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Detection and molecular analysis of circulating tumor cells for early diagnosis of pancreatic cancer

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ABSTRACT

Circulating tumor cells (CTCs) have the potential to provide a surrogate for "real-time biopsy" of tumor biological activity. Enumeration and molecular characterization of CTCs in pancreatic cancer could play an important role in diagnosis, predicting the risk for tumor recurrence, and providing novel target therapy biomarkers. CTCs can disseminate into peripheral blood in the preinvasive and early stages of pancreatic cancer. In view of these facts, we propose that identification and molecular analysis of the malignant characteristics of CTCs may serve as a "liquid biopsy" in pancreatic cancer for early diagnosis.

Introduction

The number of deaths caused by pancreatic cancer has been gradually increasing compared with other types of cancers. Despite progress in detection and management of pancreatic cancer, only approximately 4% of patients will live for 5 years after diagnosis [1]. In recent years, important advances have been made in the understanding of molecular characteristics of pancreatic cancer, as well as in diagnosis, staging, and treatment. Some progress has been made in prevention, early diagnosis, and treatment in patients with a high mortality from pancreatic cancer [2]. Failing to diagnose this devastating disease at an early stage may have adverse consequences.

Pancreatic ductal adenocarcinomas evolve through noninvasive precursor lesions, such as pancreatic intraepithelial neoplasias, acquiring genetic mutations, and epigenetic alterations in the process of carcinogenesis. Pancreatic cancer can also evolve from intraductal papillary mucinous neoplasms or mucinous cystic neoplasms.

Circulating tumor cells (CTCs) may become a surrogate for "real-time biopsy" for tumor cells disseminating from primary tumors and distant metastasis in pancreatic cancer [3]. Many studies have focused on CTCs as predictors of metastatic progress and guiding of treatment decisions [4]. CTCs are a novel biological marker for predicting or monitoring the efficacy of systemic therapy and prognosis of metastatic breast cancer patients [4,5]. Wang et al. hypothesized that CTC hemodialysis by filtrating CTCs out of blood will rescue patients at risk of blood-borne metastasis before distant metastasis and increase the survival rate [6]. Metastatic dissemination has long been regarded as the last step in multistep primary tumor progression, which has also been proven by recent genome sequencing research for ductal adenocarcinoma [7]. However, recent evidence has suggested that cells can disseminate remarkably early from noninvasive premalignant lesions in mice and humans [8].

Disseminated cancer cells in peripheral blood and bone marrow have been found in patients with ductal breast carcinoma *in situ*, which is a preinvasive cancer lesion [9]. Similar findings have also been reported in a mouse model of pancreatic intraepithelial neoplasia (PanIN), suggesting early dissemination of CTCs in the early stage of pancreatic cancer [10]. Because CTCs disseminate into peripheral blood of cancer patients, CTCs also undergo apoptosis by the body's immune system [11]. CTCs are found in low numbers in peripheral blood of cancer patients. Therefore, many new methods have been developed to detect these rare cells in pancreatic cancer. These methods include immunological assays using antibodies directed against EpCAM antigen from CTCs, polymerase chain reaction-based DNA or RNA analysis for CTCs, and technology based on the characteristics of CTCs [3]. Enumeration and characterization of CTCs in pancreatic cancer could play an important role in diagnosis, predicting the risk for tumor recurrence, detecting chemoradiotherapy-resistant profiles, and identifying prognostic and predictive molecular features [12].

Gene testing for early diagnosis of inherited susceptibility to pancreatic cancer has not been established because the mechanism of the inherited sensitivity to pancreatic cancer remains unexplained [1]. For early diagnosis of pancreatic cancer, we should attempt to identify malignant features and perform molecular

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characterization of CTCs based on the cytomorphological level, which can provide pathological information. If an early diagnosis of pancreatic cancer by CTCs could be achieved, the sensitivity and specificity of CTCs should meet the needs of screening for early pancreatic cancer. To increase the detection of rare malignant cells in circulating blood, current common or novel methods can be strictly selected. For example, immunological assays using immunomagnetic beads coated with CD45 antibody, where blood cells are discarded, are called negative selection. An advantage of negative selection is that CTCs are not immunomagnetically labeled, and therefore are relatively unaffected by the procedure [13]. Furthermore, according to recent findings, more invasive CTCs may lose their epithelial antigens by the epithelial to mesenchymal transition (EMT) process. This suggests that the detection of CTCs cannot be based only on the expression of epithelial makers, such as cytokeratins [14], whereas, the reverse process (mesenchymal to epithelial transition) is thought to allow outgrowth of these cells at distant sites [15]. This novel finding may help explain the mechanism of metastasis of CTCs and the concept for identification of malignantly metastatic CTCs. For positive selection, magnetic beads are used that are conjugated to antibodies against epithelial antigen (e.g., EpCAM). The positive selection methods of CellSearch are approved by the Food and Drug Administration for evaluating the prognosis of metastatic breast cancer [5]. These epithelial markers are downregulated during tumor cell dissemination and are transferred to distant organs, affecting the detection of CTCs [16]. However, the CellSearch method may not be appropriate for detection of the early stage of cancer because of the low detection rate of CTCs in this stage [17]. Therefore, the negative selection of CTCs by immunomagnetic beads coated with CD45 antibody by discarding hematopoietic cells may be a good choice. Microchip technology may also be used to separate CTCs in the early stage of cancer. CTCs in 99% (115/116) of different types of solid tumors and 100% (7/7) in the early stage of prostate cancer can be identified [18]. Microfluidics offers the opportunity to create the next generation of superior CTC enrichment devices in the future [16]. Because nowadays no excellent tumor makers are used to diagnose pancreatic cancer, as to the tumor makers for the identification of CTCs in pancreatic cancer, cytokeratin positive and CD45 negative or CA19-9 and CK double positive may be a good choice [3]. CK8, CK18 and CK19 were used as makers to detected CTCs in pancreatic cancer. CTCs were detected in 6/16 pancreatic cancer patients [19]. Zhou et al. also identified CTCs by conventional RT-PCR after immunomagnetic enrichment. They identified CTCs by human telomerase reversetranscriptase(hTERT), C-MET, CK20, and CEA; the mRNA expression rates of these molecules were 100% (25/25), 80% (20/25), 84% (21/25), and 80% (20/25), respectively. tTERT positive mRNA was detected in 100% (5/5) stage I + II, 100% (8/18) stage III, 100% (12/12) stage stage IV of pancreatic cancer. By combining the markers into a multimarker approach, all 25 patients exhibited positive CTC mRNA status [20,21]. CK20-positive cells were detected in 52/154 (33.8%) blood sample. CTCs was detected in11.1% (1/9) stage I, 33.3% (6/18) stage II, 27.1% (13/48) stage III and 40.5% (32/79) stage IV of pancreatic cancer [22]. We reported that the detection rate in patients with in stage I and II stage was 46.2%(18/38) (data not published) and stage III and IV was 80.5% (33/41) [23]. In a mouse model of pancreatic intraepithelial neoplasia (PanIN), early dissemination of CTCs can be found in the early stage of pancreatic cancer [10]. So detection of CTCs may be a new tool for early diagnosis of pancreatic cancer.

The hypothesis

CA19-9 is poorly effective as a tumor marker in the early stages of tumor development [24]. CTCs may overcome the limitation of the currently available serum tumor markers, such as CA19-9. As a surrogate for "real-time biopsy", CTCs may be used as a marker for a tumor's biological activity for prognosis in pancreatic cancer. Characterization of CTCs has the potential to represent malignant cells shed from the primary pancreatic tumor and macroscopic metastasis organs, and also allows dynamic detection of CTCs at multiple sampling time points during the disease process for evaluation of the efficacy of treatment [3,23,25].

We hypothesize that enumeration and molecular analysis of CTCs in pancreatic cancer based on cytometry levels in noninvasive precursor lesions (e.g., pancreatic intraepithelial neoplasias, intraductal papillary mucinous neoplasms, and mucinous cystic neoplasms or the early stage of pancreatic cancer) can be used to detect malignant cells in peripheral blood that cannot be detected by radiography. CTCs in peripheral blood of samples are isolated by negative selection of immunomagnetic beads or microchip technology. Therefore, the enumeration and molecular analysis of malignant features of CTCs in patients in the early stage of pancreatic cancer or noninvasive precursor lesions may be helpful in the early diagnosis of pancreatic cancer.

Evaluation of the hypothesis

Less than 20% of patients present with localized, potentially curable tumors diagnosed as pancreatic cancer. Because chemotherapy and radiotherapy have failed to significantly improve the overall survival of patients with pancreatic ductal adenocarcinoma, scientists need to find better diagnostic markers of pancreatic neoplasia. This would be useful for improving the early diagnosis of patients with pancreatic cancer for curative surgical resection, and also helpful for patients at a high risk of developing pancreatic cancer at the early stage. Gene expression, DNA methylation, and proteomics alterations, which occur in pancreatic cancer, may be used as tumor markers for early diagnosis of pancreatic cancer [26]. The combination of miR-16, miR-196a in plasma, and CA19-9 in serum is more effective than with CA19-9 alone for pancreatic cancer diagnosis, especially in early tumor screening [27]. Tri-phasic pancreatic-protocol computed tomography is the best initial diagnostic test for pancreatic cancer [1]. However, these novel tumor markers and traditional clinical methods do not provide sufficient sensitivity and specificity for the diagnosis of early pancreatic cancer. Moreover, these markers cannot provide pathological information of cells regardless of whether they have a malignant origin.

Screening to detect curable precursor lesions may cause a risk of overtreatment. Therefore, isolation and molecular analysis of CTCs in patients with intraductal papillary mucinous neoplasms or mucinous cystic neoplasms may help diagnose the early stage of pancreatic cancer. If malignant features of CTCs are found in patients with these curable precursors, then treatment by surgery may be necessary to cure patients. Furthermore, currently, the purpose of screening efforts is to detect preinvasive lesions, rather than early pancreatic cancer. Once preinvasive lesions develop into invasive pancreatic cancer, its spread beyond the pancreas is probably rapid, restricting the use of traditional markers of invasive pancreatic cancer [1].

Early-stage pancreatic cancer is usually clinically asymptomatic, and disease only becomes obvious after the tumor invades surrounding tissues or there is metastasis to distant organs. Symptoms can take up to 1 year to develop in pancreatic cancer patients [28], suggesting a missed opportunity for early diagnosis. CTCs can be repeatedly detected in the peripheral blood. Some literature support for use of CTCs as a diagnostic in pancreatic cancer [3,21] or early tumor dissemination in a mouse model of pancreatic intraepithelial neoplasia [10]. CTC-chip technique will provide a robust platform at early diagnosis of cancer, including pancreatic