Detecting circulating tumor cells in peripheral blood of pancreatic cancer patients using negative selection strategy

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Methdology

◇ **Patients and Healthy Donors**. 97 peripheral blood samples were collected from the 71 patients, in which 69 samples were obtained before and 28 were taken after operation; among them 27 blood samples were analyzed from 12 patients whose specimens were collected both pre- and post-operation. Five healthy donors blood were taken as controls.

◇ **Tumor Cell Culture and Cell Spiking.** The number of spiked cells was determined by averaging the result of three enumerations of same size aliquots, using hemacytometer. To spike 2 cells, a micromanipulater was used to pick up cells under microscope.

◇ **Blood Sampling and Negative Enrichment of Circulating Tumor Cells(CTCs)** 7.5 mL of peripheral blood was drawn from individuals after discarding the first 2 mL blood. Red blood cells were lysated followed by WBC depletion with immunomagnetic beads conjugated with antibody cocktail. Cell pellet was spotted on glass slides and fixed.

◇ **Immunofluorescence(IF) Staining.** Double IF staining was performed with anti-CA19-9-Alexa 488 and anti-CK 8/18-Alexa 594.Cells were counterstained with DAPI.

◇ **Fluorescence in situ hybridyzation(FISH).** The genomic DNA probes, chromosome enumerating probes (CEP) 8 and CEP 20 were used to perform aneusomy analysis and DAPI was added to fluorescently label the nuclei of cells.

Summary of Key Findings

◇ **Recovery of Spiked Cancer Cells.**
For 2-cell spikes, we get a recovery rate of 90% to 100%, and for 10-cell spikes, 85-90% of spiked cells were recovered in 3-6 separate experiments as indicated (Fig. 1).

◇ **Criteria for Identification of CTCs.**
1). A round to oval shape with an intact nucleus;
2). Cellular sizes varied from 10 μm to 30 μm in diameter, sometimes with doublets, clusters, and irregular shapes and multinucleated cells;
3). A high nucleus to cytoplasm ratio; 4). Anti-CK8/18 and anti-CA19-9 dual-positive; 5). Some ambiguous positive cells were judged by FISH. (Fig 2)

◇ **Blood CTCs was Complementary with the Serum CA19-9.**
The study included 30 clinically diagnostic pancreatic cancer patients who were subsequently confirmed as pancreatic cancer by pathological or cytological results.
The diagnosis sensitivity of counting CTCs and serum CA19-9 were 93.3% (28/30) and 73.3% (22/30) respectively, and the combined sensitivity of serum CA19-9 and CTCs increased to 100% (30/30). As far as the two examinations are concerned, blood CTCs seems to appear earlier than increased serum CA19-9 in our pilot study(Fig. 3).
We also tested 5 blood samples from different healthy donors (7.5ml/per sample), and the positive cells on the slides were found much lower than the patients(Fig. 4).

◇ **The Dynamic Alteration of CTCs Before and After Treatment.**
Total 27 blood samples were analyzed from 12 patients whose specimens were collected pre- and post-operations (Table 1). The numbers of CTCs in post-operative blood were found to decrease in almost all cases except 2 patients. Preliminary data showed long survival time for the cases with declined CTCs in their post-treatment samples.
The CTC count of one patient who was clinically diagnosed as pancreatic cancer was observed increasing dramatically after operation(from 5 to 718).
After 2-month 5-FU chemotherapy, CTCs decreased again(from 718 to 128).

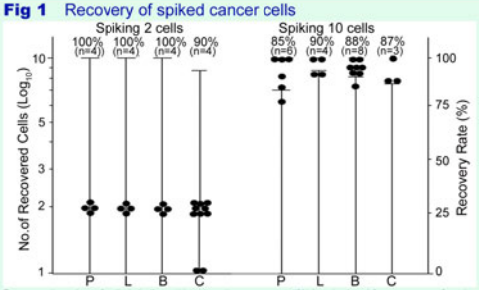
Table and Figures

Table 1 The CTCs detected in pre- and post-operative patients.

Sample #	CTC No. Pre	Post (Weeks after operation) 1	Post (Weeks after operation) 2	Stage	Treatment	Survival time(month)
31	4	0 (21)	N/A	III	Gastrojejunostomy, FU gel local chemotherapy	> 9
32	32	1 (4)	N/A	IV	Lymph node biopsy	> 7
33	5	718 (2)	128 (24)	IIB	Whipple procedure, FU chemotherapy	> 6
34	6	2 (1.3)	N/A	IB	Whipple procedure	> 3
35	12	9 (1.3)	N/A	IIA	Distal pancreatectomy, spleen resection	> 3
36	72	9 (4)	N/A	IIA	Whipple procedure	> 5
37	16	12 (1)	N/A	IIA	Distal pancreatectomy, spleen resection	> 5
38	4	29 (1)	N/A	IIA	Gastrojejunostomy, FU gel local chemotherapy	N/A
39	18	5 (1.4)	N/A	III	Gastrojejunostomy	N/A
40	9	2 (1.7)	N/A	IIA	Distal pancreatectomy, spleen resection	> 5
41	10	3 (1)	N/A	N/A	Laparotomy	N/A
42	8	1 (1.3)	N/A	N/A	Cholecystojejunostomy	N/A

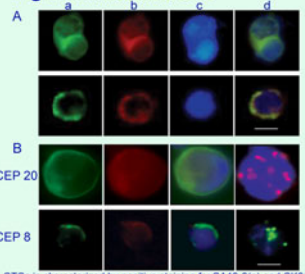
Pre: Pre-operation; Post: Post-operation; Path.: Pathology; N/A: Not available

Fig 1 Recovery of spiked cancer cells



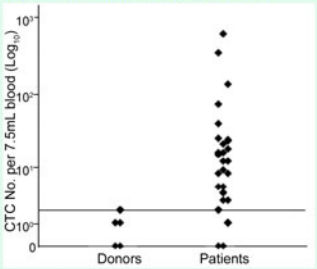
Recovered number of cells is indicated by (•) and recovery rate (%) is calculated from average of total number of experiments (n=3-10) was indicated. 90-100% of recovery rate were obtained on spiking 2 cells. For spiking 10 cells, 85-90% recovery rate were ahieved. P, L, B and C represented prostate, lung, breast and cervical cancer respectively.

Fig 2 Identification of CTC



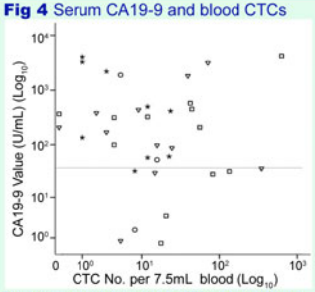
A. CTCs is characterized by positive staining for CA19-9(a) and CK8/18(b) in cytoplasma and cell membrane; a visible nucleus (c); the ratio of nuclear/cytoplast more than 0.8 (merge, d). B. Detected positive cells (CA19-9 positive, a; CK 8/18 positive, b and merged c) hybridized with CEP20(orange, d); CEP8(green, d). The white scale bar indicates 10μm.

Fig 3 CTCs in donors and patients



CTCs in 7.5ml peripheral blood of 5 health donors and 30 pre-operative pancreatic cancer patients confirmed by pathology and/or cytology. The horizontal line indicated 2 CTCs in 7.5ml peripheral blood.

Fig 4 Serum CA19-9 and blood CTCs



The abscissa and ordinate represented CTC number and CA19-9 value respectively. The gray horizontal line was added according to the recommended normal value of serum CA19-9 (37 U/mL). Stage I, II, III and IV was represented by ○,△, □ and ★ separately.

Acknowledgments

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