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Patient-derived xenograft models of squamous cell carcinoma of the uterine cervix

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ABSTRACT

Patient-derived xenograft (PDX) models of cancer are considered to reflect the biology and treatment response of human tumors to a larger extent than xenograft models initiated from established cell lines. The characterization of a panel of four novel PDX models of cervical carcinoma of the uterine cervix is described in this communication. The outcome of treatment differed substantially among the donor patients, and the PDX models were found to mirror the histology, aggressiveness, and metastatic propensity of the donor patients' tumors. Two of the models (BK-12 and LA-19) were highly metastatic, one model (ED-15) was poorly metastatic, and one model (HL-16) was non-metastatic. The primary tumors of the two highly metastatic models showed high density of intratumoral lymphatics, whereas the other two models did not develop intratumoral lymphatics. The potential of the models to metastasize to lymph nodes was associated with high expression of both angiogenesis-related genes and cancer stem cell-related genes. The models may be highly valuable for studying mechanisms linking lymph node metastasis to lymphangiogenesis, hemangiogenesis, and the presence of cancer stem cells.

Introduction

Locally advanced carcinoma of the uterine cervix is generally treated with cisplatin-based concurrent chemoradiotherapy [1]. Approximately two thirds of the patients are disease-free five years after treatment, but at the expense of a high incidence of severe treatment-induced complications [2,3]. Treatment failure is associated with severe abnormalities in the microenvironment of the primary tumor, including high lactate concentration, elevated interstitial fluid pressure, low microvascular density, low oxygen tension, and large regions with hypoxic tissue [4–8]. The development of improved therapeutic strategies may require appropriate preclinical tumor models for exploring basic biological mechanisms affecting tumor growth, metastatic dissemination, and outcome of treatment.

Most current preclinical *in vivo* studies of cervical cancer are carried out by using xenografted tumors initiated from established cell lines as tumor models. There is increasing evidence that cell line-derived tumor xenografts do not adequately mirror the biology of tumors in patients and have limited power to predict clinical treatment outcome, and thus are suboptimal models of human cancer [9]. Recently, there has been an enhanced interest in establishing novel tumor models by transplanting patient tumors directly into immune-deficient mice and maintaining the tumor tissue solely *in vivo* [10,11]. These models, usually referred to as patient-derived xenograft (PDX) models, have been suggested to be useful in several types of translational cancer research, including screening of potential treatment agents, identification and evaluation of biomarkers, and development of personalized treatment strategies [9–13].

A panel of PDX models is required to address biological and therapeutic questions related to a specific malignant disease. Four new PDX models of squamous cell carcinoma of the uterine cervix are reported in this communication. These PDX models differ significantly in molecular characteristics, and the validity of the models was assessed by comparing their growth pattern and metastatic propensity with the histology and aggressiveness of the donor patients' tumors. Our observations show that essential biological characteristics of the donor patients' tumors are recapitulated in the PDX models, and moreover, the data suggest that the four models constitute a panel that may be particularly useful for studying mechanisms linking lymph node metastasis to cancer stem cells and tumor-induced lymphangiogenesis.

Materials and methods

Donor patients and donor patients' tumors

Four models of squamous cell carcinoma of the uterine cervix (BK-12, ED-15, HL-16, LA-19) were established in athymic mice from patients with FIGO stage IIB

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disease. The patient and xenografted tumors were positive for HPV E6 and E7. The donors were treated with curative intent at the Norwegian Radium Hospital according to the chemoradiotherapy protocol for previously untreated locally advanced cervical cancer. The tumor models were derived from the primary tumors of the donor patients before the patients were subjected to treatment.

The BK-12 model was derived from a 45-year-old patient having developed a primary tumor with a volume of ~60 cm^3 and at least five pathological pelvic lymph nodes at presentation. Locoregional control was not achieved after treatment, and the patient died with an extensive tumor burden in the pelvis 32 months after the initial diagnosis. Histologically, the primary tumor was moderately differentiated (histological grade II), showed abundant keratinization, and appeared as solid sheets of carcinoma cells separated by a sparse stroma.

The donor of the ED-15 model was a 62-year-old patient with a primary tumor with a volume of ~90 cm^3. Pathological lymph nodes were not detected at presentation. The patient responded well to treatment and was disease free at the 5-year control. The primary tumor was moderately differentiated (histological grade II) and showed abundant keratinization. Nests of carcinoma cells were surrounded by a moderate stroma.

The HL-16 model was derived from a 56-year-old patient showing a 180-cm^3 primary tumor but no lymph node involvement at presentation. The treatment resulted in locoregional control, and the patient was free of disease at the 5-year control. Histologically, the primary tumor was poorly differentiated (histological grade III), appeared as solid sheets of carcinoma cells separated by a moderate stroma, and showed an inconspicuous inflammatory reaction.

The donor of the LA-19 model was a 41-year-old patient presenting with a small primary tumor with a volume of ~25 cm^3 and with extensive metastatic disease involving at least seven lymph nodes. Locoregional control was achieved after treatment; however, the patient had developed distant lymph node metastases at the 1-year control and died from metastatic disease 7 months later. The primary tumor was moderately differentiated (histological grade II) and showed prominent regions with necrotic tissue and an abundant inflammatory reaction. The carcinoma cells appeared as broad sheets separated by a sparse stroma.

Mice

Adult (8–12 weeks of age) female BALB/c *nu*/*nu* mice, bred at our institute, were used as hosts for xenografted tumors. The mice were maintained under specific pathogen-free conditions at constant temperature (24–26 °C), constant humidity (30–50%), and a 12-h light/12-h dark cycle. Sterilized food and tap water were given *ad libitum*.

Establishment of patient-derived tumor models

Tumor tissue was removed from the donor patients' tumors with a curette and cut into ~1-mm diameter pieces with a scalpel. The pieces were transplanted subcutaneously into recipient mice, and when the transplants had grown to ~1-cm diameter tumors, the tumors were excised. To expand the tumor material, cell suspensions were prepared from the excised tumors, and aliquots of ~1×10^6 cells were inoculated intramuscularly into the right hind leg of mice. Intramuscular tumors developed, and to establish a large stock of tumor material for future experiments, cell suspensions from these tumors were frozen and stored in liquid nitrogen. The frozen stock thus consists of cell suspensions from tumors in passage 2. The characterization experiments described herein were carried out with 400–600-mm^3 intramuscular tumors in passage 4 or 5. These tumors were initiated in the right hind leg of mice by inoculating aliquots of 5×10^5 cells derived from intramuscular tumors initiated from the frozen stock.

Tumor growth rate and lymph node metastasis

Tumor volume (V) and tumor volume doubling time (T_d) were calculated as $V = \pi/6 \times a \times b \times c$ and $T_d = \ln 2 \times t/(\ln V_t - \ln V_0)$, where a, b, and c represent three perpendicular tumor diameters measured with calipers, and V_t and V_0 represent tumor volume at time t and time 0, respectively. Euthanized mice were examined for external lymph node metastases in the inguinal, axillary, interscapular, and submandibular regions, and internal lymph node metastases in the abdomen and mediastinum. The presence of metastatic growth in enlarged lymph nodes was confirmed by histological examination.

Immunohistochemistry and histological examinations

Histological sections were prepared by standard procedures and stained with hematoxylin and eosin (HE) or immunostained for hypoxia, blood vessels, lymphatics, or collagen I. Pimonidazole [1-[(2-hydroxy-3-piperidinyl)-propyl]-2-nitroimidazole], injected as described earlier [14], was used as a marker of tumor hypoxia, and CD31 and LYVE-1 were used as markers of blood and lymph vessel endothelial cells, respectively. An anti-pimonidazole rabbit polyclonal antibody (Professor James A. Raleigh, University of North Carolina, Chapel Hill, NC, USA), an anti-mouse CD31 rabbit polyclonal antibody (Abcam, Cambridge, UK), an anti-mouse LYVE-1 rabbit polyclonal antibody (Abcam), or an anti-collagen I rabbit polyclonal antibody (Abcam) was used as primary antibody. Quantitative studies were carried out on preparations cut through the central regions of tumors, and three sections of each staining were analyzed for each tumor. Microvessels were scored as described by Weidner [15]. Fraction of pimonidazole-positive tissue and fraction of collagen I-positive tissue were assessed by image analysis [16] and were defined as the area fractions of the viable tissue showing positive staining.

Quantitative PCR

Total RNA was isolated from tumor tissue stabilized in RNA*later* RNA Stabilization Reagent (Qiagen, Hilden, Germany). RNA isolation and cDNA synthesis were performed as described previously [17]. The RT^2 Profiler PCR Arrays Human Angiogenesis (PAHS-024Z) and Human Cancer Stem Cells (PAHS-176Z) from SABiosciences (Frederick, MD, USA) were used for expression profiling of angiogenesis-related and cancer stem cell-related genes, respectively. Real-time PCR was performed as described elsewhere [17]. Fold difference in gene expression was calculated by using the $\Delta\Delta C_T$-method [18]. A C_T-value of 35 (15 cycles above the positive PCR control) was set as detection limit, and hence, all C_T-values above 35 were set to 35. The arrays included 5 housekeeping genes, and each C_T-value of a tumor was normalized to the mean C_T-value of these genes ($\Delta C_T = C_T^{\text{gene of interest}} - C_T^{\text{mean of housekeeping genes}}$). The normalized gene expression level of each PDX model was calculated from three tumors as $2^{-\text{mean } \Delta CT}$.

Ethics

The excision of tumor tissue for establishing PDX models of cervical cancer was approved by the Institutional Ethics Committee. Informed consent was obtained from the donor patients. Animal care and experimental procedures were approved by the Institutional Committee on Research Animal Care and were performed in accordance with the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY, USA) and the EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

Statistical analysis

Data are presented as mean ± standard deviation. Statistical comparisons of data were carried out by one-way ANOVA followed by the Bonferroni's test when the data complied with the conditions of normality and equal variance. Under other conditions, comparisons were carried out by Kruskal–Wallis ANOVA on ranks followed by the Dunn's test. Probability values of $p < 0.05$, determined from two-sided tests, were considered significant. The statistical analysis was carried out with SigmaStat statistical software.

Results

The four PDX models differed substantially in histological appearance (Fig. 1). The BK-12, ED-15, and LA-19 tumors were moderately differentiated, whereas the HL-16 tumors were poorly differentiated, consistent with the donor patients' tumors. Furthermore, the xenografted tumors were similar to the donor patients' tumors in the appearance of stroma, keratinized tissue, necrosis, and inflammatory reaction, thus reflecting the interpatient tumor heterogeneity.

All tumor models showed heterogeneous staining for pimonidazole, and the staining pattern was consistent with staining of hypoxic tissue without staining of normoxic tissue (Fig. 1). Necrotic regions were always encompassed by a rim of pimonidazole-positive tissue, a few cell layers thick, and non-necrotic tissue showed scattered foci of pimonidazole-positive tissue differing in size and shape. Perinecrotic as well as focal staining were characteristic features of the BK-12 and ED-15 tumors. The HL-16 tumors had little necrosis and showed primarily focal staining, whereas the LA-19 tumors had substantial necrotic regions and showed a predominant perinecrotic staining pattern.

The ED-15 and HL-16 tumors differed from the BK-12 and LA-19 tumors in the development of blood vessels and lymphatics, as illustrated in Fig. 2, which shows histological preparations from HL-16 (Fig. 2A and B) and LA-19 (Fig. 2C–F) tumors. Similar illustrations for ED-15 and BK-12 tumors are shown in Fig. S1. Blood vessels were seen in the central as well as in the peripheral regions of the tumors of all models; however, the vessel diameters appeared to be generally larger in the BK-12 and LA-19 tumors than in the ED-15 and HL-16 tumors. The ED-15 and HL-16 tumors showed peritumoral