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Methods

Genetic analysis of circulating tumor cells in pancreatic cancer patients: A pilot study☆

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ABSTRACT

Pancreatic cancer is one of the most aggressive malignant tumors, mainly due to an aggressive metastasis spreading. In recent years, circulating tumor cells became associated to tumor metastasis. Little is known about their expression profiles.
The aim of this study was to develop a complete workflow making it possible to isolate circulating tumor cells from patients with pancreatic cancer and their genetic characterization.
Results: We show that the proposed workflow offers a technical sensitivity and specificity high enough to detect and isolate single tumor cells. Moreover our approach makes feasible to genetically characterize single CTCs.
Conclusions: Our work discloses a complete workflow to detect, count and genetically analyze individual CTCs isolated from blood samples. This method has a central impact on the early detection of metastasis development. The combination of cell quantification and genetic analysis provides the clinicians with a powerful tool not available so far.

1. Introduction

Pancreatic cancer (PC) is considered as one of the most malignant tumors with a very high mortality rate. Although not very common, PC incidence is steadily increasing and nowadays is the fourth leading cause of death for cancer in western countries [1]. PC can be diagnosed as Pancreatic Ductal Adeno Carcinoma (PDAC) accounting for more than 85% of all pancreatic malignancy or Acinar Cell Carcinoma (ACC) [2]. In both cases, the tumor is characterized by the extremely high tendency to metastasize. The majority of the patients are diagnosed already with a progressed tumor stage characterized by spread metastasis generally to the liver and/or to the peritoneal cavity, therefore palliative surgery and/or palliative chemotherapy remain the only options [3], with a 5 year survival rate of 1–4% and a median survival rate of 4–6 months [4–6]. Standard imaging analysis such as contrast enhanced computed tomography or magnetic resonance imaging are the only available options for PC detection. However, low sensitivity makes the detection of small volume variations or small metastasis challenging. In addition they do not provide any information about PC type or malignancy. Diagnosis can also be based on the measurement of tumor biomarkers such as the carbohydrate antigen 19-9 (CA19-9) and the carcinoembryonic antigen (CEA) although their specificity is quite limited and the sensitivity is low [7]. Alternative detection methods based on RNA, DNA and tissue biomarkers are presently under investigation and still not introduced in the clinical routine. Since PC diagnosis at an early phase is technically difficult and the methods available are often not showing the required sensitivity and specificity, early detection would be highly desirable to decrease mortality, avoiding the disease progression and the metastatic invasion to secondary organs. Metastasis can be started by an occult hematogenous spreading of tumor cells originated from the primary tumor already during the early phase of the disease [8]. An increasing number of reports support the idea that disseminated tumor cells (DTCs) in bone marrow and circulating tumor cells (CTCs) in peripheral blood of cancer patients can be

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associated to distant metastasis [9]. The analysis of CTCs can offer essential information on the genetic characteristics of occult minimal residual disease (MRD) in patients with detected primary cancer but without any clinically detectable metastasis [10]. CTCs are tumor cells which after detaching from the primary tumor, intravasate and through the blood stream reach secondary distant organs, forming micrometastases [11, 12] which might then further develop giving origin to macrometastasis [13–15]. After even long times of latency [16], secondary metastasis can be initiated if CTCs enter again in the blood stream and reach new organs. Bone marrow is particularly rich of DTCs, however their isolation and characterization are possible only after needle aspiration, a quite invasive and difficult procedure [15]. In addition, till now data on their prognostic value in metastatic patients is still not completely clarified and additional clinical studies are surely needed [17]. Isolation of CTCs from blood samples looks on the contrary a more attractive option because of its limited invasiveness. Several methods for CTC detection and characterization, based on immunocytochemical or molecular techniques, are already available [8,18–24]. The Food and Drug Administration (FDA) approved CellSearch™ system (Veridex, Raritan NJ, USA) has been extensively validated in breast, prostate and colon cancer patients in clinical multicenter settings and has proven good reproducibility [23,25–27]. The prognostic relevance of CTCs was shown for the first time in breast cancer patients by Cristofanilli and collaborators [28]. The detection of ≥5CTC/7.5 ml peripheral blood, before and after a new line of treatment, was associated with a shorter progression free survival and a shorter overall survival. In this pilot study we established, at our knowledge for the first time, a complete workflow to isolate CTCs by flow cytometry from PC patients and to genetically analyze them by RT-PCR. Detection, isolation and molecular characterization of these cells might be the key for providing PC patients with a more precise diagnosis and a more targeted treatment.

2. Results

2.1. Sensitivity and specificity of MoFlo™ XDP

Due to the extremely low number of tumor cells potentially circulating in peripheral blood, the selected isolation method should be extremely sensitive and specific. To evaluate if the MoFlo™ XDP system (Beckman Coulter) used in this study could fulfill these requirements, six different dilutions of the pancreatic cancer cell line AsPC-1 (from 1×10^6 to 1×10^1) were spiked in a background of 1×10^7 peripheral blood mononuclear cells (PBMC) and stained with an antibody mix recognizing the epithelial cell adhesion molecule (EpCAM) marker, the adhesion marker α5β4-Integrin and the leukocyte surface marker CD45. In addition Hoechst dye and Propidium Iodide (PI) were added to stain nuclei and dead cells, respectively. The cytometric gating strategy included only α5β4-Integrin$^+$/EpCAM$^+$/Hoechst$^+$/CD45$^-$/PI$^-$ cells and excluded all the other combinations. The six different dilutions were analyzed to calculate the number of detected AsPC-1 cells relatively to the number of background cells (Fig. 1). The percentage of AsPC-1 cells at the different dilutions showed a linear trend over the complete dilution series, suggesting that the recovery rate follows a systematic error influenced not by cellular interactions, but rather by the technical system. Accordingly, we found a homogenous 10% loss in the total amount of recovered tumor cells due to the enrichment process (data not shown). To test the specificity of the system, a single AsPC-1 cell, stained with an antibody cocktail recognizing EpCAM, the tumor marker mucin-1 (MUC-1) and CD45, was spiked in a background of 1×10^6 PBMCs (Fig. 2). The single cell was spiked by flow sorting to avoid any counting and/or manual pipetting error. In 42% of the cases ($n = 12$) the single spiked tumor cell was detected and gated. The cell was then sorted directly on AmpliGrid (Beckman Coulter), a platform designed to perform single cell PCR analysis, and its genetic profile was confirmed by RT-PCR analysis (data not shown). In the remaining cases the gated cells were not sortable, most probably either because trapped in the cytometry tubing system or because of the stringent single cell modus applied by the sorter or finally because resulting positive to PI (therefore automatically excluded according to the chosen settings). Based on the obtained data, MoFlo™ XDP performed with the sensitivity and specificity required, even when analyzing and sorting one single tumor cell in a high background of PBMCs. Most importantly once isolated, single cells could be still genetically analyzed by RT-PCR.

2.2. CTC isolation in PC patients

The complete workflow from CTC isolation by flow cytometry to molecular profiling at single cell level was tested on blood samples drawn from PC patients. A schematic overview of the workflow is

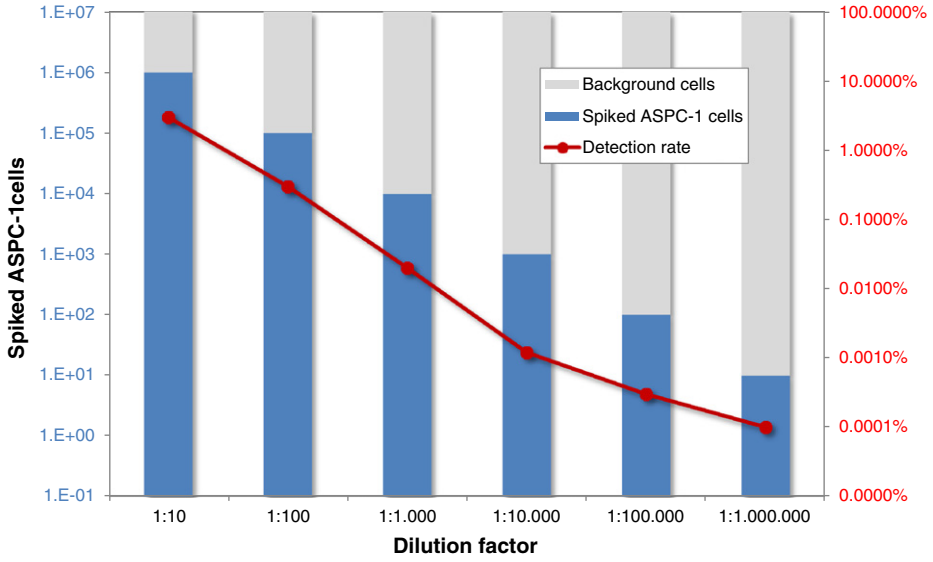

Fig. 1. Sensitivity of the MoFlo™ XDP system. Different dilutions of AsPC-1 cells (from 1×10^6 to 1×10^1) were spiked in a background of 1×10^7 PBMCs. Cells were stained with anti-EpCAM, anti-α5β4-Integrin and anti-CD45. The red curve depicts the detection rate of the spiked tumor cells; blue bars display the number of originally spiked in tumor cells in a fixed number of background cells (gray bars).