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Original Article

Correlation between the positive expression of circulating tumor cells and clinicopathological characteristics in well-differentiated nonfunctional pancreatic neuroendocrine tumors performed on CellSearch platform

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Abstract: Objective: to investigate the feasibility of the circulating tumor cells applied in well-differentiated non-functional pancreatic neuroendocrine tumors (NF-pNETs) and present the pilot study of its clinical value using CellSearch platform. Method: From September 2014 to March 2016, 15 consecutive patients (7 males) who were pathologically diagnosed as well-differentiated NF-pNETs were enrolled in this study. 7.5 ml blood from the peripheral vein were drawn, detected and analyzed through the CellSearch system. The clinical characteristics, tumor grade, CTCs counts of each patient were recorded and statistically compared. Results: Of the 15 enrolled patients, 46.67% were males. All of the patients were NF-pNETs (26.67% G1, 73.33% G2) and 6 of them had liver metastases (LM). The median size of the primary lesion was 2.14 cm, and the median ki-67 index was 6.27%. CTC were detected in 53.33% of patients, the mean count was 2.2 CTCs (median 1). For all the recruited patients, compared with the control group, patients with lymph metastases (P=0.003), tumor size larger than 2 cm (P=0.020) and elevated CgA level (P=0.001) preoperatively were likely to have higher capture rate of tumor cells. Conclusion: CTCs detection from the peripheral blood is practical in well-differentiated NF-pNETs, which has the potential to suggest the prognosis. Further detection and exploration need to be continued.

Keywords: Circulating tumor cells, pancreatic neuroendocrine tumors, clinical correlation

Introduction

Neuroendocrine tumors (NETs) are neoplasms originating from various anatomical systems, such as lung, pancreas, mid-gut, and rectum, especially occurs in the pancreatic endocrine system. Recently the number of these neoplastic diseases has appeared to be on the rise [1, 2], and the management of pancreatic neuroendocrine tumors (p-NETs) still remains a clinical challenge, because of its peculiarity of occult malignancy often makes the choice of optimal treatment option perplexing [3].

P-NETs with hormonal syndrome classified as functional tumors may easily be detected and diagnosed by determination of their serum secretory products and the associated symptoms; by contrast, there is limited experience in the detection of nonfunctional p-NETs (NF-PNETs). Beyond that and of these second-common tumors in the pancreas, according to the latest world classification of digestive tumors and Expert consensus [4], grade G1 and G2 were identified as the kind of tumor with better differentiation and low proliferation activity. Unlike tumors in high proliferation activity often found at the advanced stage or accompanied by distant metastasis, NETs in well differentiation were often neglected with non-specific symptom. Thus, a critical issue is to make the improvement of early detection and treatment in those NF-PNETs for validly ameliorate the prognosis.

Traditional imaging diagnosis techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) have the comparatively lower level of sensitivity in the tumor progression and can hardly play the predictive role in the real-time monitoring for every patients; in addition, although some serum tumor markers like chromogranin A (CgA) and Neuron Specific Enolase (NSE), to some extent, have been proved to be clinically valuable, the instability and impressionability to other factors make those detection results lonely the clinical reference index [5, 6]. A systematic review conducted by Modlin et al [7] has concluded that the heterogeneity of NET and the interaction of the tumor microenvironment result in the fact that no single biomarker were effective to guide management, monitor the efficacy of therapy and provide a prognostic assessment of disease progress. Hence, a kind of stable and easily operated detection technique are in need and has always been a pretty highlight of the research.

Circulating tumor cells (CTCs) are cells that have detached from the primary tumor and entered the blood circulation. CTCs can be migrated to other sites to form distant metastases and consequently be detected from the peripheral blood [8]. Recently the rapid advances in the field of detecting CTCs have brought some novel ideas on earlier forecasting the tumor recurrence and metastasis. So we dedicated this study to introducing a method and to preliminarily exploring the clinical correlation between CTCs and well-differentiated NF-pNETs [9].

Materials and methods

Patients and sample collection

This study was approved by the Ethical Committee of Fudan University, and it followed the ethical guidelines of the Declaration of Helsinki. Related written informed consent was obtained from each participant. From September 2014 to March 2016, 15 consecutive patients with histopathologic diagnosis of NF-PNETs, which were confirmed from both the hematoxylin-eosin and immunohistochemistry staining, were enrolled in this study.

Once the resected specimens were confirmed to be NF-pNETs through the pathological examination, a volume of 7.5 ml blood was drawn from the peripheral vein, this procedure is strictly performed under the principles of the sterilization. In order to make sure the accuracy of the final pathological diagnosis, all cases and pathological sections were re-evaluated by another independent pathologist according to the site of origin and criteria of the World Health Organization (WHO) and graded according to the latest European Neuroendocrine Tumor Society (ENETS) proposal for grading and staging of NETs [10].

The data of preoperative clinical manifestations, radio-graphical information, blood biochemical examination as well as the postoperative complications, recovery processes were all recorded in the 15 patients. After the discharge, all the patients were performed regular follow-up through the outpatient clinics and phone call as well as the common essential information of the tumor progression was also recorded accordingly. Specifically, to assess the data of tumor progression, enhanced CT or MRI were performed in clinics and were compared with the previous one by another radiologist, Furthermore, some laboratory blood tests such as CgA and NSE were periodically inspected, what to be noticed is that the period of Follow-up cycle are usually range from 2 to 8 weeks and the specific time depends on the definite situations, such as the disease status, patients' fundamental state and the pathological grade of the NF-pNETs.

Detection of circulating tumor cells in Cellsearch system

All the CTCs in this study were detected based on the Cellsearch platform, which is an immunocytochemistry (ICC)-based system, of which the detailed techniques regarding the accuracy and reproducibility have been reported already [11]. Here the brief detection procedures using CellSearch platform are listed as follows: 7.5 ml venous bloods samples were drawn into the CellSave tubes (Veridex LLC) which contains the EDTA (Ethylene Diamine Tetraacetic Acid) and some patented preservatives and maintained at the room temperature (15-30°C). Then the CellSearch platform can isolate the cells expressing EpCAM (Epithelial cell adhesion molecule) through the specific antibody-

Table 1. Clinical characteristics of patients with pancreatic neuroendocrine tumors

Patients No.	Sex	Age (years)	CTC number(s)	Path	WHO Grade	Operation method	M	CgA (PT)	Ki-67 index	Tumor Size (cm)
1	M	66	2	NET	G2	DPS	Liver	278.7	10	3.3
2	M	54	0	NET	G2	DPS	Liver	50.7	15	0.7
3	F	41	3	NET	G2	DPS	-	162.9	3	3.5
4	M	32	1	NET	G2	DPS+HL	Liver	93.9	5	3.0
5	M	57	0	NET	G1	PD	-	30.3	5	0.8
6	F	56	1	NET	G2	PD	-	125.9	10	3.0
7	F	67	0	NET	G2	DPS	-	30.5	10	3.0
8	M	51	0	NET	G1	PD	-	36.2	2	3.8
9	M	47	0	NET	G2	PD	Liver	24.6	10	1.5
10	F	46	4	NET	G2	DPS+HL	Liver	92.2	5	1.5
11	F	53	11	NET	G1	PD	-	164.3	2	2
12	M	62	10	NEC	G2	LP	Liver	74.3	10	2.2
13	F	46	0	NET	G1	DPS	-	18.1	1	0.9
14	F	58	0	NET	G2	PD	-	45.2	3	1.5
15	F	38	1	NET	G2	PD	-	89.5	3	2.5

Annotation: CTCs: circulating tumor cells; Path: pathology; DPS: distal pancreatectomy and splenectomy; PD: pancreaticoduodectomy; HL: hepalobectomy; LP: liver puncture; PT: pre-treatment.

coated Ferromagnetic nanoparticles (The CellSearch analyzer II), which is the preliminary screening, and in the next step, the epithelial cells and leukocytes were divided through the stains of fluorescently labeled monoclonal antibodies, anti-CD45-allophycocyanin (APC) and anti-pan-cytokeratin (CK)-phycoerythrin (PE), finally the CTCs were defined as 4,2-diamidino-2-phenylindole-dihydrochloride (DAPI)-stained cells lacking CD45 and expressing cytokeratin. All the detections and evaluations were performed by the 3 professional clinical laboratory doctors without the knowledge of the disease status and clinical progression.

Statistical analysis

Demographic and clinical data, details of laboratory index, pathological diagnosis and classification and the presence of CTCs were collected. All data including the follow-up status and CTC index were imported into the computer and a database was established. The statistical analysis was performed using the SPSS 19.0 for Windows statistical software. Continuous data were expressed as mean ± SD. Comparisons of continuous variables were performed using Student's t test, and comparisons of categorical variables were performed using χ^2 test where appropriate. All statistical tests were two sided, and $P<0.05$ was considered statistical significance.

Results

General condition and clinical characteristics

The clinical characteristics of 15 NF-pNETs patients were shown in **Table 1**. From September 2014 to March 2016, there are totally 15 patients participated in our study. The ratio of male to female (Gender distribution) is 7:8, namely in a similar quantity. 3 cases were found through regular physical examination, whereas the other 12 cases were found through a few non-specific symptoms, such as abdominal pain, anorexia, abdominal distension hematochezia etc. The pancreatic masses were located in the head and uncinated process of the pancreas in 7 patients and in the body/tail in 8. Metastatic lesions to the liver and lymph node were both revealed in 6 patients, respectively. On the aspects of treatment, 7 received pancreaticoduodenectomy for the sake of the primary tumor located in the head and uncinated process of the pancreas; 7 received the distal pancreatectomy and splenectomy; of which 2 patients went through the resection of the primary tumors and (or) hepatolobectomy, and the last 1 patient was performed the liver biopsy owing to the widespread metastasis.

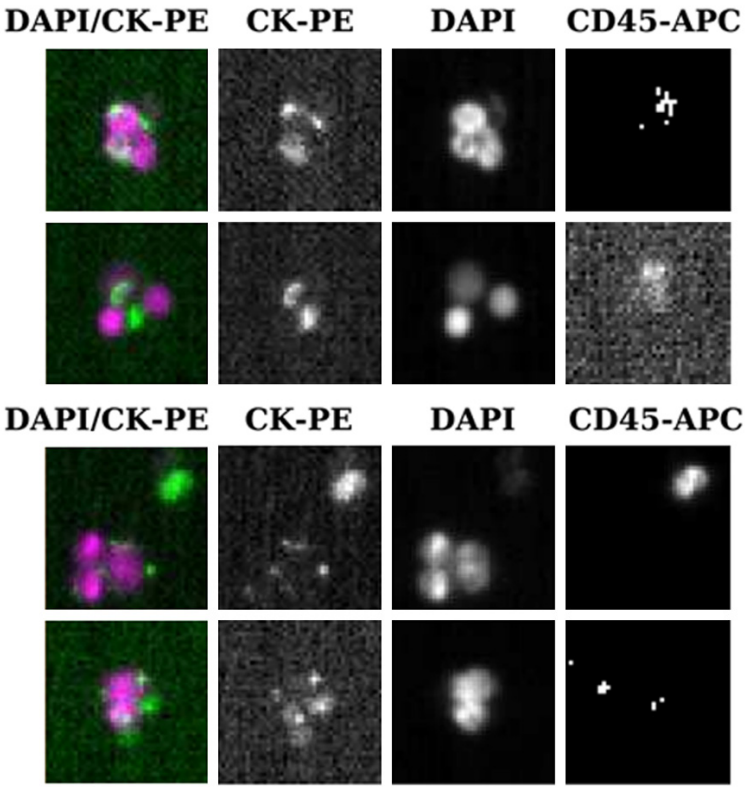

Figure 1. The CTCs captured by CellSearch system.

Correlation of positive CTCs counts with clinical characteristics

It has been reported that the expression rate of circulating tumor cells in pancreatic tumors were lower than some other kinds of neoplasms [11]. Based on these results, we considered even 1 CTC per 7.5 ml of blood to be significant, and we defined a CTC count of 1/7.5 ml of blood or higher as CTC-positive. Of the 15 enrolled patients, 8 were CTC-positive (accounting for 53.33%), ranging from 1 to 11 counts per 7.5 ml venous blood, **Figure 1** shows the common form of the CTCs captured by the CellSearch platform.

All of the patients were NF-pNETs (26.67% G1, 73.33% G2) and 6 of them had liver metastases (LM), with 3 synchronous LM and 3 metachronous LM. The median size of the primary tumor was 2.14 cm, and the median ki-67 index was 10.26%. The mean count was 2.2 CTCs (median 1). The median CTC count of p-NETs with LM and p-NETs without LM were 4.4 and 0.9, respectively.

The mean sizes of the pancreatic cancer tumors, measured by gross samples, were 2.63 cm in the 8 CTC-positive patients and 1.74 cm in the 7 CTC-negative patients. The metastasis of lymph node was noted in 6 of the 8 CTC-positive patients (75.0%). The mean Chromogranin A (CgA) level was 87.8±71.2 ng/ml (range from 18.1 to 278.7 ng/ml); 135.2±67.2 ng/ml (range from 74.3 to 278.7 ng/ml) in the CTCs-positive patients, 33.6±11.4 ng/ml (range from 18.1 to 50.7 ng/ml) in the CTCs-negative patients.

All the data variables were analyzed with SPSS19.0 for Windows statistical software. From the Chi-Squared test results of one-way analysis of variance (ANOVA) in our study, the positive reaction of CTCs were found statistically correlated with the metastasis of lymph node, primary lesion diameter (greater than 2 cm) and the CgA levels, with the *P* value 0.003, 0.020 and 0.001, separately. Due to the low volume of the statistically significant variables from the single factor Chi-Squared test, Logistic multivariate regression was not performed in the following analysis.

Discussion

Tumor recurrence and metastasis were well recognized as one of the most essential factors regarding to the survival period of patients. The surgical results of pancreatic cancer were unsatisfactory, for the pancreatic neuroendocrine tumors itself at present, no method is now available to predict, on the preoperative period, those who are likely to have postoperative distant metastasis. It is still the pathological type and definite differentiation grade from the paraffin sections that can help predict the prognosis, such as the invasion of nerve tract and (or) surrounding tissues indicating the relatively higher possibilities of postoperative recurrence and metastasis. What is more importantly, circulation system is the main way to facilitate distant metastasis as currently reported. CTCs are those cells that have detached from the primary tumor, screened

Table 2. Correlation of CTCs counts with clinical characteristics in NF-pNETs patients

	No.	No. of positive CTCs patient	No. of negative CTCs patient	*P* value
Gender distribution				0.447
Male	7	3 (42.9%)	4 (57.1%)	
Female	8	5 (62.5%)	3 (37.5%)	
Age				0.605
≥60 y	3	2 (66.7%)	1 (33.3%)	
<60 y	12	6 (50.0%)	6 (50.0%)	
Primary tumor location				0.447
Head	7	3 (42.9%)	4 (57.1%)	
Body and tail	8	5 (62.5%)	3 (37.5%)	
Lymphatic metastasis				0.003
Positive	6	6 (100.0%)	0 (0)	
Negative	9	2 (22.2%)	7 (77.8%)	
Primary lesion diameter				0.020
≥2 cm	9	7 (77.8%)	2 (22.2%)	
<2 cm	6	1 (16.7%)	5 (83.3%)	
Tumor grade				0.185
G1	4	1 (25.0%)	3 (75.0%)	
G2	11	7 (63.6%)	4 (36.4%)	
Hepatic metastasis				0.398
Positive	6	4 (66.7%)	2 (33.3%)	
Negative	9	4 (44.4%)	5 (55.6%)	
Chromogranin A level				
Elevated	8	8 (100%)	0 (0)	0.001
Normal	5	0 (0)	5 (100%)	
Depressed	2	0 (0)	2 (100%)	
Total	15	8 (53.3%)	7 (46.7%)	

from the immune system and finally entered the blood circulation, thus those cells can accordingly be migrated to form distant metastases [12]. Owing to its noninvasive, simple and accurate peculiarity, CTCs has been a highlight of the research on detecting free tumor cells in peripheral blood to indicate the kind of patients with high risk of metastasis and thereby take targeted efforts to ameliorate these patients' prognosis.

On the detection of tumors in digestive system, there are accumulating literatures demonstrating that the circulating tumor cells can be applied to peripheral blood examination in gastric [13, 14] and liver [15] cancer and are proved statistically significant to be indicative for tumor recurrence and metastasis. These years in regard to the CTCs detection and utility of pancreatic tumors, in spite of basing on various detection platforms, the EpCAM-positive expression in CTCs of pancreatic ductal adenocarcinoma have already been reported, and were statistically proved to be of great clinical utility in many published studies [16-19]. However, Challenges still exist in the CTCs detection and its clinical implications because of the heterogeneous essence of CTCs population and p-NETs itself [8], which blocks the implement of identifying, isolating and molecularly characterizing the tumor cells in liquid blood. Despite facing the same problems in the p-NETs, on the basis of our study we can still detect the CTCs from the blood sample with a good discovery rate (53.3%), which demonstrated that such a noninvasive method can be performed in the clinical research in NF-PNETs patients.

CTCs, a component of so-called "real-time liquid biopsy", have a great potential to enable the new kind of evolution in current cancer treatment. But the lack of standardizing optimal methods to detect CTCs makes the clinical utility of CTCs unlocking and in slow development [20]. With the rapid development of the CTCs detecting techniques, there are increasing number of detection methods consisting of variable biologic principles, which included the immune-cytological and PCR-based detection methods [8]. Among various CTCs detection platforms the CellSearch detection system are the most classic and the only FDA cleared method for enumeration of circulating tumor cells based on immune-magnetic theory [21]. In order to use CTCs detection method more specifically and sensitively, it would be ideal to detect as many CTC as possible in as many cancer patients as possible. Kiki C. Andree [22] has concluded in a systematic review that the CellSearch system is though the authority-validated detection technology, however, can often miss many CTCs without EpCAM expression [23, 24]. In our study CellSearch platforms was

performed to detect CTCs as shown in **Table 2** with a pretty high discovery rate, demonstrating that the feasibility of this platform, however simultaneously, the definite CTCs counts of the positive one are in a small quantity compared with breast cancer and gastric cancer. In the future, there would be many new technologies based on various CTCs enumeration theory, especially the one that has the peculiarity of much specificity and sensitivity in detecting the tumor cells in p-NETs after a multi-center clinical trial. The scientificity, utility and veracity in the fields of p-NETs about the novel detection techniques should be further verified.

P-NETs are second-most common malignancy of pancreas and the occurrence rate appears to be rising [25, 26]. With great heterogeneity in epithelial neoplasms, the pathological characteristics are commonly recognized as the vital information guiding the prognosis. Thus, the statistical comparison between certain pathological features and CTCs property can be used as the dependable indication for prediction. Through the comparison in our study, all the NF-PNETs patients with lymph node metastasis were found CTCs positive, the patients with large tumor load (diameter greater than 2 cm) and elevated CgA level were also found with tendency to positively reactive to CTCs detection. Those results reveal that the positive expression of CTCs has the significant connection with the pathological and laboratory factors indicating unfavorable prognosis, which can reflect biological essence that the expression of CTCs could be a risk factor to give rise to tumor recurrence and metastasis. These years it has been explored twice about the prognostic significance of the CTCs in neuroendocrine tumors [27, 28], but there is also insufficient information to support the clinical use of circulating tumor cells as useful prognostic markers for this disease.

The detection and identification of CTCs in p-NETs presents several novel opportunities. Its character as a forecasting biomarker of the efficacy to adjuvant therapy needs to be further explored; the concrete statistical significance of PFS and OS between the positive and negative expression of CTCs should also be incorporated in the consequent study; the detection technology to specifically and sensitively facilitate the layering of patients in different treatment strategies [18, 29]. Furthermore, therapeutic targets including human epidermal growth factor receptor-2 and epidermal growth factor receptor have been identified in CTCs, and recent studies have demonstrated the ability to undertake single-cell sequencing [30]. CTCs could be utilized as a prognostic marker in NETs to stratify therapy, and in real-time monitoring of tumor growth or treatment response.

In summary, this is the first exploration in the CTCs' clinical value in well-differentiated NF-PNETs using the CellSearch platform; CTCs can be captured through this platform from the peripheral blood with a relatively high discovery rate. Furthermore, the positive expression of CTCs has been statistically associated with poor prognosis factors. Due to the low amount of variables, the study-related conclusion needs a larger series and longer follow-up. Continued identification of the CTCs' biological characteristics should be encouraged with the goal and anticipation of understanding the disease better.

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Disclosure of conflict of interest

None.

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