Clinical Cancer Research

Tumorgrafts as In Vivo Surrogates for Women with Ovarian Cancer


Abstract

Purpose: Ovarian cancer has a high recurrence and mortality rate. A barrier to improved outcomes includes a lack of accurate models for preclinical testing of novel therapeutics.

Experimental Design: Clinically relevant, patient-derived tumorgraft models were generated from sequential patients and the first 168 engrafted models are described. Fresh ovarian, primary peritoneal, and fallopian tube carcinomas were collected at the time of debulking surgery and injected intraperitoneally into severe combined immunodeficient mice.

Results: Tumorgrafts demonstrated a 74% engraftment rate with microscopic fidelity of primary tumor characteristics. Low-passage tumorgrafts also showed comparable genomic aberrations with the corresponding primary tumor and exhibit gene set enrichment of multiple ovarian cancer molecular subtypes, similar to patient tumors. Importantly, each of these tumorgraft models is annotated with clinical data and for those that have been tested, response to platinum chemotherapy correlates with the source patient.

Conclusions: Presented herein is the largest known living tumor bank of patient-derived, ovarian tumorgraft models that can be applied to the development of personalized cancer treatment. Clin Cancer Res; 20(5); 1288–97. ©2014 AACR.

Introduction

Ovarian cancer develops in an estimated 22,240 women in the United States annually (1) and most commonly presents with advanced stage, which has a high recurrence rate (2). Although incremental advances in chemotherapy over the past four decades have improved median survival, the cure rate is essentially unchanged (3) and ovarian cancer remains the most lethal gynecologic malignancy. An important barrier to achieving better outcomes for these patients is a lack of in vivo models that accurately reflect the diverse histology and molecular biology of primary tumors and predict their response to treatment.

Cell lines and cell line–derived xenograft models have contributed significantly to our current understanding of ovarian cancer development. However, it is well known that in vitro models are prone to culture-induced genotypic and phenotypic alterations that can diverge from the parent tumor (4, 5). Thus, the extrapolation of in vitro data to patient outcomes and response to therapy is of questionable utility. Indeed, the most frequently used ovarian cancer cell lines are significantly divergent from the molecular characteristics of patients with ovarian cancer (6) and may partially explain the lack of evidence supporting the use of in vitro drug sensitivity assays (7). Although xenograft models derived from human cell lines are an improvement (8–10), they are ultimately subject to many of the same limitations.

Heterotransplantation of fresh human tumors, also referred to as “tumorgrafts,” may more accurately recapitulate the primary patient tumor. When maintained under conditions of low passage in vivo, such models maintain histologic (11–13) and genomic (14) fidelity. As a result, tumorgrafts may act as better surrogates for patients and more accurately predict responses to treatment. As proof of principle, tumorgraft models developed for sarcoma, melanoma, and adenocarcinoma demonstrated strong correlation with the patient experience when tumorgraft response was used to guide treatment for patients (15). Although ovarian tumorgrafts have been used to study the efficacy of targeted therapies in molecularly defined subgroups, such as BRCA1/2-mutated (14, 16) or HER2-upregulated (17, 18) ovarian cancer, a prospective tumorgraft-guided study has...
3 cm3 of tumor slurry was mixed with 1:1 McCoy’s media and media (#10-050-CV; MediaTech). Approximately 0.3 to 0.5 minced on ice and in limiting volumes of ice-cold McCoy’s specialized training in gross dissection. Tumors were adjacent malignant tissue was procured by clinical staff with expanding with a single passage into 10 passage, tumor from the initial founder mouse/mice is sacrificed, and to maintain each tumorgraft model as low and Use Committee. Moribund mice with tumor were accorded with the Mayo Clinic Institutional Animal Care dimethyl sulfoxide, 1% penicillin/streptomycin in McCoy’s necessary in a small cohort of mice. Cryopreserved tumors are preexpansion amplification of tumor volume may be nec-sufficient tumor volume for banking and future experi-mals. Cells were frozen at −80°C in liquid nitrogen indef-initely. Ascites harvested from moribund mice was collected by sedated paracentesis before necropsy and red blood cells were lysed using ammonium-chloride-potassium (ACK) buffer (NH4Cl 0.15 mol/L, KHCO3 10.0 mol/L, Na2EDTA 0.1 mol/L); cells were viably frozen as above.

**Tissue processing and immunohistochemistry**

Tissues collected from mice or patients were fixed over-night in buffered formalin (Fisher Scientific; #23-011-120) and processed in the tissue core facility at Mayo Clinic (Scottsdale, AZ). Deparaffinized and rehydrated 5- to 6-μm sections were unmasked for 15 minutes in EDTA Buffer (1 mmol/L EDTA, 0.05% Tween 20, pH 8.0) at 95 to 99°C. Primary antibodies purchased from Life Technologies (pan-cytokeratin clone AE1/AE3 at 1:300 and Vimentin V9 at 1:2000) and Dako North America (Ki67 clone MIB1 at 1:600, CD45 clone 2B11 + PD7/26 at 1:500) were incubated overnight at 4°C. Secondary antibody (Cell Signaling: SignalStain Boost IHC detection system #S125S) was applied for 30 to 60 minutes at room temperature. Chromogenic detection of protein expression was determined in the presence of 3,3’Diaminobenzidine (DAB) (BioCare; Beta-zoid DAB) and visualized by light microscopy.

**In vivo CA125 measurements**

Whole blood was collected by sedated cardiac puncture from moribund mice bearing tumorgrafts and at necropsy. Serum was collected after a 30- to 60-minute incubation at room temperature. centrifugation at >13,000 × g for 10 minutes, and stored at −80°C. Sera (100 μL) were assayed for human CA125 using an ELISA kit according to the manufacturer’s protocol (#CA125T; Calbiotech). The stand-ard curve (r² = 0.994) and all reference controls were within the expected range. All patient samples were analyzed by the Mayo Clinic central clinical laboratory.

**Microarray and array comparative genomic hybridization**

RNA and DNA were simultaneously extracted following the manufacturers protocol for Qiagen AllPrep DNA/RNA mini Kit (#80204). Nucleic acid concentration and purity was determined on a Thermo Scientific NanoDrop 2000c UV-Vis Spectrophotometer. Total tumorgraft DNA was ana-lyzed by Affymetrix HG U133 plus 2.0 arrays at the Mayo Medical Genome Facility according to the manufacturer’s protocol.

To assess for genomic gains and losses, array comparative genomic hybridization (aCGH) was performed on the Agilent Human Genome CGH microarray Kit 244A using matched-patient reference genomic DNA as previously described (19). Briefly, genomic DNA was collected as above and 1 μg of test (tumor) and reference (matched-patient germline) DNA was labeled with Cy5 and Cy3, respectively, by random priming PCR, hybridized over 24 hours at 65°C, and analyzed with the Agilent Technologies Genomic Workbench 6.5 Lite Edition software. Gains and losses were defined as >4 regional probes with an absolute
average log ratio of 0.26 for the region, which equates to 1.67 gene copies for losses and 2.4 gene copies for gains.

Gene expression arrays were preprocessed and normalized by robust multichip analysis (20). For differential gene expression studies, platinum-sensitive versus platinum-resistant tumorgrafts were compared using the linear models and empirical Bayes methods that share information across genes to estimate variance (LIMMA; ref. 21) and visualized with Cluster (22) and Java TreeView (23). The criteria for differential expression were defined as fold change ≥5-fold and a P value of <0.01. For molecular subtyping, single sample gene set enrichment analysis was applied to 36 tumorgrafts using the classification of ovarian cancer (CLOVAR) gene set and normalized enrichment scores (NES) were derived as previously described (24). The degree of similarity across tumorgrafts was defined by the Pearson correlation coefficient between NES profiles.

**In vivo imaging**

Mouse abdominal fur was removed with Nair Sensitive Skin (Church & Dwight Co. Inc.). Aplicare sterile jelly lubricant (#82-280; Cardinal Healthcare) was applied to bare skin and an SLAx 13-6 MHz linear transducer with a SonoSite S-Series Ultrasound was used to capture weekly images for analysis of tumor cross-sectional area in the ImageJ 1.46 platform-independent software (25).

**Tumorgraft chemotherapy treatment**

Mice with 0.5- to 1-cm tumors were randomized to either intraperitoneal (IP) saline or carboplatin (#61703-360-18)/paclitaxel (#55390-304-05) from NOVAPLUS at 50 and 15 mg/kg, respectively, on days 1, 8, 15, and 22 as described (26). The primary endpoint was change in tumor size relative to day 1.

**Statistical analysis**

Time to engraftment and engraftment rate were determined using a cumulative incidence approach to account for models still under observation for determination of engraftment. Comparisons between engraftment status and patient characteristics were assessed using $\chi^2$ tests and Wilcoxon rank sum tests. The Spearman correlation was

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<th>Table 1. Descriptive statistics of patients providing tumor for models</th>
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<td>Patient age at collection, y median (range)</td>
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<td>Time (h) from FSL to mouse, median (range)</td>
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*CFSL, frozen section lab, which is the primary point of surgical material procurement.*
used to assess association between continuous variables. The association between engraftment status and overall survival for the patient was assessed using Kaplan–Meier curves and Cox proportional hazards models; overall survival was defined as time from ovarian cancer to death or date of last follow-up.

Results

Clinical characteristics of tumorgrafts

Ovarian, primary peritoneal, or fallopian tube tumors were collected at the time of surgery from patients at Mayo Clinic (Rochester, MN) and injected intraperitoneally into female SCID mice under an approved Institutional Animal Care and Use Committee protocol. Two hundred and forty-one models have been injected through December 31, 2012; 168 models have engrafted (Table 1), 46 models failed, and 27 are still under assessment (censored for evaluation) at the time of this publication. After an initial pilot study to determine optimal conditions, the engraftment rate is 74% (Fig. 1A). Patient data are associated with each tumorgraft in accordance with Health Insurance Portability and Accountability Act regulations and maintained by the Mayo Clinic Ovarian Tumor Repository. Basic information such as stage and grade is abstracted along with more detailed data such as chemotherapy type and number of cycles, recurrence-free and overall survival, and CA125 levels over time. Histologic diversity of tumorgrafts is reflective of ovarian cancer patients, with serous comprising the most common histologic subtype (Table 1). Mixed histology was seen and rare subtypes, carcinosarcoma and transitional cell, have been engrafted. Successful mouse engraftment was associated with adverse patient characteristics such as advanced stage ($P = 0.049$), high-grade tumors ($P = 0.00087$), and presence of ascites ($P = 0.00091$).

Although ascites was more common in serous primary peritoneal (15 of 19) relative to serous ovarian (43 of 71) tumorgrafts, consistent with other studies (27–30), this difference was not significant ($P = 0.2255$) and the relative frequency of ovarian and primary peritoneal tumors in tumorgrafts (3.74 to 1) was comparable with all patients consenting for this study (3.89 to 1). In addition, patients whose tumors successfully engrafted in mice had inferior overall survival (HR, 2.14; 95% confidence interval, 0.90–5.08; $P = 0.059$) relative to patients whose tumors did not successfully engraft (Fig. 1B).

Recapitulation of metastatic pattern in tumorgrafts

Tumorgraft models reproduce clinically relevant complications of ovarian cancer. The most common site of engraftment is in the pelvis but 46% of models will have involvement of the bowel, mesentery, visceral pleura of liver, spleen, diaphragm, or omentum. Intraperitoneal dysfunction, such as bowel obstruction (Fig. 1C, left), is observed along with mesenteric engraftment (Fig. 1C, right), which
may not be obstructive but can still cause weight loss in a near-moribund mouse. Of the 17 models that developed ascites in at least one mouse, 70.6% were derived from patients who had ascites at the time of debulking surgery. Tumorgraft ascites was typically bloody or serosanguineous. When normal mouse peritoneum (Fig. 1D, left) was compared with mice developing ascites, carcinoma-tosis was evident by innumerable explants forming a continuous sheet of malignant cells (Fig. 1D, center). Discrete masses often did not form in this setting but aggregates of cells in clusters or spherules (Fig. 1D, right) were apparent within ascitic fluid and carried malignant potential when passaged. As a pilot, 0.2 mL of fresh ascites from one mouse produced carcinomatosis and ascites in 3 out of 3 mice within 44 to 67 days.

Microscopic similarity between patients and tumorgrafts

To demonstrate similarity between tumorgrafts and their primary tumors, low-passage tissues were compared with initial surgical specimens. The glandular characteristic of adenocarcinoma was conserved, along with the relative proliferation index (Fig. 2A and Supplementary Fig. S1). Expression of pan-cytokeratin confirmed the epithelial origin of each tumorgraft, which is necessary because SCID mice can develop spontaneous lymphomas (31). Although tumor-associated lymphocytes (TAL) are common in primary tumors (32), CD45-positive cells did not frequently coheterotransplant with epithelial tumors (Fig. 2A). However, persistent CD45-positive cells were observed in a subset of tumorgraft models: PH040 and PH055 harvested 110 and 78 days, respectively, after injection into mice (Supplementary Fig. S2).

To determine if tumorgraft microenvironment mimics source tumors, stromal composition was estimated with ImageJ, as previously described (33). This was performed in eight models by selecting nonepithelial tissue areas, which were negative for pan-cytokeratin expression, and plotting the percentage area per field in patient tissue versus tumorgraft tissue (Fig. 2B). Strong correlation was seen in percent stroma composition with a Spearman \( r = 0.7381 \) (\( P = 0.0458 \)), suggesting a tendency for some tumors to induce stroma de novo as the epithelial component expanded (Fig. 2C). When patient and tumorgraft tissues were evaluated for expression of human vimentin using an antibody with no reactivity against mouse protein (34), patient stroma stained strongly while tumor-graft stroma did not, indicating that the stroma is murine and a subset of tumorgrafts maintain a propensity to recapitulate the source tumor microenvironment (Supplementary Fig. S2).

Genomic aberrations and molecular subtyping of tumorgrafts

Array CGH was performed on 41 tumorgraft models. To demonstrate the fidelity of aberrations between source patients and their resultant tumorgrafts, aCGH was performed in two tumorgraft models from different patient tumors. The results show marked overlap in genetic gains and losses between the patient tumor and corresponding
tumorgraft (Fig. 3A). In addition, commonly gained/lost genes in ovarian cancer, as determined by gene copy number analysis of 489 ovarian serous carcinomas from The Cancer Genome Atlas Research Network (TCGA; ref. 35), are seen in 41 tumorgraft models (Fig. 3B and Supplementary Fig. S3).

Ovarian cancer molecular subtypes have been proposed, based on global gene expression analysis (36), which was later validated in an independent dataset supporting four distinct molecular subtypes (differentiated, proliferative, immunoreactive, and mesenchymal; ref. 35). More recent evidence suggests that individual ovarian tumors are markedly heterogeneous in terms of subtype enrichment (24). Similar to patient tumors, early-passage tumorgrafts (n = 36) do not clearly classify into a specific subtype, but do exhibit gene set enrichment of multiple ovarian cancer subtypes and high correlation within subsets (Fig. 3C).

Limited utility of clinical CA125 in sera of mice bearing tumorgrafts

In vivo monitoring of tumor burden is necessary to assess real-time response to therapy. Given its clinical utility, CA125 has been considered as a biomarker of response during treatment experiments with tumorgraft models (11). To investigate this further, a pilot study with sera from eight

Figure 3. Molecular analysis of tumorgrafts. A, the graphical representation of array CGH at the chromosome level from source patient (blue) and tumorgraft (red) in model PH015 (high-grade endometrioid). B, heat map comparison of frequently lost (green) and gained (red) genes is the TCGA dataset (right column) compared with two representative tumorgrafts (PH015 and PH013, a high-grade serous). C, top, normalized enrichment scores (NES) of 36 tumorgrafts according to the CLOVAR (classification of ovarian cancer) gene signature. Varying degrees of enrichment for signatures associated with differentiated (red circle), immunoreactive (green square), mesenchymal (blue triangle up), and proliferative (purple triangle down) molecular subtypes were observed. Bottom, a heat map of 36 tumorgraft models clustered as a function of their gene expression correlation with each other. Highest (red), inverse (blue), and zero correlation was used to cluster tumorgrafts by their overall correlation.
models was conducted. All mice were near moribund with tumor, but not in excess of standard animal care and use regulations, to maximize circulating CA125. All models were derived from patients with high preoperative CA125 levels (median, 1,507; range, 276–3130). Only three models (PH013, PH015, and PH038) had CA125 levels above the limit of detection, despite having maximum tumor burden (Supplementary Fig. S4). Even then, CA125 levels in mice were only modestly elevated.

Correlation of response between patient and tumorgraft
To assess the clinical relevance of these models, response to platinum-doublet chemotherapy was investigated. Patients PH037, PH053, PH069, and PH070 had either platinum-refractory or -resistant cancer, defined as tumor progression during or within 6 months of completing chemotherapy, respectively (Supplementary Table S1). PH013, PH015, PH039, PH077, and PH080 were derived from patients with platinum-sensitive disease. Although patient PH077 clinically recurred 4.43 months after completing chemotherapy, she was still considered platinum sensitive because postoperative complications necessitated treatment delays and dose modifications, which led to suboptimal adjuvant treatment; after recurrence, she was subsequently treated with nine cycles of carboplatin/paclitaxel and achieved a complete response by CA125 and CT scan, indicative of platinum-sensitive disease. All nine tumorgraft models were heterotransplanted in SCID mice. Because tumor diameter accounts for only one dimension of growth, weekly cross-sectional area was measured by transabdominal ultrasonography to determine response. Tumor tissue (hypoechoic in mouse abdomen) can be discriminated from surrounding bowel/stool (Fig. 4A). Ultrasound highly correlated with traditional caliper-based measurements when both modalities were compared at necropsy (Fig. 4B). Representative ultrasound images show decreased tumor area and echogenicity in a sensitive tumorgraft, whereas a resistant tumorgraft demonstrated no change in the target mass but a new (nontarget) tumor grew on treatment (Fig. 4C). Saline-treated controls grew without regression. Nine of nine tumorgrafts (100%) demonstrated in vivo platinum responses reflective of the respective patient’s clinical response. After four weeks of carboplatin/paclitaxel as described in Materials and Methods, PH037 (n = 9), PH053 (n = 6), PH069 (n = 8), and PH070 (n = 8) exhibited tumor growth, whereas PH013 (n = 9), PH015 (n = 6), PH039 (n = 6), PH077 (n = 10), and PH080 (n = 7) regressed (Fig. 5A). When untreated tumors were analyzed by Affymetrix for differential gene expression, two distinct patterns were apparent and suggestive of a molecular difference between platinum-sensitive and -resistant tumorgrafts (Fig. 5B).

Discussion
The models presented herein comprise the largest published bank of human ovarian, primary peritoneal, and fallopian tube tumorgrafts. They recapitulate key clinical and molecular characteristics of the primary tumors and are molecularly relevant. Responses to carboplatin and paclitaxel in vivo correlate well with the corresponding patient’s clinical response. Although the most common pathologic subtypes are represented, the breadth of the engrafted models includes less common subtypes, such as carcinosarcomas and transitional cell ovarian cancer, providing an opportunity to test therapies for subtypes with limited options. Taken together, these tumorgrafts are valuable surrogates for patients with ovarian cancer.

A major strength of these tumorgrafts is the clinical data that accompany each model. Tumorgrafts can be selected for preclinical testing of novel therapeutics in a clinically defined subset of patient surrogates with ovarian cancer (such as those with known BRCA mutations or platinum-
resistant disease). Moreover, the availability of pre- and posttreatment tissue for correlative studies would obviate the need for invasive procedures in patients. In addition, actual tumor response to treatment can be determined in vivo without relying on clinical outcomes to approximate clinical benefit, such as the platinum-free interval used to define clinical platinum resistance. This is an imprecise surrogate of response and may incorrectly deny a patient from, or subject a patient to, platinum therapy (37). PH077 above is a case in point and supports the use of patient-derived ovarian cancer surrogates for preclinical testing of standard and novel therapeutics. The feasibility of maintaining a viably-frozen tumor bank is demonstrated in a high rate of engraftment after thawing. Twenty-two models have been pulled from frozen stocks and all were successfully reconstituted in mice. Across all models, the reengraftment rate was 97.7% (n = 307).

The applicability of these tumorgraft models to ovarian cancer is demonstrated by the molecular diversity seen between models, a reflection of the diversity seen in primary patient tumors (35, 36). The observation that individual ovarian cancers exhibit characteristics of multiple subtypes underscores the difficulty with assigning a single molecular subtype to a tumor as this does not fully account for the genetic complexity that may complicate therapeutic response. Designing future studies to target specific pathways associated with enriched molecular subtypes gives rise to the potential for personalized therapies and the models presented herein provide a mechanism to accomplish this goal. They may also help investigators design better clinical trials through discovery of predictive biomarker as well as testing combination therapies to find optimal drug doublets or triplets. As proof of principle, all nine tumorgraft models responded to chemotherapy commensurate to the corresponding patient’s clinical response and distinct gene expression patterns differentiate platinum-resistant and -sensitive tumors. The molecular mechanisms of resistance are multifactorial and studies are currently under way to better understand the specific genes and/or pathways involved.

The histologic conservation of primary tumor morphology and proliferation rate is consistent with other orthotopic tumorgraft models of ovarian cancer (10–14, 16, 38, 39). Maintenance of each model entirely in vivo would seem to be an important process because some ovarian tumorgrafts, which are initially established in mice but subsequently cultured in vitro, may no longer resemble the primary tumor and have altered response to chemotherapy (6, 38). Although TALs may play a role in human ovarian cancer progression, their significance in tumorgrafts remains to be seen. Human lymphocytes have been reported in other tumorgraft models (11, 13) but the uncommon occurrence in the present study suggests they are not necessary for engraftment or maintenance of the tumorgrafts.

Accurate assessment of in vivo tumor response is central to tumorgraft-based experiments and SCID mice pose unique challenges in this regard. For instance, small animal computed tomography, positron emission tomography, or MRI scanners may not be conveniently located in an animal barrier room, exposing SCID mice to unacceptable infection risk. In contrast, portable ultrasound overcomes these
limitations and also avoids the need for general anesthesia while providing accurate pre- and posttreatment tumor measurements, thereby allowing each animal to function as its own reference. We recognize that ultrasound is not used clinically because intraperitoneal tumors can be obscured by bowel and subcutaneous adipose tissue but mice are not limited by similar structural obstacles or large surface area as seen in the human intraperitoneal cavity. Moreover, when ultrasound measurements were compared with traditional caliper measurements at necropsy, correlation was strong. On the other hand, serial CA125 measurements have been proposed for ovarian tumorgrafts (11) but in our series, low sensitivity was limiting. This finding is consistent with the observation that human peritoneum is a source of serum CA125 (40) and its elevation in some patients may reflect peritoneal irritation from tumor burden, which is not measurable in mice using standard clinical assays (41).

In summary, ovarian cancer remains the most lethal malignancy in women, but there is hope that novel therapies or combinations of existing therapies will extend disease-free and overall survival. The development of accurate surrogates for patients with ovarian cancer enables investigators to discover new treatments and advance the vision of individualized therapy. As this tumor bank continues to grow, we hope to gain a better understanding of the mechanisms involved in chemotherapy resistance and develop new strategies to overcome barriers to better outcomes in our patients.

References

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