 and β -hCG-transfected variants, and human MDA-MB-435hCG-transfected variants (10), as well as HT-29 human colorectal carcinoma and green fluorescent protein (GFP)-transfected variants (11) were cultured as previously described.

Plasmid Transfection

The full coding β -hCG sequence in the pCIneo vector, as described by Shih et al. (8), was excised by XhoI and XbaI digestion and cloned into the corresponding region of the multiple cloning site of the pIRES plasmid (a gift from Steve Hobbs, Institute for Cancer Research, London, U.K.), thus generating the pIRES.hCG vector. Human 231-H2N cells, and murine EMT-6 breast cancer and B16 melanoma cells were transfected with the pIRES.hCG vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, stable β -hCG-expressing variants (H2N.hCG, EMT-6.hCG, and B16.hCG, respectively) were obtained by puromycin selection. Human MDA-MB-231 cells were transfected with β -hCG. pCIneo (8) using Lipofectamine 2000; two high β -hCG-expressing clones were isolated, following from G418 selection, and subsequently combined to generate the MDA-MB-231.hCGneo variant line.

β -hCG Measurements

To assay β -hCG in tissue culture medium and in the mouse urine, we used the commercially available Free β -hCG ELISA kit from OMEGA DIAGNOSTICS Ltd., which allows for quantitative determination of β -hCG. Results obtained were consistent with those obtained from independent assay kits from UBI. United Biotech, Inc., or from ALPHA DIAGNOSTIC INTERNATIONAL, as well as from

Sunnybrook Hospital Biochemistry services. Urine β -hCG levels were normalized by concomitant measurement of urine creatinine levels (using QuantiChrom™ Creatinine assay kit from BioAssay Systems) as detailed by Shih et al. (8). Urine was collected by placing mice in empty, aerated, tip boxes for 2 to 3 h. Typically, 0.1 to 0.4 mL of urine would be collected per mouse in this manner, of which (depending on the tumor burden) 1 to 50 μ L would be used for β -hCG detection and, 4 μ L would be used for Creatinine measurement. In some experiments, before the mice were sacrificed, blood was collected by cardiac puncture into heparinized tubes and placed on ice. Plasma was then isolated by centrifugation and used to measure plasma β -hCG levels.

Experimental Metastasis Assays

Experimental metastases were generated by injection of cells into the lateral tail vein (13) of 6- to 8-wk-old female mice. B16F1.hCG cells were washed and resuspended in PBS to 2.5×10^6 cells/mL, and C57Bl/6 mice (obtained from Charles River Canada) were each injected with 500,000 cells. Similarly, human cancer cell lines (one million cells per 200 μ L) were injected i.v. into female CB17 SCID or nude mice as indicated. Mice were sacrificed when any one of the following criteria were observed as follows: cachexia (defined by >15% weight loss), moribund state (lethargy and reduced mobility), or observable lymph node metastases.

Spontaneous (Orthotopic) Metastasis Assays

Human H2N.hCG (Her-2-positive) cells were injected orthotopically (2×10^6 cells in a 50- μ L volume, as fully detailed and described in du Manoir et al.; ref. 12) into the inguinal mammary fat pad of female CB17 severe combined immunodeficient (SCID). Tumors were removed when they reached an average size of 500 mm³, and the mice were thereafter monitored for β -hCG readings and body weight.

Derivation of Metastatically Competent Sublines

Upon autopsy, the mammary fat pad was inspected to confirm the absence of a primary tumor recurrence, and thereafter, any tissues found to have evidence of metastatic disease were rapidly excised and placed in serum-free medium at 4°C. Tissues were washed in ice cold PBS and briefly iced and mixed 1:1 with sterile 2 \times digestion mixture (Collagenase 3 at 4 mg/mL, Hyaluronidase at 2 mg/mL, and Collagenase IV at 2 mg/mL in PBS), and placed at 37°C for 30 min with occasional swirling. The digested tissue was then washed twice in PBS, passed through autoclaved gauze to remove undigested debris, and plated in growth medium containing Pennicillin/Streptomycin and Fungizone.

Treatment Regimens

Trastuzumab (Herceptin) was provided by Genentech and administered twice weekly at 20 mg/kg i.p., as described (12). Metronomic administration (20 mg/kg/d) of cyclophosphamide was carried out by addition of the drug to the drinking water as originally described (10), and later modified (14) by the addition of an upfront i.p. bolus dose (150 mg/kg). DC101 is an antiangiogenic endothelial

growth factor receptor-2 (VEGFR-2) blocking antibody obtained from Imclone, and administered i.p. at 800 μ g per mouse twice weekly as previously detailed (10).

Western Blotting

Cells were washed in PBS and lysed in ice cold Lysis Buffer [10 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, 2 mmol/L Na_3VO_4 , 1 mmol/L phenyl methylsulfonyl fluoride, 10 μ g/mL aprotinin, and 10 μ g/mL leupeptin]. Lysates were placed on ice for 10 min and then cleared by centrifugation. Protein concentration was measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories). Samples were run on 8% SDS-PAGE (30 μ g per lane) and then transferred onto Immobilon-P membrane (Millipore; Canada Ltd.). Membranes were blocked in (excess) 10% milk in TBST for 1 h at room temperature and then probed with primary antibody (at 1:1,000 dilution) overnight at 4°C. Membranes were then washed in TBST and probed with 1:5,000 dilution of anti-rabbit IgG–horseradish peroxidase or anti-mouse IgG–horseradish peroxidase (Promega). Detection was carried out using enhanced chemiluminescence reagent (Amersham Biosciences). HER2/ErbB2 antibody was from Cell Signaling Technologies, and anti- β -actin was obtained from Sigma Aldrich Canada.

Results and Discussion

β -hCG Monitoring of B16 Melanoma Experimental Metastases in Mice Undergoing Metronomic Administration of Cyclophosphamide and Antiangiogenic Drug Treatment Regimens

Previously, we have reported the antitumor effects of metronomic cyclophosphamide combined with the anti-VEGFR2 antibody DC101 in several experimental primary tumor models (10). To test if such a regimen was also effective against “artificial” (experimental) B16 melanoma metastases, B16.hCG cells (Table 1) were injected i.v. into

syngeneic mice. On day 9 after injection, after the first confirmed positive detection of β -hCG (indicating established metastases), the mice were randomized into four groups. These were treated with control PBS i.p., or administered a bolus plus oral low-dose metronomic cyclophosphamide regimen (as described in ref. 14), or given DC101 i.p. (15). The fourth group received the combination of bolus dose plus metronomic cyclophosphamide together with DC101 administration. As shown in Fig. 1A, DC101 on its own had no appreciable effect on urine β -hCG levels compared with controls. Surprisingly, the bolus plus low-dose cyclophosphamide alone group and the combination group showed a (delayed) superimposable β -hCG curve throughout the first 12 days of therapy (up to day 21; Fig. 1B), and only thereafter did the combination group show a slower increase in urine β -hCG levels (Fig. 1A). These results suggest that the effect of DC101 in the setting of established metastatic disease may be more important in blunting tumor repopulation as the therapy starts to fail, rather than contributing to an initial antitumor cytotoxic effect of this treatment. Figure 1C shows the corresponding survival curve (generated as mice had to be sacrificed due to cachexia), showing increased survival in the combination group compared with bolus dose plus metronomic cyclophosphamide, which is concordant with the observed β -hCG data. Figure 1D shows values for individual mouse urine sample analysis (on days 8 and 14) that are consistent with values for the pooled urine (shown in Fig. 1A and B).

β -hCG Analysis of Experimental Metastasis of Human Tumor Cells

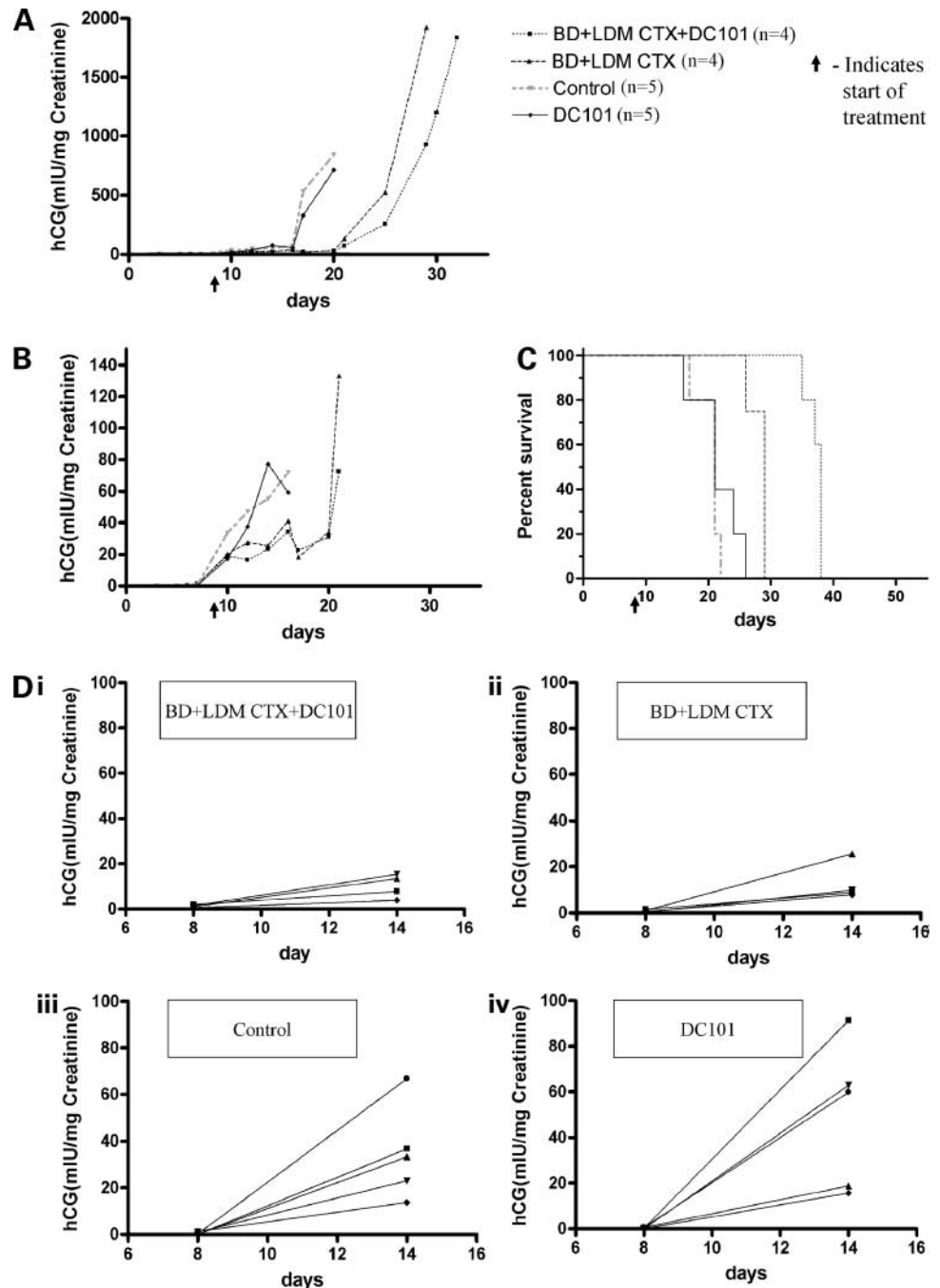
We next attempted to monitor experimental metastasis of β -hCG–tagged human tumor cells. To evaluate the kinetics of urine β -hCG readouts in experimental metastasis assays of human tumor cells, MDA435.hCG cells were generated and injected i.v. into female nude mice. Monitoring continued for 150 days thereafter, which revealed negative β -hCG readings for the first 40 days after injection followed

Table 1. Derived hCG expressing cell lines

Parent line	Derived line	Notes
MDA231 (<i>human breast</i>)	MDA231.hCGneo	Lung and kidney metastasis observed after i.v. injection
231H2N (<i>Her2+ve</i>)	231H2N. hCG	Spontaneous metastasis to liver, lung, and bone
H2N. hCG	H2N.hCG.met1	Spontaneous metastasis to brain, lung, bone and kidney
HT29 (<i>human colon</i>)	HT29.hCG (10, 11) HT-GFP.hCG	Metastatic after i.v. injection in SCID mice but poorly metastatic in nude mice
MDA-MB-435 PC3 (<i>human prostate</i>) EMT-6 (<i>mouse breast</i>)	MDA435.hCGneo PC3.hCGneo (10, 13) PC3.hCGneo.VEGF EMT-6. hCG	Highly metastatic after i.v. injection but poorly metastatic from orthotopic primary tumors
B16F1 (<i>mouse melanoma</i>)	B16F1. hCG	Refer to above EMT-6.hCG notes

NOTE: Numbers in parentheses refer to the relevant references.

Figure 1. Growth behavior of B16.hCG metastases under metronomic-based regimens. B16hCG cells were generated by transfection of pIRES-(*XhoI/XbaI*) β -hCG followed by puromycin selection, and injected i.v. (5×10^5 cells in 200 μ L) into female C57BL/6 mice. Treatment began on day 9 (arrow), after β -hCG detection in the mouse urine. Treatments involved bolus plus low-dose metronomic cyclophosphamide (BD + LDM CTX; bolus was 150 mg/kg on days 9 and 30; low-dose metronomic cyclophosphamide was 20 mg/kg/d in the drinking water) alone or combined with DC101 (anti-VEGFR2 Ab; 800 μ g per mouse twice per week, i.p.). Controls were given PBS (i.p.). β -hCG levels (in mIU/mg of Creatinine) in the urine was used to monitor growth of metastases—data are shown from pooled urine from each group shown in (A), and with the y axis magnified in (B)—this was done to highlight the overlap of the curves in the first 20 d). C, survival in this experiment. D, individual mouse urine β -hCG levels as measured on days 8 and 14. Using a log-rank test analysis for the survival data shown in C, bolus plus low-dose metronomic cyclophosphamide showed a significant increase in survival compared with controls ($P = 0.0047$), as did bolus plus low-dose metronomic cyclophosphamide plus DC101 ($P = 0.002$). DC101 alone did not significantly affect survival.



by a gradual appearance of detectable β -hCG (between 40 and 100 days after injection; Fig. 2A). Positive β -hCG readings were eventually observed in 6 of 18 mice injected. Most importantly, over the course of 160 days of this experiment, no mouse with a negative urine β -hCG reading ever developed metastatic disease. All the mice that developed detectable β -hCG levels had to be sacrificed eventually (due to cachexia), and all these mice had visible metastatic disease at autopsy. Furthermore, in all cases where β -hCG became detectable, levels could be monitored

for 4 to 8 weeks before any other or additional indications that the mouse had metastatic disease. Therefore, urine β -hCG measurement permitted early, "asymptomatic" detection of human tumor experimental metastases (Fig. 2A), growth of which could thereafter be monitored. Similar observations were made with human MDA-MB-231 breast cancer cells transfected with β -hCG (for which initial β -hCG detection took some 50 days; Fig. 2A), or with HT20.GFP.hCG colon cancer cells (for which 1 month was necessary for β -hCG detection in SCID mice but >100 days

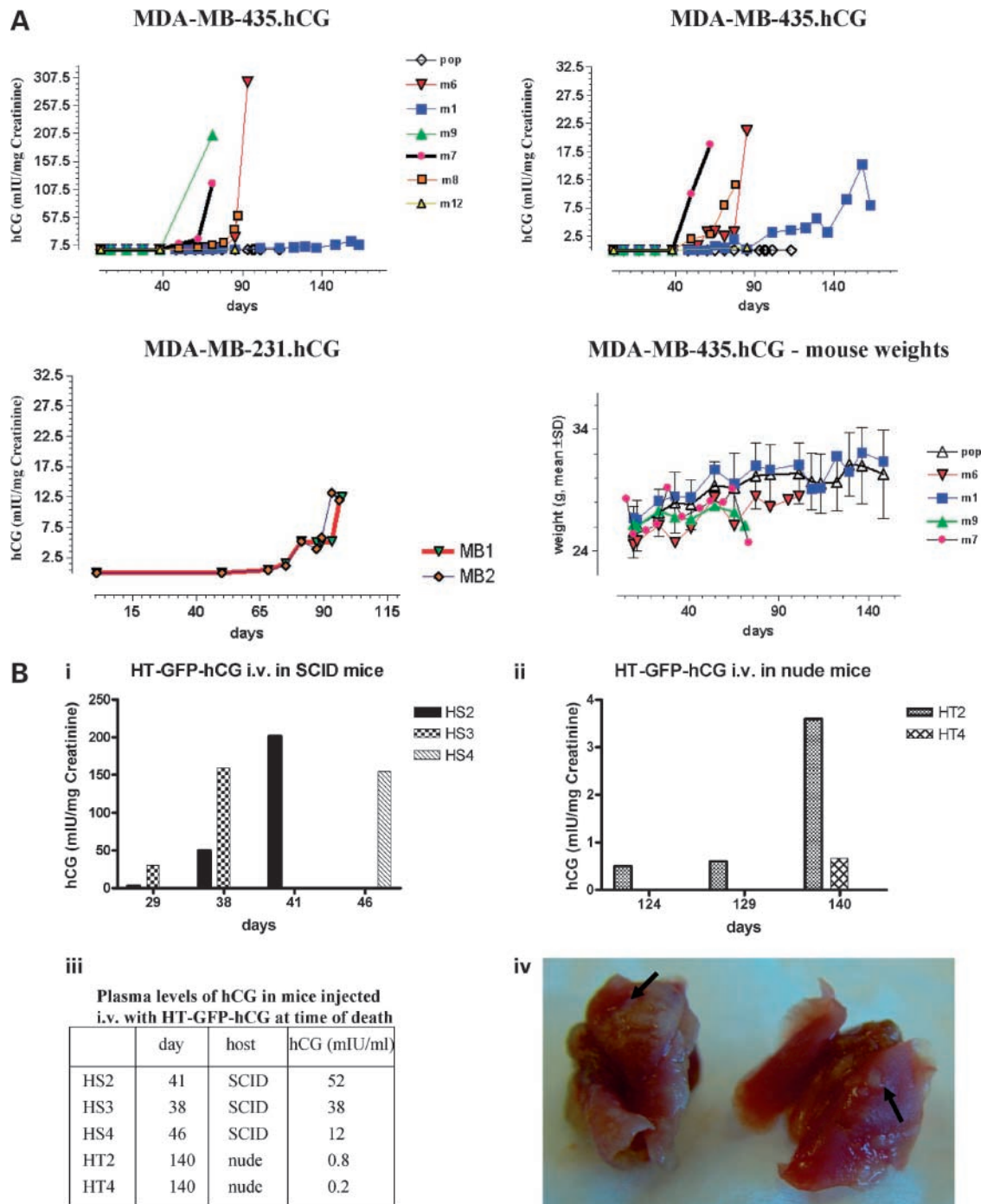


Figure 2. A, examples of β -hCG readouts in the urine of individual mice to monitor experimental metastasis of human MDA-MB-231.hCGneo (data for MB1 and MB2) or MDA-MB-435.hCGneo (all other data points shown) in female nude mice. Cells were injected i.v. (1×10^6 cells in 200 μ L), and urine was collected and assayed for β -hCG every 1 to 2 wk. *Top left*, data for mice ($n = 18$; m1–m18) injected with MDA-MB-435.hCGneo cells, of which 6 mice eventually developed metastasis [the remaining mice did not develop metastasis or detectable β -hCG levels, and are collectively presented as a negative population (pop) in the figure] as initially indicated by detectable β -hCG levels in the mouse urine; the same data are shown on the top right with a magnified Y-axis. *Bottom right*, examples of mouse weights for the corresponding mice injected with MDA-MB-435.hCGneo that developed metastasis; β -hCG detection provided early indication of the establishment of growing metastases whose relative growth could thereafter be monitored for 4 wk or longer in the absence of other indicators of metastatic disease such as weight loss or cachexia. *Bottom left*, examples of nude mice injected with MDA-MB-231.hCGneo. B, examples of β -hCG readouts from (i) SCID mice (HS2, HS3, and HS4) or (ii) nude mice (HT2 and HT4) injected i.v. with HT-GFP-hCG cells (1×10^6 cells in 200 μ L)—note that for nude mice, > 100 d of monitoring were necessary before β -hCG levels became detectable in the mouse urine. The results were confirmed by (iii) β -hCG measurement in plasma obtained at the time the mice were sacrificed and by autopsy; iv, examples of visible lung metastases of HT-GFP-hCG cells.

in nude mice; Fig. 2B). In HT20.GFP.hCG experiments, plasma was collected at the time the experiments were terminated, and used as a source of independent measurement of β -hCG levels (Fig. 2B).

Analysis of a Spontaneously Metastatic Variant of MDA-MB-231 Engineered to Overexpress erbB-2/Her-2

We next used a ErbB2/HER-2-transduced MDA-MB-231 variant, termed 231-H2N. The 231-H2N line, which we

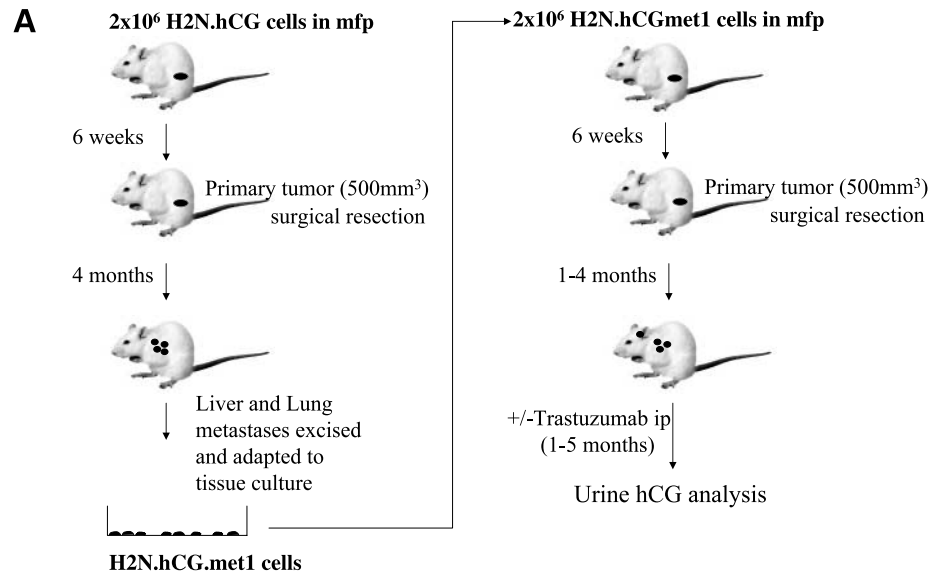
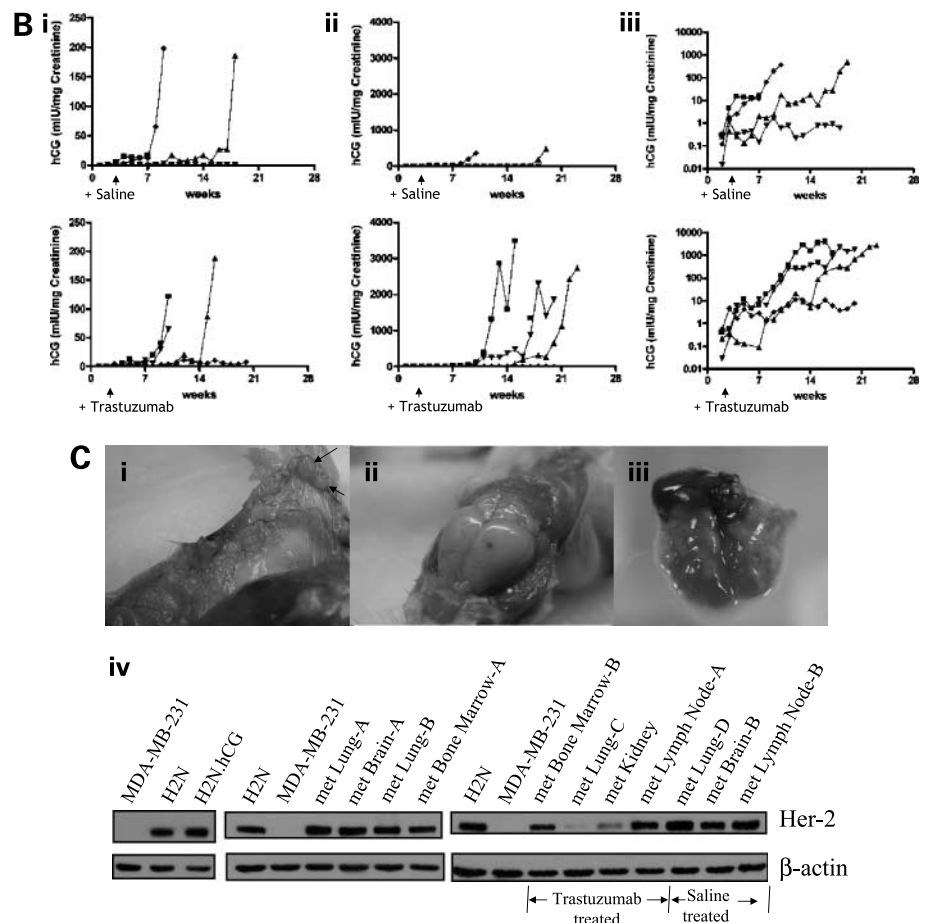


Figure 3. **A**, schematic of the derivation of spontaneously metastatic variants of MDA-MB-231 engineered to overexpress erbB2. H2N.hCG cells were generated by transfection of pIRES-(*Xho*//*Xba*) β -hCG followed by puromycin selection, and injected orthotopically into the inguinal mammary fat pad of female SCID mice. Primary tumors were removed when they reached an average size of 500 mm³. Metastases eventually resulting from this experiment were adapted to grow in tissue culture and combined to form the H2N.hCG.met1 population. H2N.hCG.met1 cells were then injected into the inguinal mammary fat pad of female SCID mice and, after removal of the primary tumor, some mice were used to test the effect of trastuzumab monotherapy on the growth of metastases. **B**, β -hCG readouts in female SCID mice with H2N.hCG.met1 metastases treated with saline (*top*) or trastuzumab (*bottom*, given twice weekly at 20 mg/kg i.p.) treatment initiated 2 wk after β -hCG was first detected in the urine. *i*, β -hCG values in the scale 0 to 250 mIU/mg Creatinine, and no difference in the β -hCG curves between the two treatment groups; *ii*, β -hCG values in the full range detected in this experiment (0–4,000 mIU/mg Creatinine); *iii*, same data on a log plot of β -hCG values. **C**, examples of H2N.hCG.met1 metastases to the (*i*) lymph nodes, (*ii*) brain, and (*iii*) lungs. *iv*, Western blot analysis for Her-2 in cells adapted to tissue culture from H2N.hCGmet1 metastases (*met*) to different tissues in mice under no treatment, or saline or trastuzumab monotherapy as indicated. H2N cells (used as a positive control) were included together MDA-MB-231 (parental cells as negative control) and the H2N.hCG transfectants. β -actin was used as a loading control.



previously described (12), was chosen for analysis for a number of reasons. Previously, we used this line to show the efficacy of combining the anti-Her-2 antibody trastuzumab (Herceptin) with metronomic low-dose cyclophosphamide to suppress the growth of orthotopically implanted 231-H2N primary tumors (12). The derivation of a metastatic variant of 231-H2N would therefore allow us to test if the same therapy is effective when treating metastatic disease. In addition, a metastatic model responsive to trastuzumab whose growth kinetics could be monitored (i.e., via β -hCG readings) would allow us to study conditions where resistance to trastuzumab-based regimens eventually develops. This, in turn, would permit proactive initiation of second-line therapies (12) when the first-line therapy begins to fail.

H2N.hCG cells were implanted in the mammary fat pad of female SCID mice. Primary tumors were then removed when they reached an average size of 500 mm³, and urine β -hCG dropped to undetectable levels. It took between 1 and 4 months after surgery for mice to develop macroscopic metastatic disease (Fig. 3A), and this occurred with 3 of 5 mice over a monitoring period of 8 months. Upon autopsy, metastases were found in the lungs and liver, from which lines were generated and adapted to tissue culture. The lines were then mixed (to maximize the heterogeneity of these metastatic variants), thus generating the H2N.hCG.met1 population (Fig. 3A), and injected orthotopically into 10 female SCID mice. Once again, primary tumors were removed when they reached 500 mm³ (see Fig. 3A). In this second round of *in vivo* selection, two mice were found to have positive urine β -hCG readings immediately after surgical removal of the primary tumors (presumably because they already had established metastases). For the other 8 mice, it took from 1 to 4 more months for β -hCG to become detectable in urine, and eventually all mice were found to have detectable β -hCG levels. As each of these 8 mice became β -hCG positive, it was placed into one of two groups. As shown in Fig. 3, 2 weeks after β -hCG detection, each mouse was randomized into a control-treated (PBS i.p.) group or a trastuzumab treatment group. Under these conditions, the growth of metastases (as solely determined by β -hCG readings) was found to be similar between trastuzumab treatment and control (Fig. 3B), suggesting trastuzumab monotherapy is ineffective in this setting of advanced metastatic disease. This stands in contrast to what we reported with respect to treatment of primary tumors using the parent (231-H2N) tumor line, i.e., an initial response to trastuzumab monotherapy lasting ~3 to 5 weeks followed by the emergence of resistance (12). We did however note that at the time the mice had to be sacrificed (due to cachexia), β -hCG levels were orders of magnitude higher in the trastuzumab-treated group than for controls (Fig. 3B). This observation suggests that trastuzumab monotherapy allowed the mice to tolerate a much higher tumor cell burden, the significance of which remains to be determined. Furthermore, some sublines from metastases in the trastuzumab treatment group showed reduced expression of erbB2 (Fig. 3C), as had

previously been noted for some 231-H2N tumors treated with trastuzumab (12). As detailed above, 2 of the 10 mice were found to have high levels of β -hCG immediately after surgery. These were excluded from the therapy experiment and had to be sacrificed (due to cachexia) 3 weeks after surgery; individual tissues were minced and placed in tissue culture. One week later, analysis of the tissue culture medium for β -hCG confirmed the presence of metastasis to the lungs, bone marrow, and brain. These results show that a spontaneous metastasis model was generated, capable of being monitored, which will be of considerable use for combination therapy experiments.

In summary, we have described examples of therapeutic strategies applied to different metastasis models using urine β -hCG levels to monitor progression of disease and response to therapy. In all the models tested, β -hCG proved to be a stable indicator of disease burden. Because of this stability, another application of the β -hCG monitoring system could be the study of emerging drug resistance to established therapeutic regimens. For example, using the H2N tumor model, we recently showed that primary tumors could be induced to regress using a combination of trastuzumab plus metronomic cyclophosphamide. This effect could be followed for up to 2 months, after which the tumors started to regrow while still under therapy. When such resistance emerged, tumor growth could be inhibited by the addition of anti-vascular endothelial growth factor antibody (12). To test if such an approach is also effective against metastatic disease, it would be ideal to use the β -hCG system as a means to detect the emergence of resistance (i.e., as a sudden upsurge in urine β -hCG levels) to the first-line therapy—at which point, second-line therapies could be proactively initiated.

One additional feature of the β -hCG methodology should be stressed. After initial experiments, we noted that for each tumor model, there was an upper end β -hCG value, which would routinely predict that the mice would need to be sacrificed within a week, even in the absence of any independent indicator of metastatic disease (e.g., cachexia). Such empirically determined β -hCG levels could therefore be set as an end point for survival studies where institutional, ethical, or other guidelines dictate that symptoms associated with advanced metastatic such as cachexia are not permissible or unacceptable. The availability of the lines described in this study should also facilitate the experimental analysis of different aspects of the biology and therapy of metastatic disease, including the testing of new strategies against drug-resistant disseminated disease.

Disclosure of Potential Conflicts of Interest

Z. Zhu and L. Witte: employees of ImClone Systems, Inc.; R.S. Kerbel: consultant for ImClone Systems, Inc. and Genentech/Roche. The authors hereby declare that there are no actual, potential, or apparent conflict of interest with regard to publication of this manuscript.

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