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Original article

Identification of high independent prognostic value of nanotechnology based circulating tumor cell enumeration in first-line chemotherapy for metastatic breast cancer patients

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ABSTRACT

Enumeration of circulating tumor cells (CTCs) is a promising tool in the management of metastatic breast cancer (MBC). This study investigated the capturing efficiency and prognostic value of our previously reported peptide-based nanomagnetic CTC isolation system (Pep@MNPs). We counted CTCs in blood samples taken at baseline (n = 102) and later at patients' first clinical evaluation after starting firstline chemotherapy (n = 72) in a cohort of women treated for MBC. Their median follow-up was 16.3 months (range: 9.0-31.0 months). The CTC detection rate was 69.6 % for the baseline samples. Patients with ≤2 CTC/2 ml at baseline had longer median progression-free survival (PFS) than did those with >2 CTC/2 ml (17.0 months vs. 8.0 months; P = 0.002). Patients with ≤2 CTC/2 ml both at baseline and first clinical evaluation had longest PFS (18.2 months) among all patient groups (P = 0.004). Particularly, among patients with stable disease (SD; per imaging evaluation) our assay could identify those with longer PFS (P < 0.001). Patients with >2 CTC/2 ml at baseline were also significantly more likely to suffer liver metastasis (P = 0.010). This study confirmed the prognostic value of Pep@MNPs assays for MBC patients who undergo firstline chemotherapy, and offered extra stratification regarding PFS for patients with SD, and a possible indicator for patients at risk for liver metastasis.

1. Introduction

The TNM (tumor size [T], regional lymph node status [N] and presence of distant metastases [M]) staging system for breast cancer remains the gold-standard guide for clinical decisions. However, about 25.0% of breast cancer patients eventually develop distant metastases after surgery and adjuvant treatment, despite recent medical achievements [1]. This dilemma indicates an urgent need for a more reliable evaluation system for breast cancer. Accumulating evidence suggests that circulating tumor cells (CTCs) spread metastases [2]. Counting CTCs is considered to be a promising predictor of treatment efficacy, progression-free survival (PFS) and overall survival (OS) [3], and several novel technologies for CTC detection have emerged in recent years.

CTCs can be found in bloodstreams of breast cancer patients. However, they occur at extremely low concentrations—as few as 1 CTC per 10^{7-8} white blood cells—and comprise a highly heterogeneous population with different biological features and biomarker expression [4]. Thus, their identification and characterization require an ultra-sensitive and specific analytical system.

Although numerous CTC-detecting techniques have been developed, few have been broadly used in sufficiently large clinical cohorts [5]. Currently, two major types (based on their working principles) of CTC capture-and-analysis methodologies are available [6]. The first type of CTC-detection methods utilizes the unique physical features of CTCs to enrich them from peripheral white blood cells (WBCs), such as CTC density (including OncoQuick®, Histopaque® and Ficoll®), size (CellSieve®, ScreenCell®, ISET® and Circulogix®) or surface charge [4, 7]. The second method utilizes the

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unique biological features of CTCs— mainly membrane protein expression of CTCs or WBCs— to achieve enrichment. In the light of the different membrane protein expression patterns between CTCs and WBCs, CTCs can be enriched by using antibodies against the epithelial-associated proteins such as EPCAM (positive selection), or by antibodies against specific proteins of WBCs (negative selection) [5]. Some commercial systems that use this second method are CellSearch®, CytoTrack®, ImageStream®, IsoFlux®, Cynvenio® [5]. Although many of these systems seem promising, clinical validation is required for any new medical technology. At present, the only method approved by the Food and Drug Administration (FDA) for monitoring MBC patients is CellSearch® [8,9]. Most CTC-detection technologies report that higher CTC frequencies contribute to more effective capture and detection results [10].

Our team at the National Center for Nanoscience and Technology pioneered a novel nanotechnology-based modification that captured EPCAM$^+$ CTCs, using a self-designed peptide combined with iron oxide magnetic nanoparticles [11]. This novel CTC-detecting platform, Pep@MNPs, enabled us to enrich CTCs with high efficiency. We thought the powerful capture efficiency originated from the high affinity ($K_D = 1.98 \times 10^{-9}$ mol/L) of the peptide with the EPCAM molecules. As the self-developed assay uses less blood, and efficiently captures CTCs, we intended to expand it into clinical practice and prospectively verify its predictive value for MBC patients who undergo first-line chemotherapy.

2. Materials and methods

2.1. Patients and samples

For this study, we enrolled 102 consecutive patients who were treated for breast cancer at the Peking University Cancer Hospital & Institute from March 2014 to October 2016. The study protocol was approved by the Medical Ethical Committee of Peking University Cancer Hospital & Institute (Approval No.: 2013KT29). Our eligibility criteria were (a) MBC, (b) undergoing first-line chemotherapy, (c) expected survival time of at least 3 months, (d) measurable lesions and (e) patient's written informed consent. We collected peripheral blood samples from 102 patients had complete follow-up information, using their first blood draws at baseline (time of inclusion), and 72 out of 102 patients for whom we had the second blood draw from their first clinical evaluation (FCE). Clinicopathological information was recorded for all the patients at the time of blood collection. CTCs were analyzed at two different times: at inclusion and at the time of first clinical evaluation. For each patient, 1 tube containing 8.0 ml peripheral blood were collected in CellSave® (Immunivest Corporation, Wilmington, DE, US) collection tubes for the Pep@MNPs assay. Samples were maintained at room temperature and processed within 96 h; only 2.0 ml blood was used for Pep@MNPs enumeration. Details of the Pep@MNPs technique have been described previously [11].

2.2. CTC isolation and enumeration

CTCs were counted by the Pep@MNPs method as previously described. In brief, an EPCAM recognition peptide is attached with iron oxide magnetic nanoparticles (MNPs) via biotin–avidin interaction. For ease of detection, 5.0 μl pre-vortexed Pep@MNPs (10 mg Fe/ml) was added to 2.0 ml peripheral blood samples, and incubated with gentle shaking at 37 °C for 30 min. The Pep@MNPs–CTC complexes were subsequently isolated and washed with PBS at least 3 times under a magnetic field; the captured CTCs were then stained by multiple color immunocytochemistry: 4′,6-diamidino-2-phenylindole (DAPI) for cell nuclei, CD45-phycoerythrin for leukocytes (negative selection) and CK19-fluorescein isothiocyanate for cytokeratin (CK; positive selection, which was used to distinguish epithelial cells from leukocytes). Further identification and counting of CTCs were performed using ZeissVert A1 fluorescent microscope (Carl Zeiss Microscopy GmbH, München, Germany).

2.3. Statistical analysis

The main objective was to test whether PFS and imaging evaluations were associated with CTC status at baseline alone, or with changes between baseline and FCE CTC levels; and secondarily to find any correlations between CTC count, metastasis status, and breast cancer subtype (with regard to ER, PR and HER2 status). PFS was calculated from date of inclusion until the date of tumor progression (per clinical or imaging diagnosis) or death. Imaging evaluations were made according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria.

Data were expressed as means or numbers (%). Categorical variables were compared by chi-square or Fisher's exact test. Correlations between CTC detection and other factors were evaluated with Kruskal-Wallis H test, with CTC detection as the outcome variable of interest. Kaplan–Meier survival curves were computed according to CTC status and were compared using log-rank tests.

3. Results

3.1. Patient characteristics

The median age at diagnosis of the 102 patients who were included in this analysis was 46 years (range: 27–81 years). Their median PFS was 10.0 months (range: 1.0–28.0 months). Their main chemotherapy regimens included taxanes (71.3%), capecitabine (15.8%), gemcitabine (15.8%) and anthracyclines (4.0%). At last follow-up (Oct 31, 2016), 102 patients with complete follow-up information were still alive. Among the 102 patients, 72 patients had CTC levels at their first examination after undergoing chemotherapy; 22 of these patients (30.6%) had confirmed disease progression. Detailed information for these 102 patients is shown in Table 1.

3.2. Identification of patient derived EPCAM$^+$ CTC

We used the conventional definition of CTCs accepted by most studies: cells that were DAPI$^+$/CK$^+$/CD45$^-$ and met the phenotypic morphological characteristics were designated as CTCs, whereas DAPI$^+$/CD45$^+$/CK$^-$ cells were designated as leukocytes. By using fluorescence staining, we imaged cells of interest using 400 × magnification on an inverted microscope. Fig. 1 shows typical immuno-fluorescence staining results for isolated CTCs (upper two rows) and leukocytes (bottom rows) by Pep@MNPs assay.

3.3. Baseline CTC counts and their clinical relevance

Patients' characteristics with stratification by baseline CTC status, are summarized in Table 2. Of 102 patients, 71 patients (69.6%) had > 0 CTC/2 ml, 69 (67.6%) had >1 CTC/2 ml and 41 (40.2%) had >2 CTC/2 ml at the time of inclusion. At their FCEs, 58 out of 72 patients (80.6%) had >0 CTC/2 ml, 39 (68.4%) had >1 CTC/2 ml and 35 (48.6%) had > 2 CTC/2 ml. We gradually set >0 CTC/2 ml, >1 CTC/2 ml, and >2 CTC/2 ml as thresholds to stratify the patients in many aspects. The analytic results reached best performance at >2 CTC/2 ml in various tests. Therefore, we designated >2 CTC/2 ml (>2 CTC) as CTC$^+$, and ≤2 CTC/2 ml (≤2 CTC) as CTC$^-$ in this study.

Briefly, 27.8% of patients had up-regulated CTCs (CTC$^-$ at baseline and CTC$^+$ at FCE), 20.8% had down-regulated CTCs (CTC$^+$ at