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Model for paraaortic lymph node metastasis produced by orthotopic implantation of ovarian carcinoma cells in athymic nude mice

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Abstract

Lymph node metastasis through the lymphatic vessels is a critical step in determining the outcome of ovarian cancer patients, and prognosis should be improved by preventing lymph node metastasis. However, experimental models for lymph node metastasis of ovarian carcinoma are not available. We developed an orthotopic transplantation model to study this process in nude mice using the human ovarian carcinoma cell lines, KF and MH. Highly metastatic sublines (KF-LN3 and MH-LN3) were selected *in vivo* in nude mice by repeated orthotopic transplantation, lymph node metastasis formation and culturing the tumour cells *in vitro*. Because this model seems to correspond to the advanced clinical stage of ovarian carcinomas, it should be useful in understanding the molecular biology of ovarian carcinomas and in the development of therapeutic modalities against lymph node metastasis.

Keywords: Ovarian cancer; Lymph node metastasis; Animal model

1. Introduction

One of the most troublesome impediments in the treatment of ovarian carcinoma is metastasis to paraaortic (PANs) or pelvic retroperitoneal lymph nodes [1–3]. To prevent lymph node metastases, a novel therapy based on the biological feature of the cancer is required for ovarian cancer, in addition to conventional surgery and chemotherapy. However, the mechanism of this process is not well understood at the cellular and molecular levels [4]. One of the reasons is the lack of animal models that can be used to examine the entire process of lymph node metastasis [5–7]. While a carcinogenesis and metastasis model of the specific organ may be most favourable, orthotopic transplantation models in athymic nude mice or severe combined immunodeficient (SCID) mice should also have advantages because they permit examination of biological behaviours of human carcinoma cells *in vivo*. Furthermore, these models can be constructed to reflect the specific pathology of the organ [8].

Therefore, we developed a novel experimental model of lymph node metastasis from ovarian carcinomas.

2. Materials and methods

2.1. Examination of patients' records

Between 1983 and 2000, 325 Japanese women with primary ovarian cancer were initially treated at the Department of Obstetrics and Gynecology of the Keio University Hospital. Between 1983 and 1989, our standard radical operation for patients with early stage cancer (stages I and II) was total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. Since 1990, pelvic and paraaortic lymphadenectomy has been added to the list of possible surgical treatments. Clinical data were collected from the patients' hospital records in the Keio University Hospital.

2.1.1. Animals

KSN nude mice (KSN/nu, 5 weeks old, female) were purchased from JAPAN SLC Inc. (Hamamatsu, Japan) and kept under pathogen-free conditions. This study

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was conducted in accordance with the standards established by the Guideline for the Care and Use of Laboratory Animals of the University of Tokyo.

2.1.2. Human ovarian cancer cell lines used in this study

MH [9–11] and KF [12–14] were kindly provided by Dr Yoshihiro Kikuchi (Department of Obstetrics and Gynecology, National Defense Medical College, Saitama, Japan). These cells were maintained at 37 °C in 5% CO_2 on plastic tissue culture dishes (Falcon, Lincoln Park, NJ) in a 1:1(v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY, USA).

2.1.3. Paraaortic lymph node metastasis by orthotopic implantation of human ovarian carcinoma cell lines

KSN/nu mice were anaesthetised with avertin (2,2,2-tribromoethanol; Aldrich, Milwaukee, WI, USA). The lower abdomen was swabbed with 70% alcohol and a small skin incision of about 20 mm in length was made in the left lower abdominal wall. A 30-gauge needle attached to a 1.0 ml syringe was directly inserted into the left ovary. Viable tumour cells (3×10^6) suspended in 50 μl of Hanks Balanced Salt Solution (Nissui, Tokyo, Japan) were injected. The skin incision was closed with a soluble thread. After confirming that the animals had recovered from bradycardia and had a stable spontaneous respiration, they were returned to their cages. Eight weeks after the inoculation of the cells, the mice were sacrificed and examined to see whether they had developed PAN metastasis.

2.1.4. Establishment of highly metastatic sublines from MH and KF cells

Formation of a primary lesion in the ovary was observed in all mice with intraovary inoculation of MH and KF cells. To obtain a highly metastatic variant subline from MH and KF cells, we performed the following sequence of operations: inoculation of these cells (3×10^6) into the ovary of mice, removal of the draining PANs that were positive for metastasis, cultivation of tumour cells from these lymph nodes from 2 mice, and re-inoculation of the tumour cells derived from lymph node metastasis into another group of mice ($n=3$–5). After repeating this procedure 3 times, we obtained sublines (MH-LN3 and KF-LN3) from MH and KF cells that had an increased capacity to metastasise to PANs.

2.1.5. Histological examination of tumours at the inoculation sites and lymph node metastases

Light-microscopic examinations of the primary lesions and metastases were performed on the mice inoculated with MH-LN3 and KF-LN3 cells ($n=10$) 5–6 weeks after treatment. The tumour specimens were fixed with 10% neutral formalin solution and embedded in paraffin after dehydration. Sections were stained with haematoxylin and eosin and examined under a light microscope.

3. Results

3.1. Correlation of PAN involvement with poor survival of ovarian carcinoma patients

Regional lymph node metastasis from the ovarian cancer is thought to be relevant to tumour spread. When we examined 325 patients who had undergone surgical treatment at the Keio University Hospital between 1983 and 2000, the following features were observed. (a) As the disease status progresses from T1 (TNM classification, tumour limited to the ovary) to T3 (tumour spread including the extra-pelvic cavity), the rate of lymph node metastasis from the ovarian cancer increased (Fig. 1). (b) There is an inverse correlation between patient survival and the presence of PAN metastasis (Fig. 2(a–d)). Significant differences between the survival rate of pT1N0M0 patients and that of pT1N1M0 patients, and between pT(1+2)N0M0 patients and pT(1+2)N1M0 patients, were observed. Thus, we established an experimental model to study PAN metastasis in nude mice.

3.1.1. Metastatic potential of MH cells and KF cells after intraovarian transplantation

Orthotopic transplantation of human ovarian carcinoma cells were employed with athymic nude mice. Tumours developed at the site of direct implantation of MH or KF cells in 100% of the animals in 3 separate experiments. Metastatic growth of tumour cells was

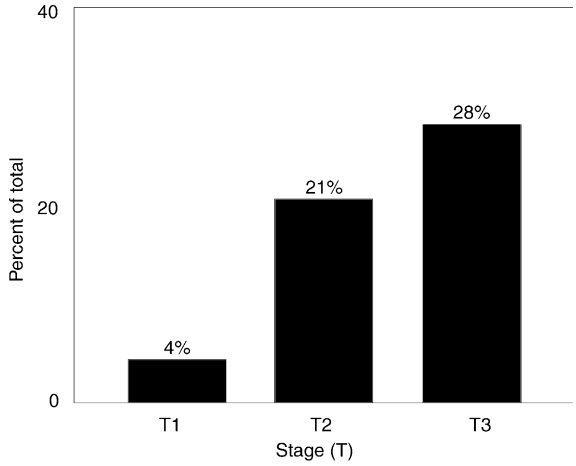

Fig. 1. The percentage of lymph node metastasis in ovarian carcinomas initially treated at the Keio University Hospital (1983–2000). The TNM classification was applied. Ordinate shows the incidence of regional lymph node metastases.

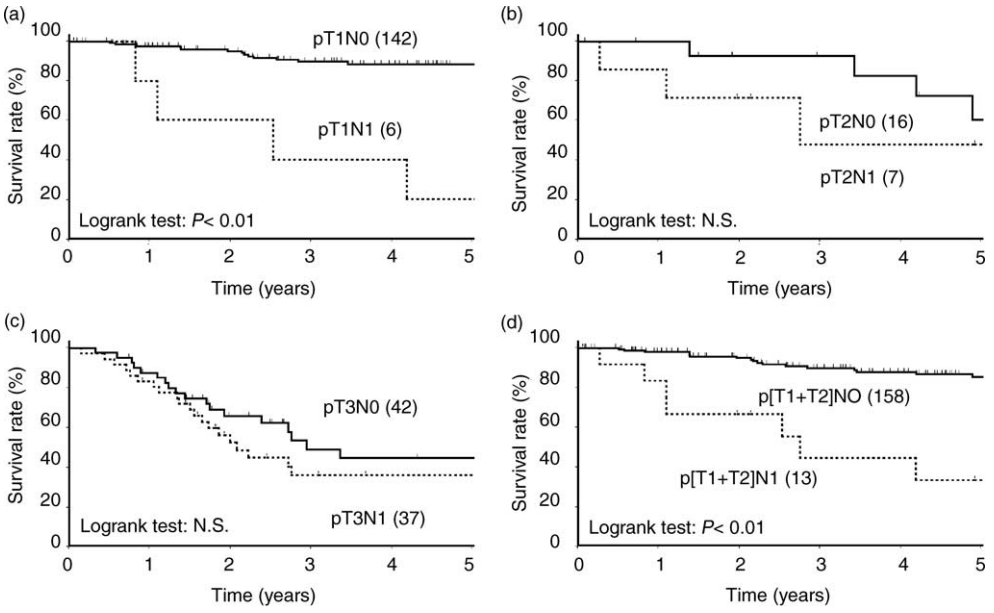

Fig. 2. The survival of ovarian carcinoma patients at each stage. (a) pT1M0, (b) pT2M0, (c) pT3M0, (d) pT(1 + 2)M0. Statistical significance of the differences was examined by the logrank test. NS, non-significant.

observed in PANs in most of the cases. Swelling of the bilateral PANs was also observed 8 weeks after implantation. The incidence of lymph node metastases was 4/4 for left side PAN and 3/4 for the right side PAN at 8 weeks after inoculation of MH cells. In the case of KF cells, the incidence was 3/4 for the left side PAN and 2/4 for the right side PANs at 8 weeks after the inoculation. When metastases to PANs did not occur, PANs were usually invisible.

Tumours derived from MH cells and KF cells developed at the implantation sites and lymph node metastases revealed similar histological features to a poorly differentiated serous adenocarcinoma, as shown in Fig. 3.

When six animals were transplanted intraperitoneally (i.p.) with 3×10^6 cells of the MH or KF cell line, tumours grew very fast and all the animals died 5 weeks after transplantation. Both cell lines formed tumours associated with various intraabdominal organs, although PAN metastases, ascitic tumour cells, or tumours at extraabdominal organs were not observed. Mesenteric lymph node involvement, which did not belong to primary regional lymph nodes for the ovarian cancer, was observed (data not shown). In the orthotopic intraovary implantation that we developed in the present work, the tumour spread was relatively limited in comparison to intraperitoneal transplantation, which mimics clinical situations in ovarian cancers. Thus, we wanted to establish highly metastatic sublines to PANs from these original parental cell lines.

3.1.2. Selection of highly metastatic sublines

In vivo selections of highly metastatic variant sublines were performed using orthotopic implantation methods (Fig. 4). In the animals injected with MH cells after the second selection, the incidence of lymph node metastases in the left PAN was 3/3 cases, and that in the right PAN was 2/3 cases, 7 weeks after the orthotopic implantation. The incidences in the animals derived from MH cells after the third selection was 4/4 for the left side and 4/4 for the right side, respectively, after 6 weeks.

After the second selection, metastasis of KF cells to PANs was observed in 4/5 cases in the left side, and 1/5 cases in the right side after 7 weeks. After the third selection, we observed 3/3 and 1/3 cases in the left and right sides, respectively, after 6 weeks. We aimed to establish variant sublines that were metastatic to PAN by harvesting metastatic tumours at early periods after orthotopic transplantation. These cells after the third selections were named as MH-LN3 cells and KF-LN3 cells, respectively.

3.1.3. Characterisation of the metastatic variant sublines

As shown in Fig. 5(a, b), MH-LN3 or KF-LN3 can metastasise to PANs. The weight of PAN after inoculation of MH-LN3 cells was 14.08 ± 10.71 mg (mean ± standard deviation (S.D.)) for the left side and 5.00 ± 7.10 for the right side. The weight of PAN after inoculation of MH cells was 3.14 ± 4.34 for the left side and 0.91 ± 0.90 for the right side. The difference in the weights of lymph nodes between MH cells and MH-LN3 cells was statistically significant at 5 weeks after the orthotopic implantation. The weight of PAN after inoculation of KF-LN3 cells was 6.80 ± 4.06 (mg ± S.D.) for the left side and 3.60 ± 1.45 for the right side. The weight after inoculation of KF cells was

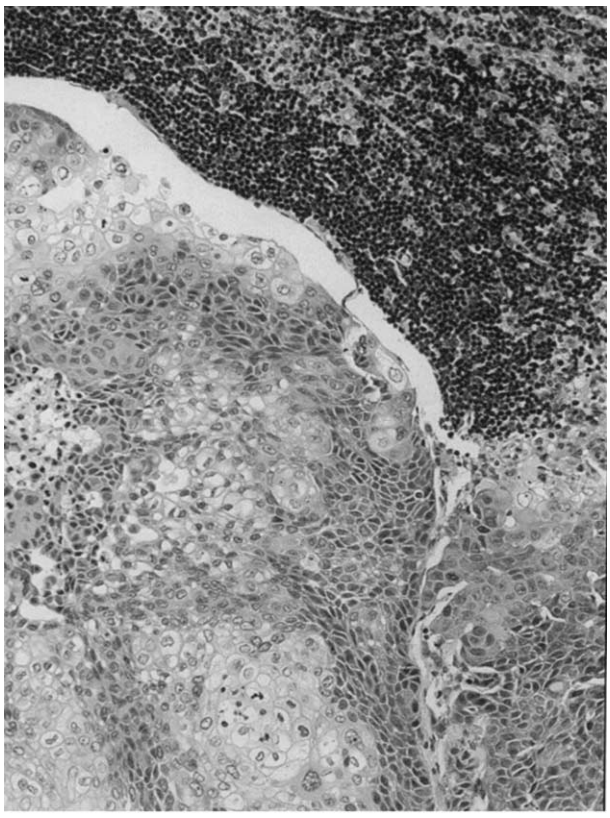

Fig. 3. Histology of a lymph node metastasis derived from MH-L3 cells orthotopically transplanted into ovary. The tumour represents a poorly differentiated serous adenocarcinoma.

3.00±1.54 for the left side and 0.87±0.83 for the right side. In addition, KF-LN3 cells were more metastatic to PANs than KF cells, and the difference in the weight of lymph nodes was statistically significant at 6 weeks after the orthotopic implantation. PAN metastases seemed to require tumour growth at the primary sites, at least to some extent. The *in vitro* growth rate of KF-LN3 cells was not significantly different from that of the KF cells.

4. Discussion

In ovarian carcinomas, the incidence of pelvic and PAN involvement is high. In 1985, the International Federation of Gynecologists and Ostetricians (FIGO) recommended the examination of retroperitoneal lymph nodes for the accurate staging of ovarian carcinomas. Since then, systemic aortic and pelvic lymphadenectomy has been recommended in the treatment of early stage of ovarian carcinomas in Japan. Although gynaecologists often use FIGO staging rather than the TNM classification in the staging of ovarian carcinomas, we reviewed our hospital data using the TNM classification. The FIGO stage IIIc corresponds to stage p(T1, T2, T3)N0M0 and T-factor is supplemented with N-factor. The incidence of lymph node metastases increased in parallel with advancing clinical stages; i.e., 4% with stage pT1M0, 21% with stage pT2M0, 28% with stage pT3M0. In our clinical data, the incidence of lymph node metastases with stage pT1M0 was approximately 20% lower than in other reports [1–3]. However, the clinical stage correlated with the increase of lymph node metastases in all of the previous reports.

Onda and co-workers reported that serous and clear cell types of ovarian carcinomas showed a significantly higher incidence of lymph node metastases than endometrioid or mucinous types [2]. Thus, we chose serous type cell lines, MH and KF, to establish a model to study lymph node metastasis of ovarian cancer. In the present study, we established a useful model for metastasis to PANs by orthotopic intraovary implantation of MH or KF cells in athymic nude mice. One advantage of this model is the simple implantation procedure with a small skin incision at a predetermined site followed by direct injection into the ovary. The whole implantation process was performed in approximately 2 min for each mouse, and the operative mortality was less than 5%. Tumours developed at the site of direct implantation in 100% of the animals. According to clinical analyses of lymphatic metastases, PAN is the most frequent site of metastasis in ovarian carcinomas [2]. Furthermore, there are patients who had positive contralateral lymph nodes [2,3], and our model with nude mice reflects those clinical situations.

Studies have shown that the biological behaviour of human tumour cells is influenced by the implantation site [15] and that the orthotopic implantation of human tumour cells into relevant organs of nude mice can provide an *in vivo* model to study the biology and therapy of these tumours [15,16]. Orthotopic nude mouse or nude rat models have been developed for a number of human cancers including those of the lung, colon, kidney and pancreas [17–20]. Kiguchi and colleagues developed a peritoneal dissemination model by the orthotopic implantation of human ovarian cancer cells into the ovary of nude mice [21]. However, our study, utilising MH and KF cells, is the first model of lymph node metastasis of ovarian carcinomas. This model should be useful to study the dissemination and metastasis of ovarian carcinomas and should also be useful to elucidate the mechanism of lymph node metastasis of carcinomas.

Several investigators have determined some of the molecules that are involved in lymph node metastasis. For example, the following molecules are down-regulated during lymph node metastasis; intercellular adhesion molecule-1 (ICAM-1) in endometrial cancers [5], thrombomodulin in oesophageal cancers [22] and KAI1/CD82 in pancreatic cancers [23]. Upregulated molecules include urokinase-plasminogen activator (u-PA), u-PA receptor, plaminogen activator inhibitor (PAI)-1 and PAI-2 in ovarian cancers [24], CD44 var-

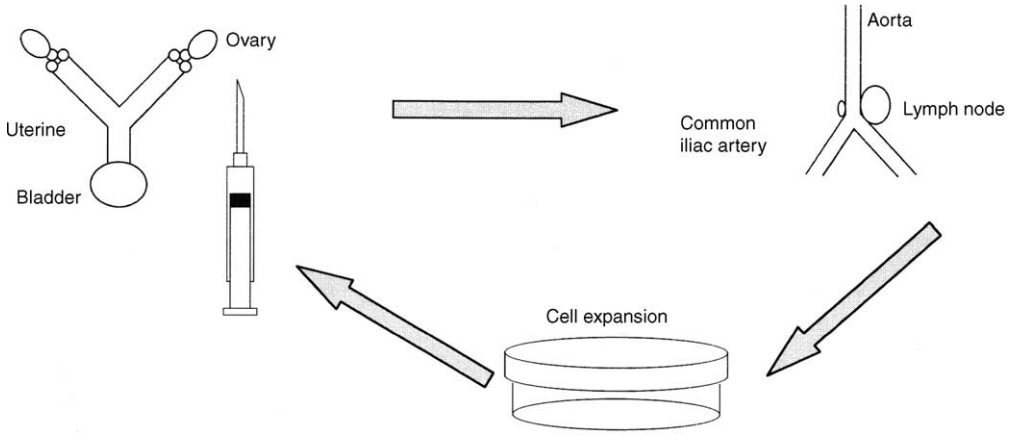

Fig. 4. Scheme for the *in vivo* selection of variant cell lines that are highly metastatic to the lymph nodes.

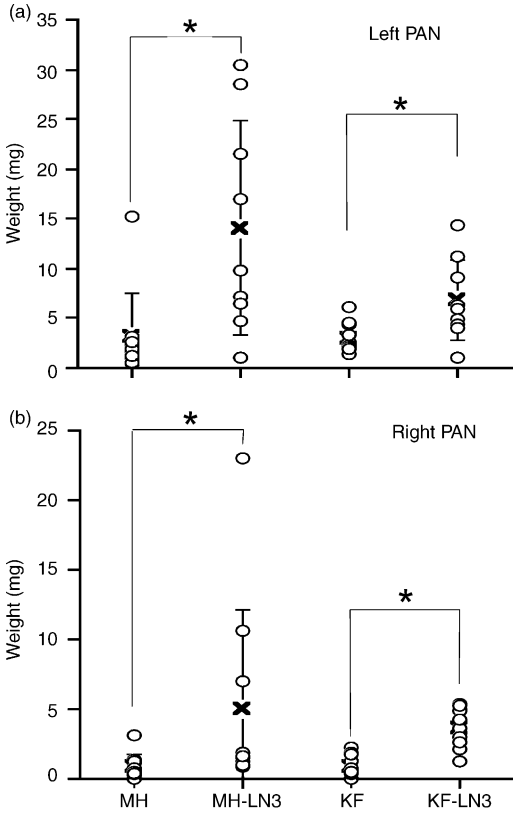

Fig. 5. The weights of PANs 5 weeks after orthotopic implantations of MH and MH-L3 cells (left portion of the panel). The weights of PANs 6 weeks after the orthotopic implantation of KF cells and KF-L3 cells (right portion of the panel). a and b represent left- and right-sided PANs, respectively. An open circle indicates the value for each mouse. X-marks indicate the mean values. Statistical significance of the difference in each comparison was determined by the Mann-Whitney U-test. Asterisks indicate that the differences were statistically significant ($P < 0.05$).

iant exon [25], c-erbB2 [26], receptor for hyaluronan-mediated motility (RHAMM) [27], membrane-type 1 matrix metalloproteinase (MT1-MMP) and activated matrix metalloproteinase-2 (MMP-2) [28] in breast cancers, MMP-7 [29,30] and cyclooxygenase-2 [31] in gastric cancers, c-jun and c-myc in lung cancers [32], and MMP-7 [33] in colon cancers. The mechanism of lymph absorption by the vessels may also closely correlate with the occurrence of lymph node metastasis [34]. Down-regulation of the target molecules that mediate killing by cytotoxic lymphocytes is involved in the escape from immunosurveillance and in the final formation of the metastatic lesion in the lymph nodes. The model developed in our study should be useful to determine which one of these or other mechanisms is involved in PAN metastasis of ovarian carcinomas.

Co-injection of tumour cells with Matrigel was previously reported to lead to the enhancement of lymph node metastasis, as well as to the formation of primary tumours [6]. However, we used simple cell suspension to form lymph node metastases of ovarian carcinoma cells in athymic nude mice. Currently available chemotherapy seems to be ineffective to eradicate tumour deposits in lymph nodes [35,36], this model with human ovarian carcinoma cells in athymic nude mice may also prove useful for evaluating the efficacy of some biological response modifiers and other therapeutics. This procedure is simple and reproducible with a low mortality from the implantation procedure.

Acknowledgements

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