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Exploring alternative ovarian cancer biomarkers using innovative nanotechnology strategies

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Abstract

Our increased understanding of ovarian cancer's blueprints (mediated by DNA and RNA) and behavior (mediated by proteins) points to wide differences across patients that cannot be depicted by histology alone. Conventional diagnosis usually entails an adequate tissue biopsy, which limits serial testing. There is thus a motivation to shift towards easier to obtain clinical samples (e.g. ascites or blood). In response, investigators are increasingly leveraging alternative circulating biomarkers in blood or proximal fluids and harnessing novel profiling platforms to help explore treatment related effects on such biomarkers in serial fashion. In this review, we discuss how new nanotechnologies we developed intersect with alternative ovarian cancer biomarkers for improved understanding of metastases and therapeutic response.

Keywords

ovarian cancer; biomarkers; circulating tumor cells; ascites; exosomes; microfluidics; nuclear magnetic resonance; nanotechnology

I Introduction

The adage “Never judge a book by its cover” is relevant in ovarian cancer. Genomic and proteomic analyses on ovarian cancer specimen revealed significant differences among patients that cannot be appreciated by histology alone. As such, the promise of “smart or targeted therapies” unique to specific tumor can only be realized with improved profiling approaches. The dynamic nature of tumor growth and its response to treatment, requires tools that would allow us to readily measure specimens for pharmacodynamic changes. Understanding when and how they change and their implications for the patient would be important steps for managing this highly lethal disease that was historically treated with “a one size fits all” approach.

Conventional testing (e.g., immunohistochemistry, ELISA) usually entails an adequate biopsy – this is not a limitation with initial surgery where ample tissue is available for

analyses. This approach, however, becomes impractical for serial analyses. There is thus a motivation to shift towards easier to obtain clinical samples (e.g. ascites or blood) to avoid missed therapeutic opportunities. In this review, we discuss how novel nanotechnologies we developed in our laboratories intersect with alternative ovarian cancer biomarkers, specifically circulating tumor cells, ascites tumor cells, and exosomes / microvesicles, for improved understanding of metastases and therapeutic response.

II Ascites Tumor Cells in Ovarian Cancer

Ovarian cancer often spreads to peritoneum and causes massive accumulation of fluid (ascites) [1]. We hypothesized that isolating and molecularly profiling cancer cells in ascites would yield unique and diagnostically useful information. After all, drainage of ascites (via paracenteses) is routinely performed in ovarian cancer patients for symptomatic relief and thus provides a source of abundant clinical material. While the precise cellular composition of ascites tends to vary across patients, the fraction of ascites tumor cells (ATC) is generally believed to be <0.1% of harvested cells, with the remainder being host cells (37% lymphocytes, 29% mesothelial cells, and 32% macrophages) [2]. Up until our recent study, cellular profiling in ovarian cancer ascites remained limited. We therefore began our investigation by performing a comprehensive analysis of ATCs. This study identified unique diagnostic (coined ATC_{DX}) and treatment-response marker sets [3].

II.A ATC Chip

To translate the above-mentioned findings for potential clinical application, we designed an ascites-specific microfluidic chip (ATC chip) that isolates ATCs from their highly inflammatory milieu (Figure 1) [3]. The end objective sought to leverage simple and rapid ATC profiling approaches to increase the reach of point-of-care strategies and potentially guide therapeutic clinical trials in ovarian cancer. Prior to the passage of ascites fluid through the ATC chip, the sample is: 1) labeled with magnetic nanoparticles that bind to noncancerous, yet ubiquitous, inflammatory cells (via CD45 labeling) and 2) labeled with a mixture of antibodies to the ATC_{DX} proteins. The sample is then introduced into the ATC chip through a filter intended to trap clumps of debris at the onset. Any fluid passing through is then exposed to a magnet that captures the CD45-nanoparticle labeled cells, thus removing inflammatory cells from following forward (i.e. negative selection). After the magnetic sorting, the sample passes over a series of microwells of successively smaller size, which collect the ATCs. The concentration of captured ATCs is 1,000 times greater than it was in the original fluid sample. As such, the amount of ascites needed to derive a proteomic readout is reduced from liters to microliters.

Our results showed that viable ATCs are commonly found in ovarian cancer patients. The unique panel of diagnostic markers (ATC_{dx}) was highly specific to identify cancer cells. By using the ATC chip, we were able to monitor these markers in serial samples over the course of tumor progression/treatment. We demonstrated that this approach could serve as a convenient strategy for mapping treatment response, thus extending the function of paracenteses as merely a method for symptomatic relief to a valuable means of monitoring therapy.

III Circulating Tumor Cells in Ovarian Cancer-derived Blood

Circulating tumor cells (CTCs) have emerged as a promising surrogate for tissue-based markers in molecular profiling of cancer, enabling non-invasive, real-time disease detection, including ovarian cancers [4–6]. Yet the detection and characterization of CTCs remains a significant challenge due to the rarity of CTCs (~10–100 per mL of blood). The most commonly used method for identifying CTCs, CellSearch, is based on the enumeration of epithelial cells using anti-epithelial cell adhesion molecule (EpCAM) antibodies and subsequent staining for visualization [7]. CellSearch's low sensitivity to low EpCAM expression cancers and EpCAM-negative cancers, which account for 40% and 20% of cancers, respectively, has created a need for new methods to detect CTCs with greater sensitivity.

III.A μ NMR Technology

We have established a novel sensing technology termed micro-nuclear magnetic resonance (μ NMR), which exploits magnetic resonance to detect cells labeled with magnetic nanoparticles (MNPs; Fig. 2a) [8, 9]. Samples containing MNP-labeled cells exhibit faster relaxation of NMR signals due to local magnetic fields created by MNPs. With its basis in magnetic interactions, μ NMR can be performed with minimal purification steps, which reduces cell loss and simplifies assay procedures.

MNP-labeled objects cause faster decay of NMR signal, or shorter transverse relaxation time T_2 , which is how targets can be distinguished from non-targets. μ NMR-sensitivity can be increased by making magnetically stronger MNPs, increasing the number of MNPs per cell, and/or decreasing the NMR detection volume.

III.A.1 Magnetic Nanoparticles (MNPs)—We developed a new type of Fe-MNP construct that maximizes particle magnetization, Fe@MFe₂O₄ MNPs [10]. These MNPs have an elemental Fe-core (chosen for its stronger magnetization) and a protective ferrite shell, which provide high stability against oxidation with negligible changes in particle shape and magnetic properties.

III.A.2 BioOrthogonal Nanoparticle Detection (BOND) Labeling—We developed a new targeting strategy, BOND, that amplifies MNP-binding to biological targets (Fig 2c) [11]. BOND is based on a {4+2} Diels-Alder cycloaddition between tetrazine (Tz) and trans-cyclooctene (TCO). The reaction is fast, irreversible, and can be performed at room temperature without a catalyst. Cells are pre-targeted with TCO-modified antibodies and subsequently incubated with Tz-loaded MNPs. BOND chemistry allows multiple TCO tags to be incorporated onto an antibody without loss of affinity and consequently promotes multiple attachment of Tz-MNPs. BOND yields ~15-fold improvements in MNP cell loading, thus improving performance.

III.A.3. Clinical Applications

III.A.3A. Multiplexed Testing: Due to the absence of single universal cancer markers, the focus has shifted towards multiplexed detection. Based on fine needle aspirates (FNA) from

50 patients, we quantitated the protein expression levels of nine well-established cancer markers and one leukocyte marker [8]. We identified a four marker panel consisting of MUC-1, EGFR, HER2 and EpCAM, coined quad-marker, that conferred a 96% diagnosis accuracy compared with clinical pathology interpretation of corresponding patient biopsies.

III.A.3B More Rapid and Sensitive CTC Detection: Integrating the above-mentioned quad-marker assay into μ NMR resulted in rapid and sensitive CTC detection through minimal cell loss (Fig 3) [12]. The labeling process takes 30 minutes without compromising detection sensitivity of quad- μ NMR (400% fold higher than CellSearch). In addition, we found that the average quad- μ NMR recovery rate was 38% compared with a CellSearch recovery rate of 9.1% across all concentrations assessed.

III.A.3C Detection of EMT Cancer Cells: The μ NMR system is able to address one of the significant challenges to ovarian cancer CTC detection: the epithelial-to-mesenchymal (EMT) transition of cancers cells [12]. During this transition, CTCs may express little or no EpCAM. We tested the influence of EpCAM expression on the performance of quad- μ NMR by comparing EpCAM-only versus quad-marker labeling in 12 different cell lines with variable levels of EpCAM expressions. μ NMR signal was highest for quad- μ NMR across all cell lines irrespective of the EpCAM expression levels (Figure X). The application of quad- μ NMR is thus especially helpful for detecting cancer cells with variable EpCAM expression levels.

III.A.4C Minimally Invasive Monitoring in Ovarian Cancer: Quad- μ NMR's broader dynamic range of CTC enumeration correlated well with clinical metrics. In a pilot study of 15 patients with advanced serous ovarian cancer, quad- μ NMR detected higher average CTC counts in patients with more advanced disease (stages III, IV), platinum resistant and progressive disease, and in patients not pursuing active therapy [12]. Compared with EpCAM-based CellSearch, quad- μ NMR also reported higher CTC counts in all patient samples. Studies are under way to explore the utility of quad- μ NMR for minimally invasive monitoring of response or early recurrence.

III.B Micro-Hall Detection Technology

We have also developed a miniaturized magnetic cytometer, the micro-Hall detector (μ HD), that can rapidly and quantitatively screen individual cells in unprocessed clinical specimens (Fig 4) [13]. The system detects the magnetic moments of cells 'in flow' that have been immune-labeled with MNPs.

By targeting cells with MNPs, using the BOND method detailed above, and subjecting them to an external magnetic field B_0 , each cell acquired a magnetic moment m , directly proportional to both the number of biomarkers N and the magnetic moment of the MNPs m_p ($m = N \times m_p$). The microfabricated Hall sensors measure the magnetic moments of each MNP-labeled via the Hall voltage (Fig 4). Cells with non-specifically bound MNPs can be accurately excluded by gating the measured signals above a particular threshold value.

II.B.1. Clinical Applications

III.B.1A. Molecular Profiling: The μ HD system can be used to detect different cellular markers by using a panel of MNPs, which are distinguishable by their magnetization properties. The quantity of each MNP type, and hence the expression level of a target biomarker in a single cell, could be obtained using the particles' distinctive magnetization properties. In a model study on human breast carcinoma cell line MDA-MB-453, the profiling results of the μ HD correlated with those by fluorescence flow cytometry ($R^2 > 96\%$) [13].

III.B.1B More Rapid and Accurate CTC Detection: We applied the μ HD to the detection of rare CTCs in whole blood samples spiked with different amounts of MDA-MB-468 breast cancer cells. The μ HD results showed excellent agreement with expected cell numbers ($R^2 > 99.9\%$) as well as a large dynamic range (10^1 to 10^5 cells). At high cell numbers ($>10^3$ cancer cells), flow cytometry errors were caused by false negatives (81% for flow cytometry and 10% for μ HD with 10^6 spiked cells). At low cell numbers ($<10^3$ cancer cells), flow cytometry reported false positives (900% with 20 spiked cells) predominantly because of competing autofluorescence signals from surrounding leukocytes, a problem that is not encountered through the magnetic detection of CTCs with the μ HD. The μ HD can rapidly detect target cells ($\sim 10^7$ cells/minute with the current device and potentially up to 10^9 cells/minute) and does so among a vast number of background cells ($\sim 10^6$ white blood cells and $\sim 10^9$ red blood cells).

III.B.1C Minimally Invasive Monitoring in Ovarian Cancer: The μ HD has shown higher sensitivity and diagnostic accuracy than CellSearch system. In a study of 20 ovarian cancer patients with advanced disease, CellSearch detected CTCs in only 5 of 20 ovarian cancer cases with a diagnostic accuracy of 25% [13]. The μ HD enumerated a higher number of CTCs across all patient samples ($P < 0.001$). In contrast to CellSearch, the μ HD successfully identified CTCs in 100% of patients with evidence of clinical progression as well as stage IV disease, where only 18% of cases were detected with CellSearch.

IV Ovarian Cancer Exosomes

Exosomes are membrane-bound phospholipid nanovesicles (50–150 nm in diameter) actively secreted by cancer cells from multivesicular bodies. Exosomes have emerged as promising novel biomarkers for clinical diagnostics since they are present abundantly in easily accessible bodily fluids (e.g. 10^7 - 10^{11} vesicles/ml of peripheral blood), are remarkably stable over time [14, 15] and carry a variety of biomolecules (e.g. extracellular and intracellular proteins, DNAs and RNAs) representative of their originating cells [16–18]. Despite the clinical potential of exosomes, detecting and molecularly profiling exosomes remains a challenging task. Conventional analytical methods such as Western blotting, enzyme-linked immunosorbent assay (ELISA) and mass spectrometry often require large amounts of sample, time-consuming purification and post-labeling processes, during which exosomes can be lost or degraded [19–21]. These limitations constrain in-depth study of exosome composition and their roles in disease progression and therapy response, and are

often impractical in clinical settings where serial analyses of large patient cohorts are crucial.

IV.A Nano-plasmonic Exosome Detection Platform

Surface plasmon resonance (SPR) is an analytical technique that has been widely used to characterize binding interaction between molecules in a label-free manner. The technique is based on strong electromagnetic fields, called surface plasmons, excited on a metal surface. When molecules bind to the sensing surface, they change the local refractive index near the surface, resulting in a shift of resonance condition. Because molecular binding itself induces the shift, it can omit post-labeling process for detection, rendering the process ‘label-free’.

Recently, we developed a novel SPR sensing platform, termed **nPLEX** (nano plasmonic exosome), for label-free detection and molecular profiling of exosome with high sensitivity and throughput (Fig 5) [22]. The system comprised multiple arrays of periodic nanoholes (200 nm diameter) patterned in a 200 nm-thick Au film on a glass substrate. Compared to conventional SPR systems [23, 24], nPLEX is uniquely suited for exosome analysis, because the sensing range is tuned to overlap with the exosome size for improved sensitivity. In addition, nPLEX operates in a ‘transmission mode’ rather than in a ‘total internal reflection mode’ commonly used in conventional SPR platforms [23]. The transmission configuration enables the use of 1) dense arrays for high-throughput measurement and 2) simple, miniaturized optical setup for signal readout. Through the integration with multi-channel microfluidics, the system allowed for parallel and multiplexed measurements up to 12 markers on small sample volumes (< 10 µl).

IV.A.1 Preclinical testing of nPLEX—In a proof-of-concept study, the nPLEX platform was used to detect and molecularly profile exosomes derived from various ovarian cancer cell lines. In nPLEX, target-specific binding of exosomes on the nanohole array changed its local refractive index, detected by monitoring either spectral shifts or intensity changes at a fixed wavelength. Such changes were proportional to the number of bound exosomes and correlated with overall abundance of cancer antigen (i.e. quantitative molecular profiling). The expression levels of a target protein marker were determined by scaling the protein-associated change to that of CD63, which represent the concentration of exosomes. Such normalization accounted for differences in exosome counts among samples and reported an average expression level of a target marker per exosome. The nPLEX assay showed excellent agreement with conventional ELISA, but the nPLEX demonstrated much higher sensitivity — about 10,000-fold more sensitive than Western blotting and 100-fold more sensitive than conventional chemiluminescence ELISA [22]. Moreover, the label-free detection scheme in nPLEX enabled fast assay (< 60 min). Importantly, the *in vitro* screening with nPLEX demonstrated that exosomes indeed reflect the molecular profiles of their originating ovarian cancer cells. In addition, it was identified that EpCAM, CD24 or both were highly expressed in most of ovarian cancer cell lines with the potential for reliable detection of ovarian cancer exosomes in clinical specimens.

IV.A.2 Clinical testing of nPLEX in human specimens—The nPLEX was subsequently applied to detect cancer exosomes within patient-derived ascites. Exosomes

were collected by passing specimen through a membrane filter with 0.2 μm size cut-off, rather than by ultracentrifugation. nPLEX analyses of 30 patient samples (20 with ovarian cancer and 10 with ascites from noncancerous causes), demonstrated that cancer-derived exosomes can be identified through elevated expression of EpCAM and CD24. Based on the expression of EpCAM and CD24, the nPLEX assay identified cancer cases with diagnostic accuracy of 97% [22]. Our approach also revealed the potential use of exosomal protein profiles as indicators of treatment response: the levels of exosomal EpCAM, CD24 or both decreased among responding patients, whereas levels of these markers increased in non-responding patients.

These results supported the clinical potential of exosomes as surrogates for primary cancer cells, and have motivated the rigorous and comprehensive testing of exosomes as relevant diagnostic and prognostic biomarkers. With its capacity for sensitive, label-free molecular detection, the nPLEX could be a promising technology for comprehensive exosomal molecular analyses, for exploring exosomes as a cancer biomarker, for diagnostics and for evaluating tumor response to therapy. To improve the throughput of nPLEX and accelerate its clinical application, a second generation chip with more than 1,000 sensing sites is under development for high-throughput parallel measurements.

V Conclusion

The promise of nanotechnologies lies in their ability to query limited amounts of biospecimens without sacrificing sensitivity or specificity. This is integral to strategies that seek serial profiling to better understand biomarkers and their changes during treatment or tumor progression. We have developed various point-of-care platforms to interrogate clinical specimens derived from ovarian cancers; we also demonstrate that nanotechnology-based assays are superior to conventional methods in proof-of-concept studies. These accomplishments prompt us to conduct larger clinical studies with an emphasis on mechanistic readouts of response to targeted therapies and understanding the metastatic process in ovarian cancer.

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Fig. 1. A schematic of the ATC microchip, fabricated to enrich for ATC based on magnetic and size separation. Benign cells (Calret+ and CD45+) are initially labeled with magnetic particles and captured at the inlet of the device. The second separation is done based on size of the cells where the relatively larger ATC are captured in the capture sites ranging from 15–40 μm and the smaller leukocytes pass through the device.

Fig. 2. μ NMR platform for clinical applications. (a) The system features automatic system tuning and user-friendly interface (e.g., smartphone). (b) To improve the throughput, multiple detection coils can be integrated into a single NMR probe. (c) Bioorthogonal labeling scheme, based on the click reaction between transcyclooctene (TCO) and tetrazine (Tz), has been developed. The method provides maximize MNP loading on target cells, thereby improving the overall detection sensitivity.

Fig. 3. Translational development of μ NMR platform. The feedback between preclinical and clinical research accelerated the translation of the μ NMR platform for sensitive protein detection in human diseases.

Fig. 4. Micro-Hall detector (μ HD) for single cell detection. (a) Each cell is labeled with target-specific MNPs, and its magnetic fields are detected by the μ Hall sensor. The measured Hall voltage (V_H) is linearly proportional to the MNP counts. B_0 , external magnetic field. (b) A photo of a packaged μ HD. The footprint of the sensor is $\sim 1.5 \times 2 \text{ cm}^2$.

Fig. 5.

Nano-plasmonic exosome (nPLEX) sensor. (a) An electron micrograph of periodic nanoholes in the nPLEX sensor. The nanohole structure was patterned in a gold film (200 nm thick) deposited on a glass substrate. The inset shows a zoomed-in image (b) The SPR sensor chip was integrated with a multi-channel microfluidic cell for parallel analyses. (c) Representative spectral shifts of transmission spectra induced by specific binding of exosome to the nanohole surface. (d) An electron micrograph of exosomes captured on the nanohole surface.