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# Cytology-based Detection of Circulating Tumour Cells in Human Pancreatic Cancer Xenograft Models With *KRAS* Mutation

YUKAKO ITO[1], ERIKO INOUE[1], YUKI MATSUI[1], SHINJI KOBUCHI[1], CHIAMI MOYAMA[2], KIKUKO AMAGASE[3], MAYUMI YOSHIMURA[4], YUZURU IKEHARA[5], SUSUMU NAKATA[2] and HAYAO NAKANISHI[4]

[1]*Department of Pharmacokinetics, Kyoto Pharmaceutical University, Kyoto, Japan;*
[2]*Department of Clinical Oncology, Kyoto Pharmaceutical University, Kyoto, Japan;*
[3]*Department of Pharmacy, College of Pharmaceutical Sciences, Ritsumeikan University, Shiga, Japan;*
[4]*Department of Gastroenterological Surgery, Graduate School of Medicine, Nagoya University, Aichi, Japan;*
[5]*Department of Molecular and Tumor Pathology, Graduate School of Medicine, Chiba University, Chiba, Japan*

**Abstract.** *Background/Aim: To examine the dynamics of circulating tumour cells (CTCs) in pancreatic cancer (PC), new mouse CTC models from human PC xenografts were developed. Materials and Methods: Orthotopic (pancreas) and heterotopic (subcutaneous) transplantation models using GFP-tagged SUIT-2 PC cells were prepared. Using a cytology-based CTC detection platform, CTCs and metastasis were compared. Results: The two types of orthotopic models, including the surgical transplantation model and the intraperitoneal injection model, showed a similar pattern of initial pancreatic tumour formation and subsequent development of peritoneal and hematogenous lung metastases. In the heterotopic model, only hematogenous lung metastasis was observed, and the number of CTCs and lung metastases was higher than that of the orthotopic model. Furthermore, KRAS mutation (G12D) was detected in CTCs. Conclusion: These orthotopic and heterotopic models clearly differ in terms of the pattern of metastasis and CTCs and therefore, would be useful PC models to investigate the effect of drug-therapy on CTCs and the role of KRAS mutation.*

Pancreatic cancer is the fourth leading cause of cancer-related death in the USA, Europe, and Asia (1). In comparison with other types of gastrointestinal cancer, such as gastric or colorectal cancers, pancreatic cancer has remarkably poorer prognosis and higher mortality. In fact, only 15-20% of patients are considered as candidates for surgery at diagnosis, and the prognosis even among patients who are able to have surgery with negative margins remains poor (5-year survival rate is only 10-25%) (2, 3). Although the current treatment modality for pancreatic cancer has progressed to multidisciplinary therapy including preoperative chemotherapy, surgery, and chemo-radiotherapy, the survival of patients with pancreatic cancer has only improved in the order of months. This is mainly because of the difficulty in obtaining an early diagnosis and the biologically aggressive nature of the cancer, which is characterized by high peritoneal, hematogenous, and lymph node metastatic potential from an early stage (4, 5).

Circulating tumour cells (CTCs) are a new type of potential biomarkers capable of resolving diagnostic and therapeutic problems associated with pancreatic cancer. They offer a minimally invasive and easily repetitive method for the detection of tumour cells at an early stage and to monitor changes in tumour cell number and genetic alterations before and after therapy. Therefore, CTCs have multimodal potential as early diagnostic and prognostic markers as well as predictive markers for therapy response in various epithelial malignancies (6, 7). Several clinical studies in pancreatic cancer have shown that high levels of CTCs are associated with tumour progression and are correlated with the patient's short survival, indicating the usefulness of CTCs as prognostic markers (8). However, the effectiveness of CTCs as an indicator of therapeutic response remains unclear in pancreatic cancer patients. This is partly because of some difficulty in the evaluation of morphological changes such as apoptosis and degeneration of CTCs after therapy by most antigen-dependent and fluorescence visualization-based CTC detection methods (9). To resolve

*Correspondence to:* Yukako Ito, Ph.D., Department of Pharmacokinetics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto, 607-8412, Japan. Tel: +81 755954626, Fax: +81 755956311, e-mail: yukako@mb.kyoto-phu.ac.jp

*Key Words:* CTC, pancreatic cancer, *KRAS* mutation, mouse model, cytology, CTC detection platform.

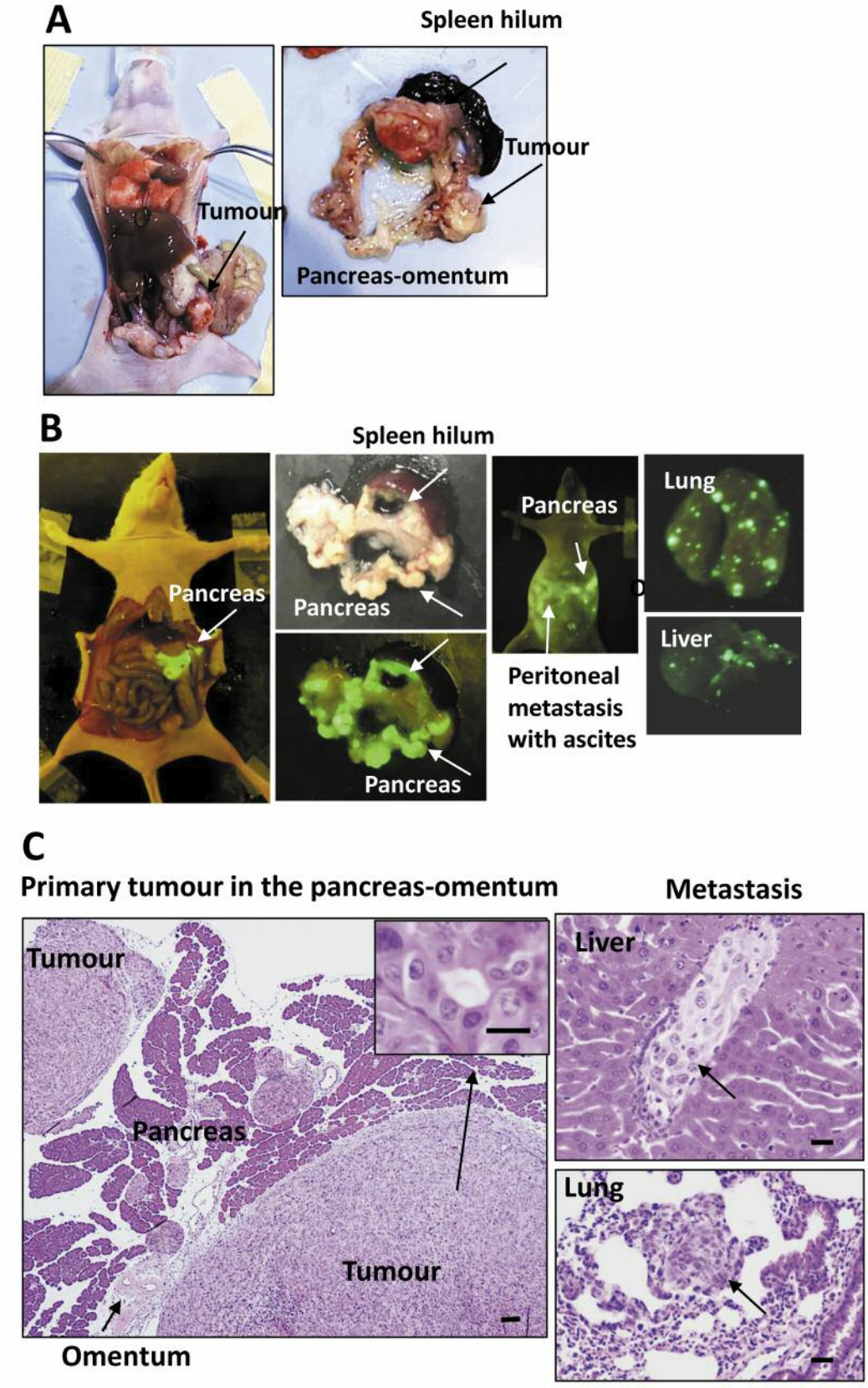


Figure 1. *Continued*

**D**

**Orthotopic transplantation models**

| Sites | Surgical transplantation model | Ip injection model |
|---|---|---|
| Pancreas - omentum | + | + |
| Spleen hilum | + | + |
| Mesenterium | + | + |
| Peritoneum | + | + |
| Ascites | + | + |
| Liver | +/- | +/- |
| Lung | +/- | + |

Figure 1. *Characteristics of two types of orthotopic transplantation CTC mouse models to the pancreas. A. The first type is the surgical transplantation model. SUIT-2 cells in HBSS were directly injected into the pancreas pulled out through surgical incision of the abdominal wall. Primary tumours are located along the pancreas-omentum-spleen hilum. B. The second type is the intraperitoneal (ip) injection model. SUIT2-GFP cells in HBSS were ip injected into the near-pancreatic region by a 27G needle. Primary tumour was similarly located at the pancreas-omental-spleen hilum. Subsequent progression such as peritoneal metastasis with ascites formation and liver/lung metastasis are clearly observed using GFP fluorescence. C. Histological examination shows primary tumours located at the pancreas-omental region and liver/lung metastasis. Inset: atypical glandular formation. Arrows indicate the primary and metastatic tumour. Bars indicate 20 μm. D: Comparison of the primary tumour and metastasis pattern between the two types of orthotopic CTC models. +; always present. +/–; sometimes present.*

this problem, we recently developed a marker-free and cytology-based CTC detection method using a glass slide under a light microscope (10, 11).

The use of a mouse xenograft model bearing human pancreatic cancer for the detection of CTCs in preclinical studies has been quite limited except for patient-derived xenograft mouse CTC models (12). Therefore, the detailed dynamics of CTCs in mice with pancreatic cancer xenograft in terms of natural history and therapeutic response to drug remains unclear. We have previously established several xenograft mouse CTC models using breast, gastric, and lung cancer cell lines (13, 14). In the present study, we developed orthotopic (pancreas) and heterotopic (subcutaneous) xenograft models using a pancreatic adenocarcinoma cell line (SUIT2) with or without GFP gene transfection and evaluated the feasibility of CTC detection in a mouse model as a diagnostic and therapeutic biomarker for preclinical study in combination with a new CTC detection platform.

## Materials and Methods

*Reagents*. Rabbit polyclonal antibody to wide spectrum human cytokeratin (Ab9377) and mouse monoclonal antibody to GFP were purchased from Abcam (Cambridge, UK). Meyer's haematoxylin was used for nuclear counterstaining of the cells.

*Cell lines*. The SUIT-2 cell line was obtained from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), and was established from the liver metastasis of Japanese pancreatic cancer patients. The SUIT2-GFP cell line was established by the transfection of the SUIT-2 cell line with the GFP gene using the pLKO.1-puro-CMV-TurboGFP and puromycin selection (Sigma-Aldrich, St. Louis, MO, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum (GIBCO, Grands Islands, NY, USA) with 100 units/ml penicillin and 100 units/ml streptomycin sulphate and cultured in a humidified 5% $CO_2$ incubator at 37˚C.

*Animals*. Seven- to nine-week-old female athymic nude mice (KSN strain, 23-25 g) were obtained from Japan SLC (Hamamatsu, Japan). Mice were maintained and used under specific pathogen-free (SPF) conditions. All animal experiments were performed according to the experiment protocol approved by the Ethics Review Committee for Animal Experimentation of Kyoto Pharmaceutical University (approval number: PKPD-18-002) and met the standards defined by the recently reported international guidelines (15).

*Mouse CTC models*. Three types of mouse CTC models including two orthotopic and one heterotopic transplantation methods were examined as follows: 1) Orthotopic surgical transplantation model to the pancreas. Cultured SUIT-2 cell line ($1\times10^5$/20 μl) suspended in Hank's balanced salt solution (HBSS) were injected into the pancreas pulled out through surgical left flank incision of the abdominal wall. 2) Orthotopic intraperitoneal injection model to the near-pancreatic region. Cultured SUIT2-GFP cells ($5\times10^5$/0.1 ml) in HBSS were intraperitoneally injected into near-pancreatic region through the left upper abdominal wall with a 27G syringe. 3) Heterotopic transplantation model. Cultured SUIT2-GFP cells ($3\times10^6$/0.1 ml) in HBSS were subcutaneously (sc) injected into the back region of mice with a 27G syringe.

*Fluorescent monitoring of peritoneal, liver and lung metastasis by SUIT2-GFP cells*. The stereomicroscope model SZ40-GFP (SZ4045, Olympus, Tokyo, Japan) equipped with a halogen lamp (LG-PS), was used to monitor peritoneal metastasis in real time in the whole body of living mice on post-injection day 1 onwards. The peritoneum, liver, and lung with small metastasis were resected after killing the mice and were observed under fluorescent lightning (excitation: 450-490 nm). GFP in metastatic tumour was visualized through a long-pass cut filter (for emission: >520 nm) of a digital camera (D1H, Nikon, Tokyo, Japan) (16). Lung and liver metastases were quantitated by counting bright small nodules with GFP fluorescence on their surface. After the experiment, the metastasized peritoneum, liver, and lung were fixed in 10% formalin for 24 h and haematoxylin-eosin (HE) staining was performed for histological confirmation.

*Blood sampling*. Blood was harvested by cardiac puncture in a tube with EDTA under sedation with isoflurane. As a result of preliminary experiments for choosing the blood route including lateral tail vein, retroorbital venous plexus, and the heart, we adopted the heart for the route of blood collection for CTCs. Cardiac puncture has advantages in terms of safety, repetitiveness, and no risk for contamination with normal epithelial cells. The volume of the blood sample used in our study was based on the body weight and interval period, as recommended by the guidelines (17). Cardiac puncture of mice was safely performed using a 27G syringe under anaesthesia without any significant side effect. Usually, we collected approximately 0.20 ml blood per mouse. In some cases, blood was further collected after an interval of several weeks.

*CTC isolation from mouse blood by a filtration-based microfluidic device*. CTC detection was conducted using a previously reported method (10). Briefly, blood (0.2 ml) was diluted 10-fold with PBS containing 0.5 mM EDTA (PBS/EDTA) and then CTCs were enriched using a semiautomated isolation apparatus (Maruyasu Industry Co., Okazaki, Japan) with 3-dimensional (3D) metal filter device (Optnics Precision Co., Tochigi, Japan). After filtering, enriched CTCs trapped on a filter were fixed with 10% formalin for 30 min and washed with PBS/EDTA in the device. The filter detached from the device was placed upside down onto a glass slide (MAS coat, Matsunami, Osaka, Japan) and the CTCs trapped in the filter were then quickly transferred to a glass slide using a mechanical pressure device (Maruyasu Industry Co.). The resultant CTCs attached to the glass slide were immediately fixed in 95% ethanol for Pap staining or fixed in 95% ethanol, followed by further 10% buffered formalin for cytokeratin immunocytochemistry (ICC) with haematoxylin nuclear counterstaining.

*Cytological and ICC staining of CTCs*. Pap staining of CTC glass slides was conducted by an automatic stainer (Sakura Fintec, Tokyo, Japan). For immunostaining, after blocking with 1% BSA, the specimen was incubated with rabbit anti-pan-cytokeratin antibodies for 2 h. After washing, the specimens were incubated with HRP-labelled polymer conjugated goat anti-rabbit antibody (EnVision+System) (DAKO, Carpinteria, CA, USA) for 30 min. After washing with PBS, the color was developed with the Liquid DAB+substrate chromogen system (DAKO). The nuclei were stained with Meyer's haematoxylin.

*Detection of KRAS mutation*. DNA from cultured cells was extracted and purified using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. The DNA of CTCs from mouse blood was extracted on the glass slide using the pinpoint slide DNA isolation system (Zymo Research, Irvine, CA, USA). Briefly, cells containing CTCs on glass slide were incubated in 1-5 μl of pinpoint gel solution and dried for 30 min at room temperature to form film and remove the cells embedded in the film from the slide. DNA was then extracted using extraction buffer containing proteinase K at 55˚C for 4 h. After heat inactivation at 95˚C for 10 min, DNA with or without column purification was used as template for polymerase chain reaction (PCR) targeted the *KRAS* mutation at codon 12 (G12D) using a cycleave PCR core kit (Takara, Ohtsu, Japan) (18). For direct PCR sequencing, the amplified *KRAS* exon 12 was sequenced directly using an ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, CA, USA) as described previously (19). For *KRAS* mutation analysis of CTCs, we used the droplet-based digital PCR (ddPCR) platform (Qx200 ddPCR system, Bio-Rad, Hercules, CA, USA) with PrimePCR product targeted the *KRAS* mutation G12D as reported previously (20).

*Statistics*. All values are expressed as the mean±S.D. Differences were assumed to be statistically significant at $p<0.05$ calculated by Student's unpaired *t*-test and/or Mann-Whitney *U*-test.

## Results

*Pancreatic cancer cell line-derived mouse xenograft models for CTC analysis*. We developed three pancreatic cancer mouse CTC models by injecting human pancreatic adenocarcinoma cells (SUIT-2 or SUIT2-GFP) into the pancreas, the pancreas-omental region and the subcutaneous tissue. The first orthotopic model (surgical transplantation model) was based on the direct transplantation of SUIT-2 cells into the pancreas, exposed through the abdominal wall (Figure 1A). In this model, the tumour forms along the pancreas-omentum-spleen hilum axis. The second orthotopic model [intraperitoneal (ip) injection model] was based on the ip injection of SUIT2-GFP cells into the near pancreas-omental region using a 27 G needle (Figure 1B). In this model, the tumour was also initially formed along the pancreas-omentum-splenic hilum axis and thereafter spread into the peritoneal cavity and finally formed ascites. This pattern of tumour progression can be externally monitored using a GFP fluorescence detection system. These two orthotopic models are essentially the same in terms of primary tumour formation pattern and metastatic progression, indicating that the ip injection model is a more rapid and practical method to generate a pancreatic cancer CTC mouse model than the surgical transplantation model. Histological analysis showed that the tumour initially formed at the omentum and the pancreas. These models also show hematogenous metastasis to the liver and lung, in addition to peritoneal metastasis (Figure 1C and D).

The third heterotopic model was the subcutaneous (sc) transplantation model with SUIT2-GFP cells. This model shows spontaneous lung metastases 1-2 months after sc injection, which is easily visualized and quantitated by GFP fluorescence. The number of lung metastases formed in the orthotopic (pancreas) model and the ectopic (sc) model was different and significantly higher ($p<0.05$) in the ectopic model than in the orthotopic model (Figure 2A). Histological analysis demonstrated that the subcutaneous tumour formed by SUIT2-GFP cells exhibits ductal adenocarcinomatous morphology (Figure 2B).

*Detection of CTCs in mouse blood*. The CTC detection procedure, using our recently developed method, is

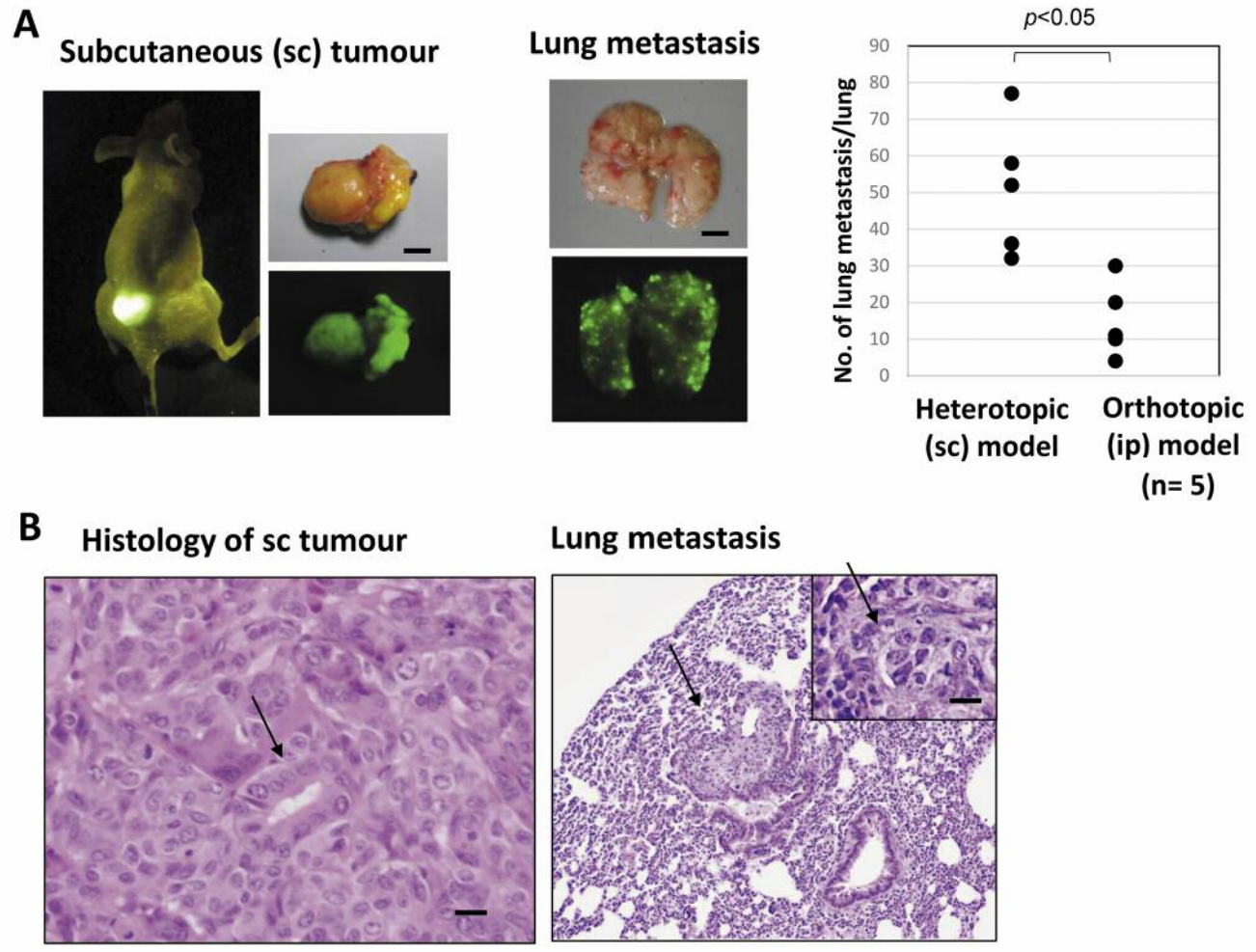


Figure 2. *Characteristics of the heterotopic transplantation CTC mouse model. SUIT2-GFP cells in HBSS were injected subcutaneously. A. primary subcutaneous tumour on the back and lung metastases visualized using GFP fluorescence. The figure shows significantly higher number of lung metastases in heterotopic (sc) model than in orthotopic (pancreas) transplantation model. B. Histological examination confirms that the primary sc tumour and lung metastases are adenocarcinoma with glandular formation (arrows). Bars indicate 20 μm.*

summarized in Figure 3A. It includes 1) a microfluidic CTC filtration device with a 3D metal filter for enrichment of CTCs, 2) transfer of CTCs to a glass slide using a mild mechanical pressure device, followed by fixation in 95% ethanol and 3) subsequent cytokeratin immunocytological staining and/or Papanicolaou staining (Figure 3A). This procedure can be easily carried out in a clinical pathology laboratory. CTCs can be visualized by ICC using a wide-spectrum antibody against cytokeratin or an anti-GFP antibody (Figure 3B). Representative cytokeratin-positive CTCs counter-stained by haematoxylin and Pap stained CTCs in orthotopic and heterotopic models are shown in Figure 3C. Both single CTCs and clustered CTCs are shown (Figure 3C). The number of CTCs tended to be higher in the heterotopic (sc) than that in the orthotopic (pancreas) mouse model (Figure 3D).

*Analysis of KRAS mutation in CTCs*. DNA was extracted from the cultured SUIT-2 cells and CTCs that were isolated from the blood of mice bearing SUIT-2 subcutaneous tumours on a glass slide (Figure 4A). *KRAS* mutations in cultured cells were analysed using the cycleave PCR method and subsequent direct sequencing of the PCR product for confirmation (Figure 4B). *KRAS* mutations in CTCs from CTC-rich blood of some xenograft mice could be successfully analysed by droplet-based digital PCR method. These results showed that SUIT-2 cells and CTCs carry the *KRAS* mutation G12D at codon 12 (G to A) (Figure 4B and 4C).

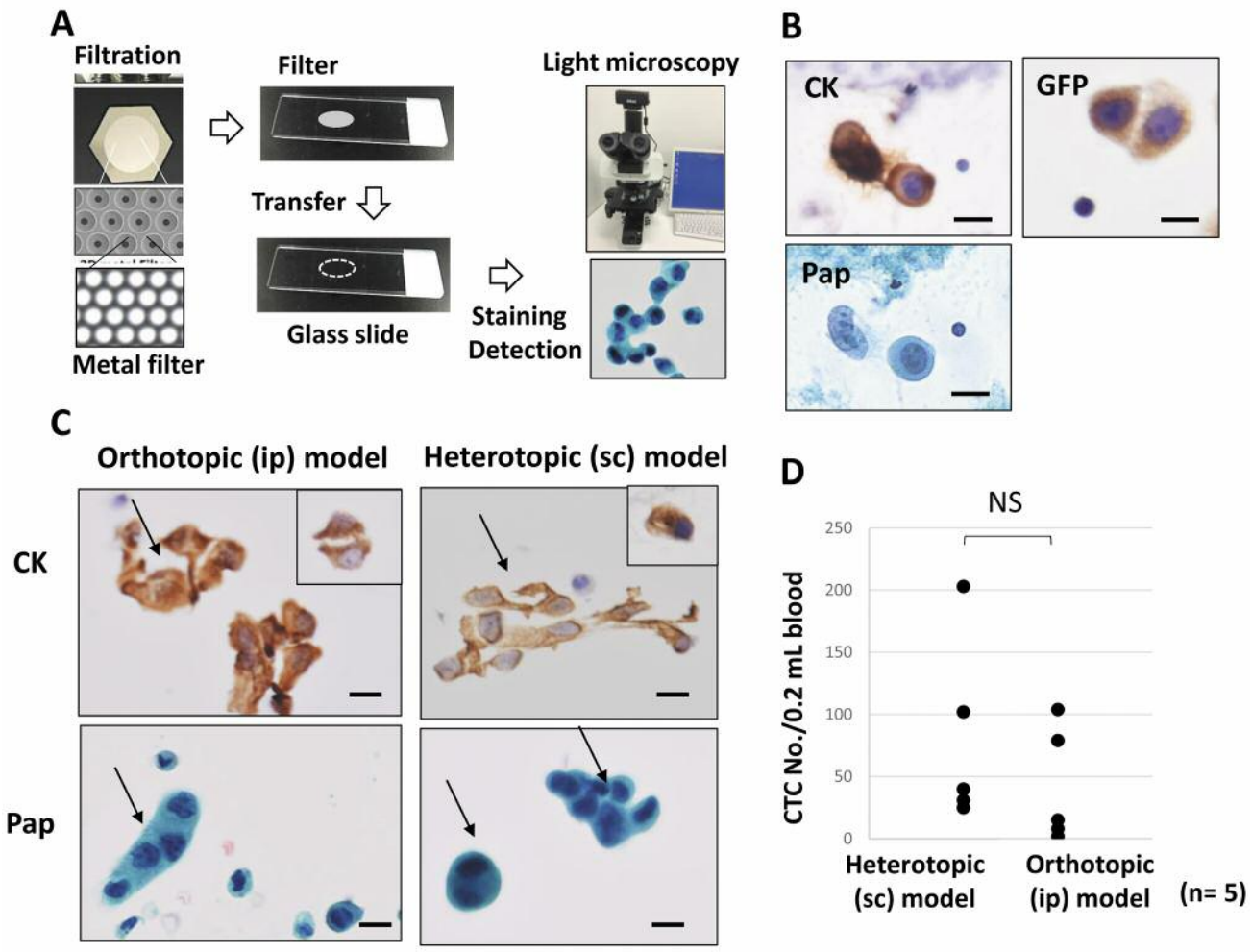


Figure 3. *Detection of CTCs in the blood from the orthotopic and heterotopic mouse CTC models using a cytology-based CTC detection platform. A. Overview of the CTC detection procedure including filtration of CTCs using a 3D metal filter, transfer of CTCs to a glass slide, subsequent staining of CTCs, and the detection of CTCs using light microscopy. B. CTCs detected using cytokeratin (CK) or GFP immunocytochemistry and Papanicolaou (Pap) staining. Bars=20 μm. C: Representative CTCs from the orthotopic and heterotopic mouse models stained for CK and Pap. Arrows indicate a CTC cluster. Inset: single CTC. Bars=20 μm. D: Comparison of the CTC number between the two types of models. The CTC number in the heterotopic (sc) model tends to be higher than that of the orthotopic (pancreas) model, but not statistically significant (NS).*

## Discussion

In the present study, we developed a pancreatic cancer mouse CTC model where CTCs can be stably detected for preclinical study. This mouse CTC model has the following unique characteristics. 1) The use of GFP-tagged SUIT-2 cell line enabled real-time monitoring and external visualization of the development of peritoneal metastasis of orthotopically transplanted pancreatic cancer in living mice. The use of the GFP-tagged cell line also allowed clear visualization of hematogenous lung and/or liver metastasis after orthotopic (pancreas) and heterotopic (sc) transplantation, leading to the accurate quantification of the number of hematogenous metastases. 2) The orthotopic (pancreas) transplantation mouse model exhibited both peritoneal metastasis and hematogenous (liver/lung) metastasis, whereas the heterotopic (sc) transplantation mouse model showed only hematogenous lung metastasis. The number of lung metastases and CTCs detected in the heterotopic model showed a tendency to be higher than that in orthotopic model, indicating that these two CTC models can be used differently depending on the purpose of the preclinical study. For example, regarding basic research on CTC dynamics associated with hematogenous metastasis, a simple heterotopic (sc) model showing only lung metastases may be preferable. 3) This SUIT-2 human pancreatic cancer mouse CTC model carries the *KRAS* G12D mutation, which is detected most

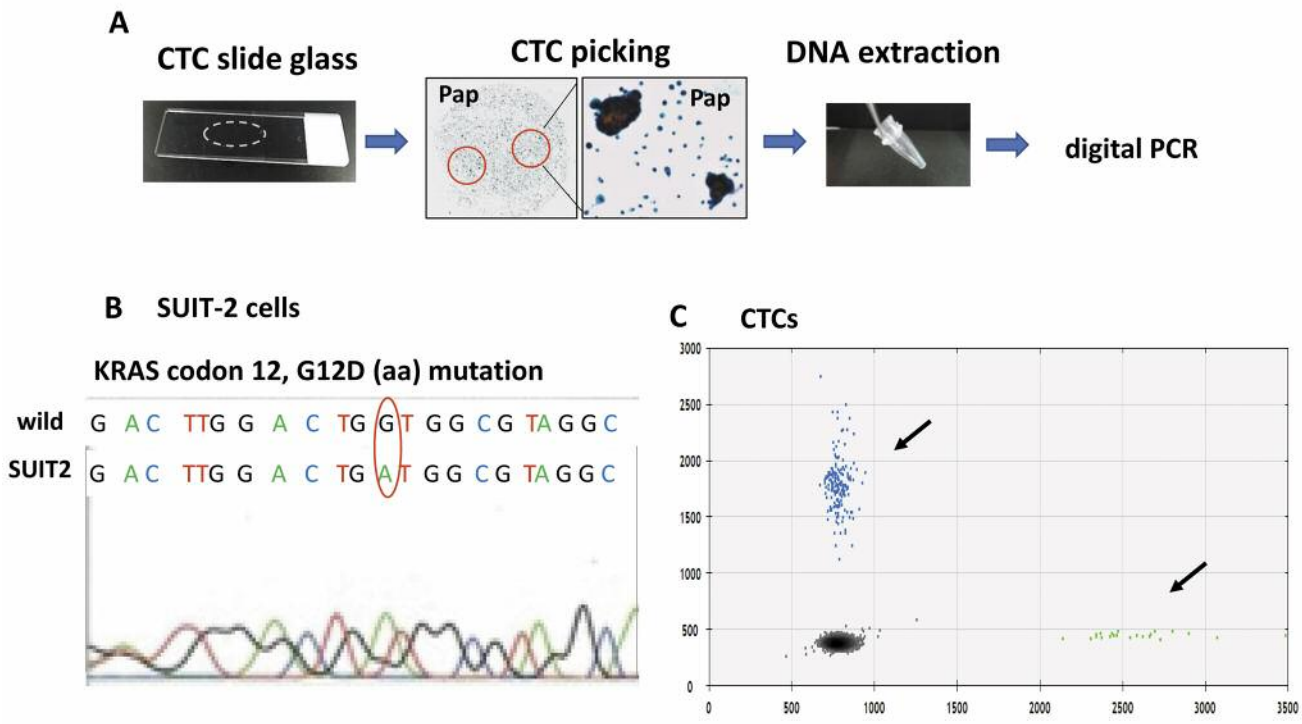


Figure 4. *Analysis of the KRAS mutation in SUIT-2 cells and CTCs from the blood of the heterotopic (sc) xenograft mouse model. A. The procedure of CTC analysis includes pinpoint picking of the CTCs from a glass slide stained by Pap, extraction of DNA, and PCR analysis. B. Analysis of KRAS mutation in SUIT-2 cells using the cycleave PCR and direct sequencing. The result showed that SUIT-2 carry the KRAS G12D mutation at codon 12. C. Analysis of the KRAS G12D mutation of CTCs from the heterotopic mouse xenograft model by droplet-based digital PCR method. The blue and green dots (arrows) indicate droplets containing the amplified KRAS G12D mutant and wild-type alleles, respectively. The grey dots correspond to droplets without any mutant and wild-type alleles.*

frequently in patients with pancreatic cancer. To date, the study of CTC using pancreatic cancer mouse models has been limited to a patient-derived xenograft model (12) and genetically engineered, endogenous pancreatic cancer mouse models (21, 22). Several cell line-derived mouse CTC models such as breast, gastric, and lung cancer cell lines have been reported (13, 14); however, human pancreatic cancer cell line-derived CTC model has not been reported (23). To our knowledge, this CTC model is the first *KRAS* mutation-positive, human pancreatic cancer mouse CTC model for preclinical study.

To estimate the CTC burden in the current CTC mouse model, we used a cytology-based CTC detection platform recently developed in our laboratory. In most of the previous studies, CTCs have been identified as Keratin+/EpCAM+/CD45-/Hoechst 33342+ cells using immunofluorescence under the dark field (24). In contrast, our cytology-based system uses CTC-attached glass slides, followed by cytokeratin (or GFP) immunocytochemistry and/or conventional Papanicolaou staining. CTCs are then observed using light microscopy, by a pathologist/cytologist, as cytokeratin-positive cells with atypical nuclear and cytoplasmic morphology (10, 11). Therefore, the current GFP-tagged pancreatic cancer CTC model in combination with our CTC detection platform enables sensitive, specific, and quantitative detection of both metastases and CTCs from as little as 0.2 ml of mouse blood. Furthermore, our cytology-based CTC detection method using glass slides allows a clearer morphological estimation of CTCs than the other filtration-based CTC detection methods such as the one using polycarbonate membrane (25, 26). Therefore, our CTC model with a cytology-based detection system would be useful for monitoring in detail the effect of chemotherapy on CTCs.

Another interesting finding was that the SUIT-2 pancreatic cancer cell line has *KRAS* mutation (G12D). In PDAC patients, 70-90% of PDACs are reportedly known to have a *KRAS* mutation mostly at codon 12, codon 13, and codon 61. The *KRAS* G12D, detected in the SUIT-2 pancreatic cancer cell line, is the most frequent *KRAS* mutation in clinical pancreatic cancers. In addition, CTCs detected in the SUIT-2 model form many clusters in blood (venous plus arterial) collected from the heart (27) and the CTC number was relatively high, ranging from 20 to 200 CTCs/0.2 ml, allowing genetic analysis of CTCs in some xenograft mice. These findings indicate that the SUIT-

2 CTC mouse model is a very useful pancreatic cancer CTC model that allows monitoring genetic changes of CTCs in pancreatic cancer in response to anti-cancer therapy. Recently, cell-free circulating tumour DNA (ctDNA) has become another prominent method of liquid biopsy, and in clinical studies, ctDNA can be relatively easily measured in pancreatic cancer patients (28). To date, however, in a preclinical mouse model, the detection and measurement of ctDNA is still not easy, partly due to the small volume of blood that is collected. Therefore, the CTC number in combination with genetic analysis of CTCs would be useful for monitoring the therapeutic response to various treatment modalities for pancreatic cancer.

## Conclusion

In conclusion, our findings suggest that this pancreatic cancer mouse CTC model is a useful and powerful tool for the research on CTC dynamics before and after various treatment modalities. Research on the relationship between the dynamics of CTCs and the pharmacodynamics of chemotherapy using the current mouse CTC model are now ongoing in our laboratory.

## Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

## Authors' Contributions

Yukako Ito: Conception and design of this study, animal model development, drafting of the article, and critical revision of the article for important intellectual content; Eriko Inoue and Yuki Matsui: Animal experiment; Shinji Kobuchi: CTC detection and Analysis and interpretation of data; Chiami Moyama and Susumu Nakata: GFP-tagged SUIT-2 cell preparation; Kikuko Amagase: SUIT-2 cell culture preparation and CTC detection; Mayumi Yoshimura and Hayao Nakanishi: CTC detection and *KRAS* mutation; Yuzuru Ikehara: Support of animal model development and genetic analysis.

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## References

1 Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. CA: Cancer J Clin *69(1)*: 7-34, 2019. PMID: 30620402. DOI: 10.3322/caac.21551

2 Rahib L, Smith BD, Aizenberg R, Rosenzweig, AB, Fleshman JM and Matrisian LM: Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res *74*: 2913-2921, 2014. PMID: 24840647. DOI: 10.1158/0008-5472.CAN-14-0155

3 Becker AE, Hernandez YG, Frucht H and Lucas AL: Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection. World J Gastroenterol *20*: 11182-11198, 2014. PMID: 25170203. DOI: 10.3748/wjg.v20. i32.11182

4 McGuigan A, Kelly P, Turkington RC, Jones C, Coleman HG and McCain RS: Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. World J Gastroenterol *24(43)*: 4846-4861, 2018. PMID: 30487695. DOI: 10.3748/wjg.v24.i43.4846

5 Collisson EA, Bailey P, Chang DK and Biankin AV: Molecular subtypes of pancreatic cancer. Nat Rev Gastroenterol Hepatol *16(4)*: 207-220, 2019. PMID: 30718832. DOI: 10.1038/s41575-019-0109-y

6 Tan Y and Wu H: The significant prognostic value of circulating tumor cells in colorectal cancer: A systematic review and meta-analysis. Curr Probl Cancer *42(1)*: 95-106, 2018. PMID: 29277243. DOI: 10.1016/j.currproblcancer.2017.11.002

7 Hardingham JE, Grover P, Winter M, Hewett PJ, Price TJ and Thierry B: Detection and clinical significance of circulating tumor cells in colorectal cancer-20 Years of progress. Mol Med *21*: S25-S31, 2015. PMID: 26605644. DOI: 10.2119/molmed.2015.00149

8 Gall TMH, Jacob J, Frampton AE, Krell J, Kyriakides C, Castellano L, Stebbing J and Jiao LR: Reduced dissemination of circulating tumor cells with no-touch isolation surgical technique in patients with pancreatic cancer. JAMA Surg *149*: 482-485, 2014. PMID: 24599353. DOI: 10.1001/jamasurg.2013.3643

9 Pimienta M, Edderkaoui M, Wang R and Pandol S: The potential for circulating tumor cells in pancreatic cancer management. Front Physiol *8*: 381, 2017. PMID: 28626429. DOI: 10.3389/fphys.2017.00381

10 Tsutsuyama M, Nakanishi H, Yoshimura M, Oshiro T, Kinoshita T, Komori K, Shimizu Y, Ichinosawa Y, Kinuta S, Wajima K, Sakakibara Y, Yatabe Y, Ito S and Kodera Y: Detection of circulating tumor cells in drainage venous blood from colorectal cancer patients using a new filtration and cytology-based automated platform. PLoS One *14(2)*: e0212221, 2019. PMID: 30811434. DOI: 10.1371/journal.pone.0212221. eCollection 2019.

11 Hattori M, Nakanishi H, Yoshimura M, Iwase M, Yoshimura A, Adachi Y, Gondo N, Kotani H, Sawaki M, Fujita N, Yatabe Y and Iwata H: Circulating tumor cells detection in tumor draining vein of breast cancer patients. Sci Rep *9(1)*: 18195, 2019. PMID: 31796846. DOI: 10.1038/s41598-019-54839-y

12 Torphy RJ, Tignanelli CJ, Kamande JW, Moffitt RA, Loeza SGH, Soper SA and Yeh JJ: Circulating tumor cells as a biomarker of response to treatment in patient-derived xenograft mouse models of pancreatic adenocarcinoma. PLoS One *9(2)*: e89474, 2014. DOI: 10.1371/journal.pone.0089474. eCollection 2014

13 Adachi Y, Yoshimura M, Nishida K, Usuki H, Shibata K, Hattori M, Kondo N, Yatabe Y, Iwata H, Kikumori T, Kodera Y and Nakanishi H: Acute phase dynamics of circulating tumor cells after paclitaxel and doxorubicin chemotherapy in breast cancer mouse models. Breast Cancer Res Treat *167(2)*: 439-450, 2018. PMID: 24586805. DOI: 10.1007/s10549-017-4532-x

14 Ito A, Nakanishi H, Yoshimura M, Ito S, Sakao Y, Kodera Y, Yatabe Y and Kaneda N: Dynamics of circulating tumor cells early after targeting therapy to human EGFR-mutated lung cancers and HER2 gene-amplified gastric cancers in mice. Anticancer Res *39(9)*: 4711-4720, 2019. PMID: 31519570. DOI: 10.21873/anticanres.13653

15 Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR and Eccles SA: Committee of the national cancer research institute: guidelines for the welfare and use of animals in cancer research. Br J Cancer *102(11)*: 1555-1577, 2010. PMID: 20502460. DOI: 10.1038/sj.bjc.6605642

16 Ohashi N, Kodera Y, Nakanishi H, Yokoyama H, Fujiwara M, Koike M, Hibi K, Nakao A and Tatematsu M: Efficacy of intraperitoneal chemotherapy with paclitaxel targeting peritoneal micrometastasis as revealed by GFP-tagged human gastric cancer cell lines in nude mice. Int J Oncol *27(3)*: 637-644, 2005. PMID: 16077911.

17 Hoff J: Methods of blood collection in the mouse. Lab Animal *29(10)*: 47-53, 2000.

18 Yatabe Y, Hida T, Horio Y, Kosaka T, Takahashi T and Mitsudomi T: A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer. J Mol Diagn *8*: 335-341, 2006. PMID: 16825506. DOI: 10.2353/jmoldx.2006.050104

19 Yokota T, Shibata N, Ura T, Takahari D, Shitara K, Muro K and Yatabe Y: Cycleave polymerase chain reaction method is practically applicable for V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)/V-raf murine sarcoma viral oncogene hosmolog B1 (BRAF) genotyping in colorectal cancer. Transl Res *156(2)*: 98-105, 2010. PMID: 20627194. DOI: 10.1016/j.trsl.2010.05.007

20 Sefrioui D, Blanchard F, Toure E, Basile P, Beaussire L, Dolfus C, Anne Perdrix A, Paresy M, Antonietti M, Iwanicki-Caron I, Alhameedi R, Lecleire S, Gangloff A, Schwarz L, Clatot F, Tuech JJ, Frébourg T, Jardin F, SabourinvJC, Sarafan-Vasseur N, Michel P and Fiore FD: Diagnostic value of CA19.9, circulating tumour DNA and circulating tumour cells in patients with solid pancreatic tumours. Br J Cancer *117*: 1017-1025, 2017. PMID: 28772284. DOI: 10.1038/bjc

21 Bardeesy N, Aguirre AJ, Chu GC, Cheng K-H, Lopez LV, Hezel AF, Feng B, Brennan C, Weissleder R, Mahmood U, Hanahan D, Redston MS, Chin L and Depinho RA: Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proc Natl Acad Sci USA *103*: 5947-5952, 2006. PMID: 16585505. DOI: 10.1073/pnas.0601273103

22 Yamaguchi T, Ikehara S, Nakanishi H and Ikehara Y: A genetically engineered mouse model developing rapid progressive pancreatic ductal adenocarcinoma. J Pathol *234(2)*: 228-238, 2014. PMID: 25042889. DOI: 10.1002/path.4402

23 Erstad DJ, Sojoodi M, Taylor MS, Ghosha S, Razavi AA, Graham-O'Regan KA, Bardeesy N, Ferrone CR, Lanuti M, Caravan P, Tanabe KK and Fuchs BC: Orthotopic and heterotopic murine models of pancreatic cancer and their different responses to FOLFIRINOX chemotherapy. Dis Model Mech *11(7)*: dmm034793, 2018. PMID: 29903803. DOI: 10.1242/dmm.034793

24 Pantel K and Alix-Panabieres C: Functional studies on viable circulating tumor cells. Clin chem *62*: 328-334, 2016. PMID: 26637479. DOI: 10.1373/clinchem.2015.242537

25 Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schütze K, Capron F, Franco D, Pazzagli M, Vekemans M, Lacour B, Bréchot C and Paterlini-Bréchot P: Isolation by the size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol *156*: 57-63, 2000. PMID: 10623654. DOI: 10.1016/S0002-9

26 Desitter I, Guerrouahen BS, Benali-Furet N, Wechsler J, Ja¨nne PA, Kuang Y, Yanagita M, Wang L, Berkowitz JA, Distel RJ and Cayre YE: A new device for rapid isolation by size and characterization of rare circulating tumor cells. Anticancer Res *31*: 427-441, 2011. PMID: 21378321.

27 Arnoletti JP, Fanaian N, Reza J, Sause R, Almodovar AJ, Srivastava M, Patel S, Veldhuis PP, Griffith E, Shao YP, Zhu X and Litherland SA: Pancreatic and bile duct cancer circulating tumor cells (CTC) form immune-resistant multi-cell type clusters in the portal venous circulation. Cancer Biol Ther *19*: 887-897, 2018. PMID: 30067440. DOI: 10.1080/15384047.2018.1480292

28 Kinugasa H, Nouso K, Miyahara K, Morimoto Y, Dohi C, Tsutsumi K, Kato H, Matsubara T, Okada H and Yamamoto K: Detection of K-ras gene mutation by liquid biopsy in patients with pancreatic cancer. Cancer *121*: 2271-2280, 2015. PMID: 25823825. DOI: 10.1002/cncr.29364

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