Establishing a peritoneal dissemination xenograft mouse model for survival outcome assessment of experimental gastric cancer

Changhua Zhang, MD, PhD,a,b,c Niranjan Awasthi, PhD,a,b Margaret A. Schwarz, MD,d and Roderich E. Schwarz, MD, PhD,a,b,*

a Division of Surgical Oncology, Department of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas
b Hamon Center for Therapeutic Oncology Research, Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas
c Department of Gastrointestinopancreatic Surgery, the First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong, China
d Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas

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Background: Peritoneal dissemination of gastric cancer is a common reason for unresectability, a frequent recurrence mechanism, and a common cause for death. The present study was performed to test peritoneal dissemination gastric cancer xenografts mouse models that would support survival outcome analyses.

Materials and methods: Human gastric cancer cell lines AGS, NCI-N87, and SNU-16 were intraperitoneally injected into nude mice and severe combined immunodeficiency (SCID) mice. The peritoneal tumor formation and mouse survival were compared among different groups. Mice were treated with oxaliplatin (5 mg/kg) and NVP-BEZ235 (10 mg/kg).

Results: The formation rate of peritoneal cancer after intraperitoneal injection of 5 \times 10^6 SNU16, NCI-N87, and AGS cells was 2/8, 6/8, and 0/8 in nude mice, and 6/6, 6/6, and 0/6 in SCID mice, respectively. Median animal survival with peritoneal dissemination was 74 d for NCI-N87 cells (10 \times 10^6), 95 d for SNU16 cells (10 \times 10^6), 78 d for SNU16 cells (20 \times 10^6), and 44 d for SNU16 cells (40 \times 10^6). In a therapeutic experiment with 40 \times 10^6 SNU16 cells, animal survival was significantly improved by oxaliplatin treatment compared with the control group (58.5 d versus 45 d, P < 0.001), but not by NVP-BEZ235 (48 d versus 45 d, P = 0.249) treatment. In the accompanying subcutaneous SNU16 mouse model, relative tumor volume compared with controls was not significantly decreased by oxaliplatin treatment (P = 0.151) but by NVP-BEZ235 therapy (P = 0.008).

Conclusions: Peritoneal gastric cancer xenografts were successfully established after intraperitoneal injection NCI-N87 and SNU16 cells. These findings provide a useful survival outcome assessment model for experimental gastric cancer research.

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1. Introduction

Gastric cancer is the second leading cause of cancer-related deaths throughout the world [1]. For locally advanced gastric cancer, radical resection is the most effective treatment component, even in the setting of multimodality therapy [2–4]. However, about one-third of patients have unresectable tumors at the time of diagnosis and about 40%–80% patients experience recurrence after gastrectomy [5,6]. Peritoneal dissemination is the prominent reason for unresectability of primary tumors; in addition, over 10% of gastric cancer patients have peritoneal disseminations at the time of diagnosis. Peritoneal dissemination is also the most frequent pattern of gastric cancer recurrence and encompasses 33%–50% of all recurrences [5,7,8]. The mechanism of peritoneal dissemination is still not well defined, but serosal involvement by the primary tumor appears to be the dominant risk factor [7,9]. There is no effective treatment for peritoneal dissemination, which therefore correlates with a poor prognosis. In a multicenter study of 370 patients with peritoneal dissemination from nongynecologic malignancies, mean and median overall survival periods were 6.0 and 3.1 mo, respectively [10].

Human tumor xenografts can be predictive of pathologic activity or treatment responses, to the point that laboratory in vivo experimentation has become an important tool to study the biology of the disease and to advance therapeutic options for a wide range of malignancies [11]. Every anticancer drug is essentially tested in animal models before being evaluated in any clinical trial. A reliable, reproducible experimental mouse model that mimics the natural progression of gastric cancer would be useful for delineating mechanisms of gastric cancer expansion and drug testing. Subcutaneous models are well established and widely used in gastric cancer experimental research for elucidating local tumor growth and drug pharmacokinetic parameters [12,13]. However, subcutaneous models may be quite different compared with primary human gastric cancer as they only reflect local tumor growth mechanisms; in addition, they do not lend themselves to survival analysis as a more important measure of experimental treatment efficacy. Previous animal studies have shown that some anti-tumor agents may well inhibit subcutaneous local tumor growth but do not affect overall animal survival to a comparable extent, suggesting that survival may be a more useful or reliable end point under such circumstances [11]. One established gastric cancer survival model reported in the literature uses MKN45 cell line that was derived from a liver metastasis in a female Japanese gastric cancer patient. The median survival of mice was 16–32 d after intraperitoneal injection of 10 × 10⁶ to 15 × 10⁶ MKN45 cells [14,15]. This model may not completely represent the biologic characteristics of peritoneal dissemination and intraperitoneal progression of gastric cancer because of its derivation from liver metastasis. In addition, due to the wide heterogeneity of gastric cancer, a single survival model may not be sufficient for in vivo therapy testing, and other lines with different origins would benefit this aspect of gastric cancer research [16]. Here we report results from our experiments with peritoneal dissemination mouse xenografts of different human gastric cancer cell lines for survival outcome analysis.

2. Materials and methods

2.1. Cell culture and reagents

The human gastric cancer cell lines AGS, NCI-N87, and SNU16 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere. AGS, a poorly differentiated cell line, had been established from primary stomach carcinoma [17]. The NCI-N87 cell line had been derived from gastric cancer metastatic to the liver [18]. The SNU-16 cell line had originated from malignant ascites of gastric cancer with a suspension growth pattern [18]. Polyethylene glycol (PEG300) and 1-methyl-2-pyrrolidone N-methyl-2-pyrrolidone (NMP) were purchased from SIGMA (St. Louis, MO). Oxaliplatin was purchased from Sanofi Aventis (Bridgewater, NJ). The dual AKT-mTOR inhibitor NVP-BEZ235 was purchased from LC Laboratories (Woburn, MA) and was dissolved into a mixture of 1:9 PEG300 and NMP.

2.2. Peritoneal dissemination model study

Animal studies were performed in accordance with the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center (Dallas, TX). Peritoneal dissemination model studies were performed using 6- to 8-wk-old female athymic nude mice or nonobese diabetic severe combined immunodeficiency (SCID) mice. Mice were randomly grouped (n = 4 to 8 per group). Nude mice were intraperitoneally injected with 5 × 10⁶ AGS, NCI-N87, or SNU16 cells per mouse, respectively. SCID mice were intraperitoneally injected with 5 × 10⁶ AGS, 5 × 10⁶ NCI-N87, 5 × 10⁶ SNU16 cells, 10 × 10⁶ AGS and 10 × 10⁶ SNU16 cells per mouse, respectively. Body weight was measured once a wk. Some mice in each group were euthanized every 30 d during the first 3 mo and peritoneal metastases were evaluated. All mice were euthanized and evaluated at the end of the 4-mo study. The peritoneal tumors were harvested, fixed in 4% formaldehyde, and embedded in paraffin. Then 5 µm thick tissue sections were obtained and stained with hematoxylin and eosin for microscopic examination.

2.3. Animal survival analyses

In the first experiment, 6- to 8-wk-old female SCID mice were randomly grouped (n = 5 to 6 per group) and intraperitoneally injected with 10 × 10⁶ NCI-N87, 10 × 10⁶ SNU16, 20 × 10⁶ SNU16, and 40 × 10⁶ SNU16 cells per mouse, respectively. Body weight was measured once a wk and animal survival was evaluated from the tumor cell injection until death. Animals were euthanized when turning moribund according to predefined criteria in order to avoid animal suffering.
Another subsequent survival study was performed with oxaliplatin and NVP-BEZ235 treatment. Each female SCID mouse (6 to 8 wk) received an intraperitoneal injection of $40 \times 10^6$ SNU16 cells. Three weeks after tumor cell injection, mice were randomly grouped ($n = 6$ to 7 per group) and treated intraperitoneally with phosphate buffered saline (PBS) (control), oxaliplatin (5 mg/kg in 100 μL PBS, two times a wk) and NVP-BEZ235 (10 mg/kg in 100 μL 1:9 PEG300 and NMP mixture, three times a wk) for 2 wk. Animal survival was evaluated from the first d of treatment until death and body weight was measured twice a wk. Animals were euthanized when turning moribund according to predefined criteria.

2.4. Subcutaneous tumor growth study

Female SCID mice (6 to 8 wk) were used for comparative modeling of subcutaneous tumor growth. SNU16 cells ($20 \times 10^6$) were subcutaneously injected into each mouse. The mice were weighed twice a wk. Fourteen days after tumor cell injection, all mice had measurable tumor. Mice were then randomly grouped ($n = 5$ per group) and treated...
intrapertonealy with PBS (control), oxaliplatin (5 mg/kg in 100 μL PBS, 2 times a wk), and NVP-BEZ235 (10 mg/kg in 100 μL 1:9 PEG300 and NMP mixture, 3 times a wk) for 14 d. The tumor size was measured twice wkly via caliper, and tumor volume (V) was calculated by using the formula [V = ½ (L x W²)], L = length and W = width [19]. Relative tumor volume was determined according to the formula Vₙ/Vᵢ, in which n equals the d of measurement, and Vᵢ represents the tumor volume on the first d. After completion of treatment, all mice were euthanized.

2.5. Statistical analysis

Survival analysis was evaluated with GraphPad Prism 5 Software (GraphPad Software, San Diego, CA). Statistical analysis for in vivo studies was performed by Student’s t-test for individual groups comparison of normally distributed data. Survival group comparison was performed via log-rank test within a Kaplan–Meier type analysis. P < 0.05 was considered to represent statistically significant group differences.

3. Results

3.1. Peritoneal metastatic tumor formation

After intraperitoneal injection of 5 × 10⁶ cells in nude mice, peritoneal tumors were found at 2 mo after implantation of NCI-N87 cells and at 4 mo of SNU16 cells; no tumor was found even 4 mo after implantation of AGS cells (Fig. 1). The formation rate of peritoneal tumor at 4 mo in nude mice was 6/8, 0/8, and 2/8 with NCI-N87, AGS, and SNU-16 cells, respectively (Table 1). In SCID mice, the tumor formation rate was 100% at 4 mo after intraperitoneal injection of either 5 × 10⁶ NCI-N87, 5 × 10⁶ SNU16, or 10 × 10⁶ SNU16 cells (Table 1, Fig. 2A and B). No tumor was observed in mice injected with AGS cells within 4-mo observation. SCID mice did develop more ascites after intraperitoneal injection of 5 × 10⁶ SNU16 cells compared with 5 × 10⁶ NCI-N87 cells (Fig. 2C and D). SNU16 cells developed numerous peritoneal nodules, grew in a loosely coherent pattern and contained microvilli (Fig. 3).

3.2. Animal survival in peritoneal dissemination model

Median survival of SCID mice in a peritoneal dissemination model was 44 d after intraperitoneal injection of 40 × 10⁶ SNU16 cells, which was significantly shorter compared with 74 d of 10 × 10⁶ NCI-N87 cells (P = 0.001), 95 d of 10 × 10⁶ SNU16 cells (P = 0.001), and 78 d of 20 × 10⁶ SNU16 cells (P = 0.001). Ranges of survival were 62 to 86 d for 10 × 10⁶ NCI-N87 cells, 90 to 120 d for 10 × 10⁶ SNU16 cells, 70 to 102 d for 20 × 10⁶ SNU16 cells, and 41 to 50 d for 40 × 10⁶ SNU16 cells (Fig. 4).

3.3. Effect of oxaliplatin and NVP-BEZ235 therapy on animal survival

In a SNU16 gastric cancer SCID mouse xenograft therapeutic study, animals were treated 21 d after tumor cell injection over a period of 14 d. The median survival was 45 d in the control group, 48 d in the NVP-BEZ235 (P = 0.249) treatment group, and 58.5 d in the oxaliplatin treatment group (P = 0.0004) (Fig. 5A). The death hazard ratio was 0.0559 (95% CI: 0.0114 to 0.2741, P = 0.002) after oxaliplatin treatment and 0.4817 (95% CI: 0.1393 to 1.666, P = 0.153) after NVP-BEZ235 treatment compared with the control group (Table 2). There was no significant difference in animal weight among these three groups over the course of therapy except for a single time point on d 38.

3.4. Effect of oxaliplatin and NVP-BEZ235 on local tumor growth

After the 14-d treatment, which consisted of similar doses of the therapeutic agents as used in the survival model, the local SNU16 tumor growth was reduced by NVP-BEZ235 and oxaliplatin treatment compared with the control group (Fig. 6A). The relative tumor volume decreased by 54.9% with NVP-BEZ235 treatment compared with the control group (Fig. 6A). The death hazard ratio was 0.151 compared with the control group (Table 2). The animal weight decreased significantly in the NVP-BEZ235 group (P = 0.036) compared with controls (Fig. 6B), but not in the oxaliplatin group (P = 0.548).

4. Discussion

Peritoneal dissemination is one predominant reason for gastric cancer-related deaths, and to date there is no highly effective treatment available [6,20]. A reliable peritoneal dissemination mouse model that represents the metastatic and dissemination tendencies of gastric cancer may benefit basic research and preclinical drug testing. In this study, three human gastric cancer cell lines were used in an attempt to establish peritoneal dissemination xenografts in nude mice as well as SCID mice. Nude mice are only deficient in T-cells, and SCID mice are also deficient in B-cells. After intraperitoneal injection of 5 × 10⁶ cells, the NCI-N87 cell line and the SNU16 cell line led to development of peritoneally disseminated tumors in both nude and SCID mice, while the AGS cell line did not develop tumors in either murine host. The peritoneal tumor formation rate of the NCI-N87 cell line was higher than that of the SNU16 cell line. It thus seemed that the NCI-N87 cell line was a better choice for a peritoneal dissemination model, but NCI-N87 cells grow very slowly and only reach up to 60% confluency in vitro. For this reason, it does not appear very feasible to use NCI-N87 cells in performing animal experiments in sufficient number to support meaningful

| Table 1 | Rate of peritoneal tumor formation in nude and SCID mice after intraperitoneal injection of different gastric cancer cells. |
|-----------------|-----------------|-----------------|-----------------|
| Mouse strain    | Number of cells (×10⁶) | Cell line |
|                 | SNU16 | NCI-N87 | AGS |
| Athymic nude    | 5     | 2/8     | 6/8 | 0/8 |
| SCID            | 5     | 6/6     | 6/6 | 0/6 |
| SCID            | 10    | 4/4     | –   | 0/4 |
| SCID = severe combined immunodeficiency. |
SNU16 cells grow quickly in a suspension pattern; therefore, these cells represented a better choice for gastric cancer mouse modeling to us. We have, therefore, resorted to the use of SNU16 cells for studying survival outcomes after peritoneal dissemination, and consider this a useful model for the investigation of experimental therapeutics targeting gastric cancer.

To reflect the diversity of tumor phenotypes, adequate models are necessary for the study of tumor heterogeneity [21]. For gastric cancer analyses, the MKN45 mouse survival model has been established and used in experimental therapy studies. Compared with MKN45 cells in a peritoneal survival model, the SNU16 cell peritoneal model has some distinguishing characteristics with theoretical experimental benefits that the MKN45 model does not possess. They are (1) the SNU16 cells originated from malignant ascites, and in the xenograft peritoneal dissemination setting also created a large amounts of ascites, which may reflect mechanisms of peritoneal progression with relevance to therapeutic targeting more appropriately; (2) SNU16 cells express and secrete

Fig. 2 — Formation of peritoneal tumor and ascites in SCID mice after intraperitoneal injection of $5 \times 10^6$ cells; (A) peritoneal tumor formation after 1 mo of implantation with SNU16 cells; (B) peritoneal tumor formation after 1 mo of implantation with NCI-N87 cells; (C) ascites after 4 mo of implantation with NCI-N87 cells; (D) ascites after 4 mo of implantation with SNU16 cells. (Color version of figure is available online.)
gastrointestinal cell associated antigens such as CEA, CA19-9, and TAG-72, all of which carry relevance to experimental gastric cancer therapy assessment [18]; (3) SNU16 cells developed a poorly differentiated in vivo phenotype with expression of microvilli and mucin, thereby maintaining the clinically encountered phenotype at greatest risk for peritoneal progression [18]. They grew rapidly in vitro, and although large cell numbers are required for reliable survival times to ensue, the nonadherent growth characteristics supported such utility for animal experimentation better than any other cell line tested. In contrast, MKN45 cells appear to have a more limited use because of the difficulties in preparing enough cells in vitro, as they appear best cultured in vivo and passaged several times for subsequent animal experiments [12]. A useful survival model, therefore, should not only lead to reliable and reproducible results regarding survival outcomes but should have a high degree of feasibility and user-friendliness. While this model, as any human cell line xenograft, carries the obvious shortcoming of being derived from a single cell line with limited ability to project applicability of therapy results to gastric cancer in general, we nevertheless consider this approach a worthwhile tool for experimental therapy of gastric cancer.

In our mouse survival model, the median survival after peritoneal injection of $40 \times 10^6$ SNU16 cells was 45 d and represents a nearly optimal time period for experimental therapeutic interventions with sufficient duration to expect outcome differences to remain detectable. Animal survival was significantly improved by oxaliplatin, a standard agent with clinical utility for gastric cancer, but not by the biological agent NVP-BEZ235 that in turn was the only of these two agents to show significant improvement.}

![HE staining of SNU xenograft mouse intraperitoneal tumors; (A) peritoneal tumor implant; (B) hepatic tumor implant.](Color version of figure is available online.)

![Survival of SCID mice with intraperitoneal injection of $10 \times 10^6$ NCI-N87 cells, $10 \times 10^6$ SNU16 cells, $20 \times 10^6$ SNU16 cells, and $40 \times 10^6$ SNU16 cells. The curve represents the animal survival time from the beginning of implantation. *Represents significant difference compared with $40 \times 10^6$ SNU16 cells at $P < 0.001$, and † represents significant difference compared with $10 \times 10^6$ NCI-N87 at $P < 0.01$.)
agents to reduce local tumor growth. Other reports in the literature show that anti-cancer agents decreased local tumor growth but did not enhance mouse survival and were subsequently found to not be effective in clinical trials [11,22,23]. In this study, oxaliplatin added a 43% survival hazard benefit compared with NVP-BEZ235, whereas NVP-BEZ235 added a 23% benefit to inhibition of local tumor growth over oxaliplatin. These results prompt a discussion on which experimental approach is superior in order to predict potential clinical benefits of an anti-cancer agent. Biological mechanisms of tumor progression in terms of local growth and peritoneal advancement are likely different, and may rely on epithelial and stromal mechanisms that potentially differ in their susceptibility to systemic treatment approaches [24]. One example would be an apparent stronger reliance on angiogenic mechanisms for local tumor growth promotion, with greater efficacy of antiangiogenic agents in local tumor models compared with survival studies [11,23,25,26].

Several anti-cancer agents that are highly effective in a mouse xenograft model fail to show any benefit in clinical trials [11,27]. One important possible reason is that most phases I, II, and III clinical trials enroll advanced and late-stage patients, whereas most mouse studies do not test therapeutic effects on advanced metastatic disease [11]. Our initial experience with this SNU16 mouse survival model supports its mimicking of peritoneal dissemination of gastric cancer and creating a later stage of gastric cancer, especially after treatment is initiated after a 2- to 3-wk interval. We believe that preclinical data of anti-cancer agents tested in this SNU16 mouse survival model carry the potential for greater relevance regarding further clinical exploration in advanced gastric cancer. The diffuse peritoneal progression of this model may even be advantageous compared with transgenic and human tissue xenograft mouse models as increasingly utilized for experimental therapy purposes [11,28].

In summary, we were able to develop a reliable and highly feasible SCID mouse gastric cancer peritoneal dissemination survival model with intraperitoneal injection of $40 \times 10^6$ SNU16 cells. Mouse survival after oxaliplatin and NVP-BEZ235 treatment showed efficacy patterns different from local tumor growth experiments using similar cell line and therapeutic agents. The findings support a novel and potentially useful model for survival outcome analysis in gastric cancer research.

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