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Exosome Transfer Between Pancreatic-cancer Cells Is Associated With Metastasis in a Nude-mouse Model

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Abstract. *Background/Aim: Cancer-derived exosomes play an important role in metastasis. In the present study, we determined whether exosome transfer between cancer cells is associated with metastasis in a mouse model. Materials and Methods: AsPC-1 human pancreatic-cancer cells expressing red fluorescent protein (RFP) and AsPC-1 human pancreatic-cancer cells transduced by exosome-specific pCT-CD63-green fluorescent protein (GFP), were co-injected into the spleen of nude mice. Results: Both pancreatic-cancer cell lines grew in the spleen and metastasized to the liver, peritoneum, and lungs, as shown by color-coded imaging. The ratio of GFP-expressing exosomes incorporated in RFP-labeled AsPC-1 cells was statistically-significantly higher in the liver, lung, and peritoneal metastases than in the spleen. Conclusion: Exosome transfer between cancer cells is associated with metastasis. Exosome transfer may play a role in increasing the metastatic capability of the recipient cells.*

Pancreatic cancer is one of the most recalcitrant cancers, with a 5-year overall survival rate less than 10% (1). The poor prognosis is due to local invasion or metastasis at the time of initial diagnosis. Therefore, more understanding of the metastatic mechanism of pancreatic cancer is urgently required.

Exosomes are extracellular vesicles, 40-100 nm in diameter, that are released from cells upon fusion of an intermediate endocytic compartment with the plasma membrane (2). Exosomes contain DNA, mRNA, microRNA, and proteins (3-5). Exosomes are transferred as a means of cell-to-cell communication (4, 6, 7).

We have previously shown by color-coding imaging that cancer-derived exosomes form a niche in metastatic-target organs, in mouse models of metastatic breast cancer (8). We also visualized by color-coded imaging that cancer-derived exosomes were present in macrophages in liver metastasis (9). In the present study, we observed that transfer of exosomes between pancreatic-cancer cells is associated with metastasis in a nude-mouse model.

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Key Words: Pancreatic cancer, nude-mouse, exosome transfer, metastasis, green fluorescent protein, red fluorescent protein, color-coded imaging.

Materials and Methods

Cell line and culture conditions. AsPC-1, human pancreatic-cancer cells were engineered to stably express red fluorescent protein (RFP), as previously reported (10). The cells were maintained in RPMI 1640 medium (GIBCO-BRL, Grand island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (GIBCO-BRL) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Lentiviral exosome labelling. AsPC-1 cells were transduced with a lentiviral vector, containing the pCT-CD63-green fluorescent protein (GFP) (System Biosciences, Palo Alto, CA, USA), which contains the tetraspanin CD63 gene fused to GFP for tracking exosomes (9).

Mouse experiments. All experiments were conducted in accordance with the institutional guidelines of Gifu University, Gifu, Japan, and approved by the Animal Research Committee and the Committee on Living Modified Organisms of Gifu University. Five-8 weeks old BALB/C-nu/nu male mice were used in this study. In order to minimize any suffering of the mice, anesthesia and analgesics were used for all surgical experiments. Mice were anesthetized by subcutaneous injection of a 0.02 ml solution of 20 mg/kg ketamine. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The mice were housed in a barrier facility on a high-efficiency particulate arrestance (HEPA) – filtered rack under standard conditions of 12-h light/dark cycles. Mice were fed an autoclaved laboratory rodent diet.

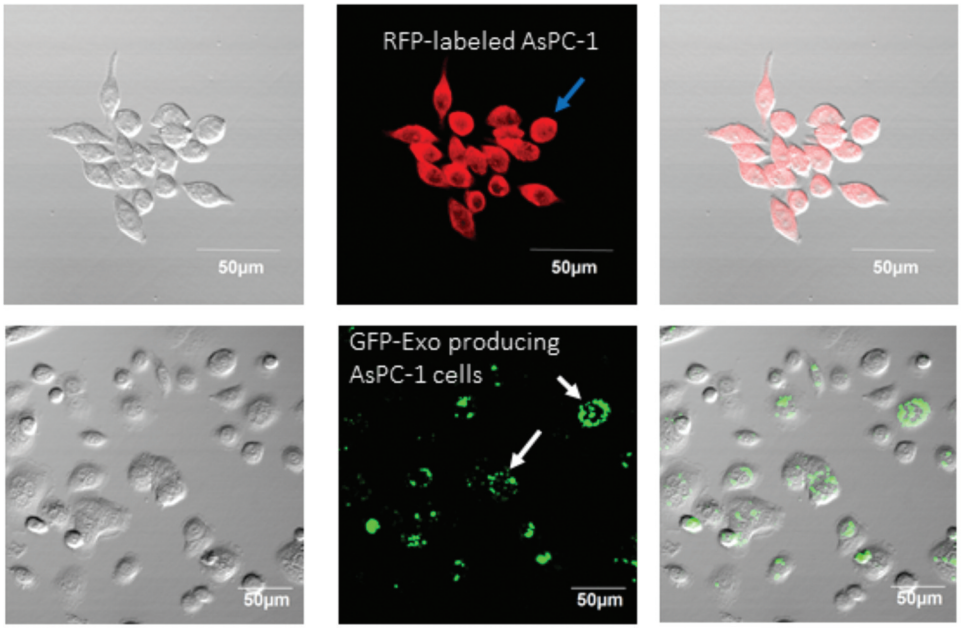

Figure 1. *Fluorescence and bright field images of pancreatic-cancer cell lines. AsPC-1 cells express RFP in the cytoplasm. AsPC-1 cells were also transduced with pCT-CD63-GFP for tracking exosomes. Blue arrow indicates RFP-labeled pancreatic cancer cells. White arrows indicates GFP-labeled-exosomes. Images were obtained with the Olympus FV1000 confocal microscope (Bar=50 μm).*

Pancreatic-adenocarcinoma experimental-metastasis model. AsPC-1 cells expressing RFP and AsPC-1 transduced with pCT-CD63-GFP were harvested by trypsinization and washed three times with cold serum-free medium, and then resuspended in serum-free RPMI 1640 medium. AsPC-1 cells expressing RFP (1.0×10^6) and AsPC-1 transduced with pCT-CD-63-GFP (1.0×10^6) were co-injected to the spleen of nude mice. Eight weeks later, mice were sacrificed and the primary tumor and metastases were harvested. The cells from primary and metastatic tumors were cultured for several weeks. The cancer cells that grew out of the organs were sub-cultured and observed by color-coded imaging.

Color-coded imaging. A SZX7 microscope and an FV1000 confocal microscope (both from Olympus Corp. Tokyo, Japan) were used for imaging the primary tumor in the spleen and the metastases, as well as the cells in culture (10).

Statistical analysis. The Fisher Exact Test was used to determine statistical significance. A two-sided *p*-value of ≤ 0.05 was considered statistically significant.

Results and Discussion

AsPC-1 cells expressing either RFP or pCT-CD63 GFP are shown in Figure 1. Upon co-culture of the two cell types, GFP exosomes were not transferred from one cell type to the other (Figure 2).

AsPC-1 human pancreatic cancer cells expressing RFP and AsPC-1 human pancreatic cancer cells transduced by exosome-specific pCT-CD63-GFP were co-injected into the spleen of nude mice. Eight weeks later, all mice were sacrificed. The primary tumor in the spleen (Figure 3A), multiple metastasis in the liver (Figure 3B), multiple metastasis in the peritoneal cavity (Figure 3C), and multiple metastasis in the lung (Figure 3D) were imaged. GFP expressing exosomes were incorporated into RFP-labeled AsPC-1 cells in the above-noted organs.

When cancer cells were sub-cultured from the metastatic organs. GFP-expressing exosomes were incorporated into RFP-labeled AsPC-1 cells in the spleen (Figure 4A), liver metastasis (Figure 4B), peritoneal metastasis (Figure 4C), and lung metastasis (Figure 4D).

The ratio of GFP-expressing exosomes incorporated into RFP-labeled AsPC-1 cells was significantly higher in the liver metastasis, peritoneal metastasis, and lung metastasis than in the primary spleen tumor ($p<0.05$) (Figure 5).

The cancer cells in the primary spleen tumor, liver metastasis, peritoneal metastasis, and lung metastasis were sub-cultured *in vitro* and observed after 7 days. The number of GFP-expressing exosomes incorporated into RFP-labeled AsPC-1 cells from each tumor was statistically-significantly higher than that before subculture ($p<0.05$) (Figure 6).

Cancer-derived exosomes influence both the primary tumor as well as distant organs to promote cancer invasion and metastasis (11-13). Cancer-derived exosomes inhibit or promote the growth of cancer cells (14-16). Exosomes contain genetic information such as mRNA, DNA, and microRNA and play an important role in forming a

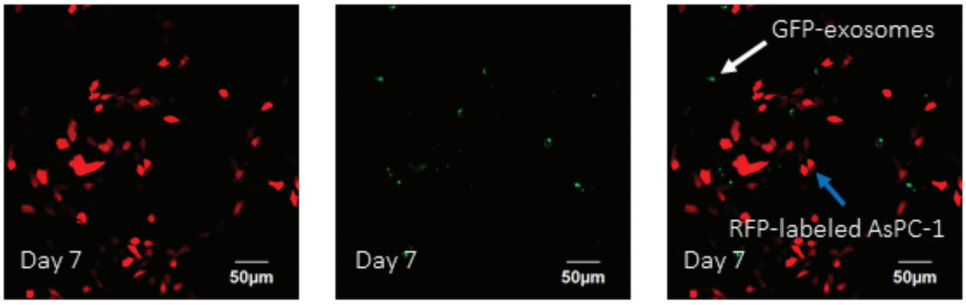

Figure 2. *Images of the two pancreatic-cancer cell lines AsPC-1 expressing RFP and AsPC-1 expressing pCT-C063-GFP co-cultured in vitro before implantation in mice. Blue arrow indicates AsPC-1 cells expressing RFP. White arrow indicates GFP-exosomes. GFP-exosomes were not incorporated in AsPC-1 expressing RFP in vitro.*

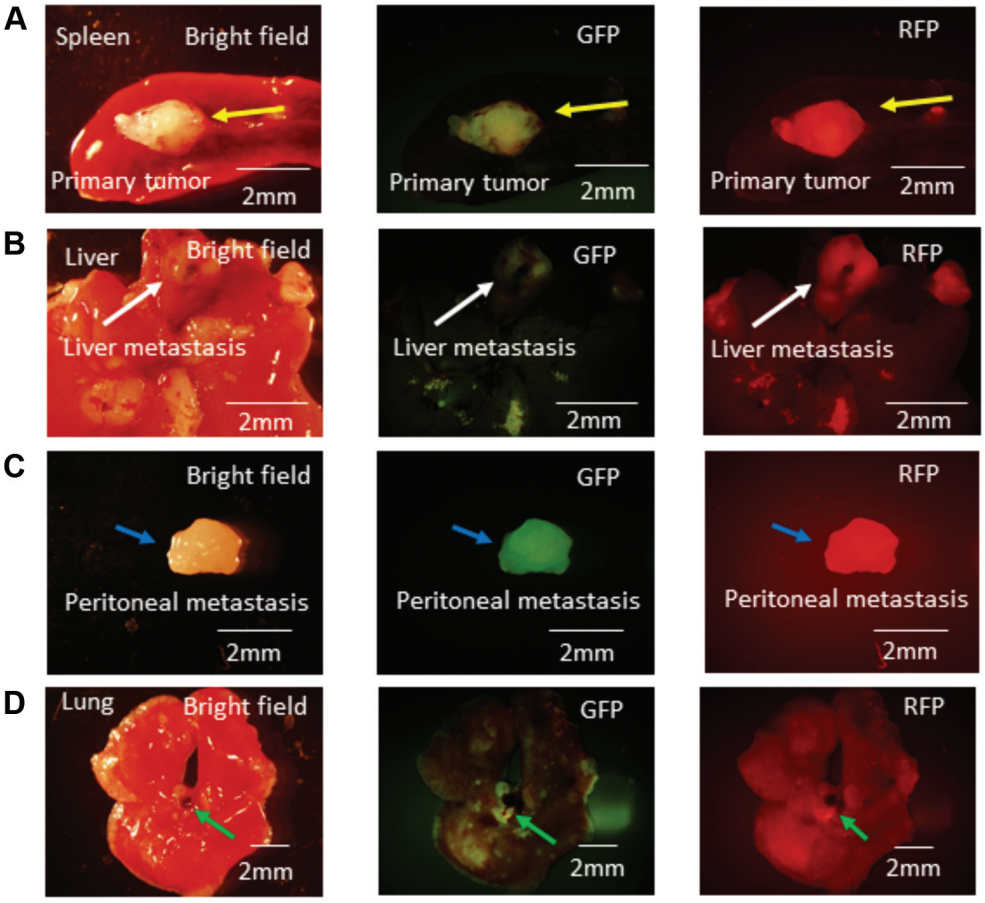

Figure 3. *Pancreatic-cancer experimental-metastasis model co-implanted in the spleen with AsPC-1 cells expressing RFP and AsPC-1 cells expressing pCT-CD63-GFP. Bright-field and fluorescence images of the spleen, liver, peritoneal nodules, and lung. Yellow arrows indicate primary tumor in the spleen. Blue arrows indicate peritoneal metastasis. White arrows indicate liver metastasis. Green arrows indicate lung metastasis. Images were obtained with the SZX7 microscope.*

metastasis niche. Costa-Silva *et al*. (17) showed that pancreatic-cancer-derived exosomes are incorporated by Kupffer cells in the liver and promote TGF-β secretion, which contributes to pre-metastatic niche formation in the liver (17). Linton *et al*. (18) showed that exosomes in pancreatic-cancer cells stimulate macrophages to secrete various cytokines and promote the growth of pancreatic-cancer cells (18). The present study showed that the ratio of

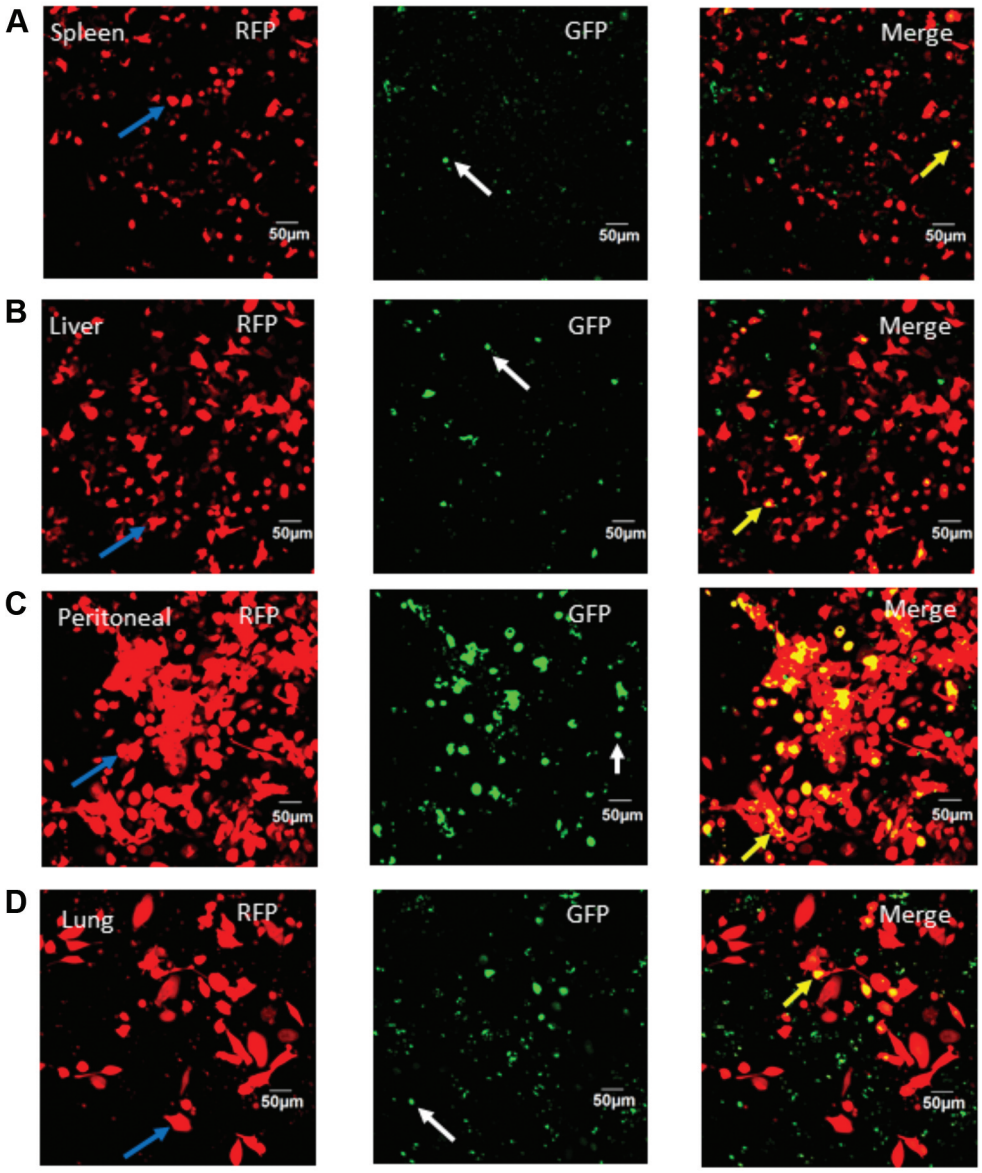

Figure 4. *Fluorescence images of cells from tumors in each organ grown in vitro for seven days. (A) Spleen. (B) Liver metastasis. (C) Peritoneal metastasis. (D) Lung metastasis. In each tissue, GFP-expressing exosomes were transferred from AsPC-1 cells expressing pCT-CD63-GFP and incorporated into RFP-labeled AsPC-1 cells. Blue arrows indicate AsPC-1 expressing RFP. White arrows indicate GFP-exosomes. Yellow arrows indicate GFP -exosomes incorporated into RFP-labeled AsPC-1 cells. Images were obtained with the Olympus FV1000 confocal microscope (Bar=50 μm).*

exosome transfer between cancer cells in the liver metastasis, peritoneal metastasis, and lung metastasis was elevated compared to the primary tumor in the spleen. RFP-labeled AsPC-1 cells incorporating GFP-exosomes increased during subculture of the various organs *in vitro* in contrast to the two cancer cell types co-cultured before transplantation, suggesting that exosome transfer between two cell types was induced *in vivo*. The present study suggests that transferred exosomes promote metastasis. Further experiments are needed to determine how cancer-derived exosomes promote metastasis.

Conflicts of Interest

None of the Authors have any conflict of interest with regard to this study.

Authors' Contributions

Hironao Ichikawa and Atsushi Suetsugu planned this study. Hironao Ichikawa participated in the animal experiments. Hironao Ichikawa, Atsushi Suetsugu, and Robert M. Hoffman contributed to the interpretation of the results. All Authors provided critical feedback and helped shape the research and manuscript.

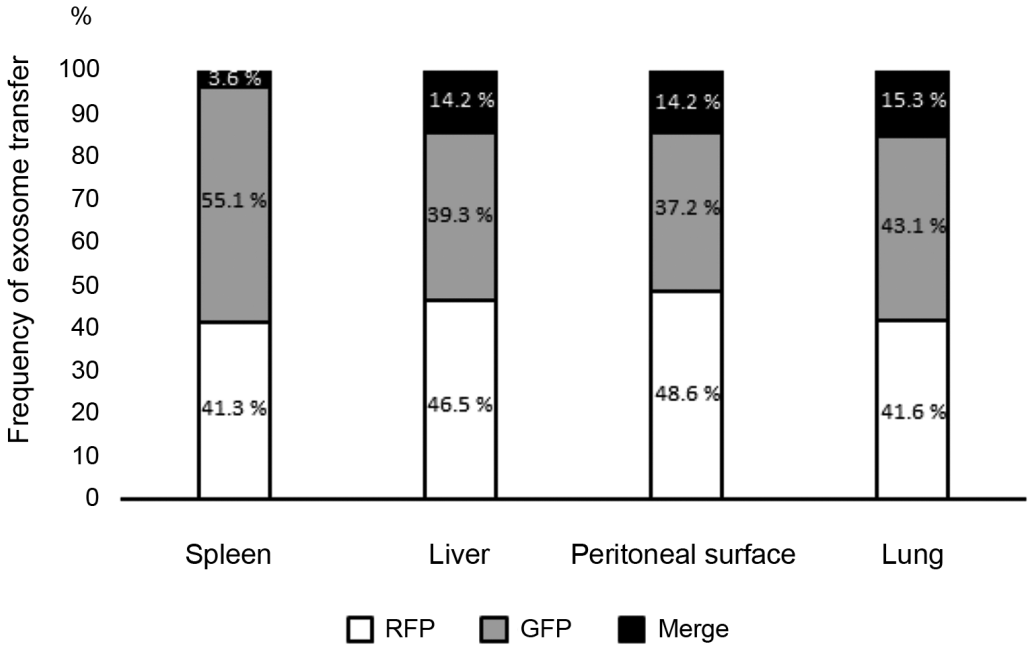

Figure 5. *Rate of GFP exosome transfer from AsPC-1-pCT-CD63 GFP cells to AsPC-1 RFP cells in primary and metastatic tumors in vivo. Ten randomly-selected, low-magnification visual fields were quantified for the number of GFP-expressing exosomes incorporated into RFP-labeled AsPC-1 cells in each organ ("merge"). The frequency of exosome GFP expression in the cells of the spleen tumor, liver metastasis, and peritoneal metastasis, as well as RFP expression, is plotted in the bar graphs. The ratio of GFP-expressing exosomes incorporated into RFP-labeled AsPC-1 cells was significantly higher in the liver metastasis, peritoneal metastasis, and lung metastasis than in the primary spleen tumor ($p<0.05$).*

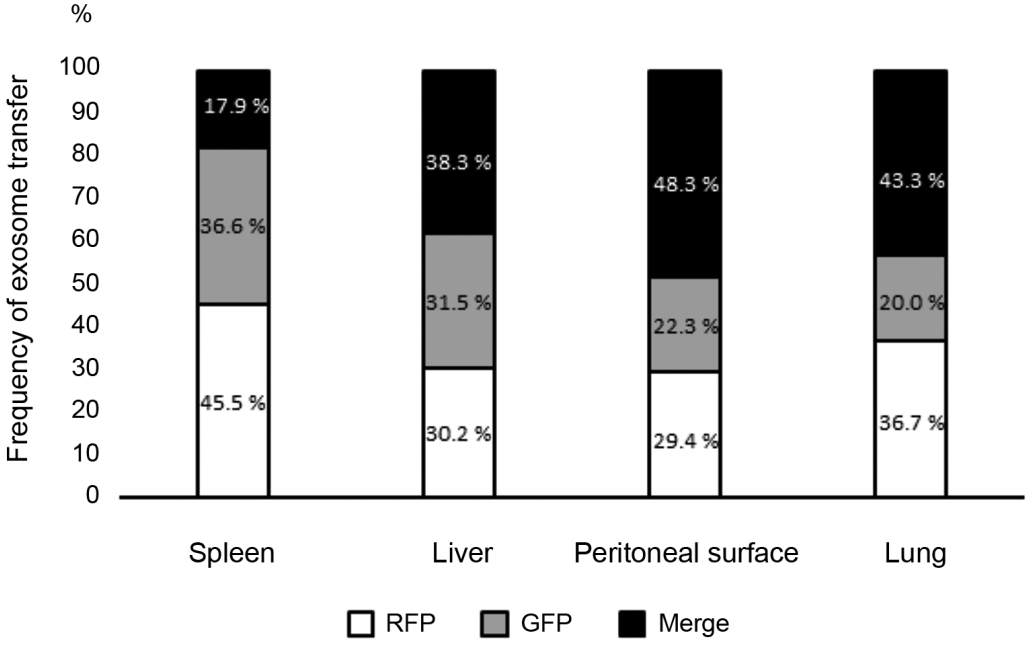

Figure 6. *Subculture of organs in vitro increases exosome transfer. Ten randomly-selected, low-magnification visual fields were quantified for the number of GFP-expressing exosomes incorporated into RFP-labeled AsPC-1 cells after organ subculture ("merge"). The ratio of GFP-expressing exosomes transferred and incorporated into RFP-labeled AsPC-1 cells was significantly higher than that before subculture, as well as higher in the metastatic organs compared to the primary spleen tumor ($p<0.05$).*

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