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Selective isolation and noninvasive analysis of circulating cancer stem cells through Raman imaging

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ABSTRACT

Circulating cancer stem cells (CCSCs), a rare circulating tumor cell (CTC) type, recently arose as a useful resource for monitoring and characterizing both cancers and their metastatic derivatives. However, due to the scarcity of CCSCs among hematologic cells in the blood and the complexity of the phenotype confirmation process, CCSC research can be extremely challenging. Hence, we report a nanoparticle-mediated Raman imaging method for CCSC characterization which profiles CCSCs based on their surface marker expression phenotypes. We have developed an integrated combinatorial Raman-Active Nanoprobe (RAN) system combined with a microfluidic chip to successfully process complete blood samples. CCSCs and CTCs were detected (90% efficiency) and classified in accordance with their respective surface marker expression *via* completely distinct Raman signals of RANs. Selectively isolated CCSCs (93% accuracy) were employed for both *in vitro* and *in vivo* tumor phenotyping to identify the tumorigenicity of the CCSCs. We utilized our new method to predict metastasis by screening blood samples from xenograft models, showing that upon CCSC detection, all subjects exhibited liver metastasis. Having highly efficient detection and noninvasive isolation capabilities, we have demonstrated that our RAN-based Raman imaging method will be valuable for predicting cancer metastasis and relapse *via* CCSC detection. Moreover, the exclusion of peak overlapping in CCSC analysis with our Raman imaging method will allow to expand the RAN families for various cancer types, therefore, increasing therapeutic efficacy by providing detailed molecular features of tumor subtypes.

1. Introduction

Cancer stem cells (CSCs) refer to a small subset of tumor cells that have the unique ability to self-renew and differentiate. Their self-renewal process typically drives tumorigenesis and their differentiation process causes tumor heterogeneity (Jordan et al., 2006; Malanchi et al., 2012). CSCs can be identified by biomarkers showing stem-like characteristics, such as CD133 and CD44, which are responsible for stemness and pleiotropic roles in cell adhesion, migration, and homing (Mizrak et al., 2008; Zöller, 2011). Even though CSCs account for a small fraction of tumor cell population (~1%), they are known to have a critical role in cancer metastasis and relapse owing to their distinctive abilities including self-renewal, differentiation, and chemoresistance (Medema, 2013; Melo et al., 2017; Moncharmont et al., 2012; Pathania et al., 2016; Visvader and Lindeman, 2008). For example, in the metastasis process, metastatic cancer cells, including CSCs, migrate along vasculature in the lymph nodes to initiate tumor growth at secondary organ sites by extravasation (Maheswaran and Haber, 2010; Riethdorf et al., 2008). While it is known that most circulating tumor cells (CTCs) obtain a migratory cell fate and lose epithelial properties through the process of epithelial to mesenchymal transition (EMT), the metastatic process is enormously complex and highly dynamic (Maheswaran and

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Haber, 2010), thereby the detailed mechanisms underlying CTC migration have not been well studied. Scientists have reported that only a small subset of CTCs, categorized and termed circulating cancer stem cells (CCSCs), are involved in successful cancer metastasis, exhibiting high invasiveness (Cristofanilli et al., 2004; Kennecke et al., 2010; Malanchi et al., 2012). Given these extraordinary characteristics, combined with the important functions of CCSCs have in cancer metastasis, a highly selective detection and analysis method for the isolation of CCSCs from a heterogeneous CTC population is essential for advancing cancer therapeutics and will providing new insights into cancer metastasis and relapse at the single cell levels (Nadal et al., 2013).

Due to the scarcity of CCSCs and CTCs among heterogeneous blood cells, as few as one cell per 10^9 hematologic cells in the blood of patients, simultaneous detection, isolation, and analysis of CCSCs and CTCs in a highly selective, sensitive, and non-invasive manner is extremely challenging (Cristofanilli et al., 2004; Lawson et al., 2015; Nagrath et al., 2007). The fluorescence-activated cell sorting (FACS) method has been utilized for CCSC isolation and detection by comparing a CSC marker (CD133 or CD44) with white blood cell (WBC) marker (CD45) expression (Al-Hajj et al., 2003; Ginestier et al., 2007; Kantara et al., 2015; Lawson et al., 2015; Liu et al., 2014). However, it is limited in validating primary cancer phenotypic characters considering that CSC markers are expressed not only on CSCs but also on normal stem cells (Pattabiraman and Weinberg, 2014). Therefore, it is necessary to combine the CCSC and CTC detection for improving accuracy in CCSC research. For example, detecting and analyzing breast CCSCs and CTCs, which have four subtypes including luminal, human epidermal growth factor receptor 2 (HER2) positive, and two triple-negative types (Basal-A and Basal-B), requires the use of a combination of at least five antibodies (four antibodies for subtyping, epithelial cell adhesion molecule (EpCAM), HER2, human epidermal growth factor receptor 1 (EGFR), and mucin 1 (MUC1), and one antibody for stemness confirmation (CD133)) (Carey et al., 2007; Eirew et al., 2008; Kao et al., 2009; Kennecke et al., 2010; Neve et al., 2006; Perou et al., 2000) (Table 1). Therefore, simultaneous detection and analysis of various subtypes of CCSCs and CTCs, showing primary cancer's heterogeneity *via* a multi-probe-based platform, requires an innovative method for efficient multiplex detection of subpopulations of heterogeneous cancer cells and the analysis of a wide range of complex analytes.

To this end, several CTC detection chips using immune-affinity-based separation methods with multiple fluorescent probes have demonstrated limited success for CCSC detection. (Lee et al., 2013a, 2013b; Yoon et al., 2013). However, given the limitations of the fluorescent probe systems, it is difficult to analyze CTC subtypes with high isolation efficiency from blood, particularly for CCSCs with significantly different quantities which require an increase in the number of probes. This is an especially critical challenge in multicolor analysis, where it is impossible to discriminate between fluorophores that spectrally overlap (Lichtman and Conchello, 2005; Zhang et al., 2016). Therefore, there remains significant room for improvement in achieving highly sensitive and specific cell detection methods that can display broad multiplexing capabilities with high reproducibility.

Raman imaging with surface-enhanced Raman spectroscopy (SERS) has significant advantages when compared to fluorescence imaging, including spectral information that shows a larger number of characteristic peaks, and crucially, distinct non-overlapping peaks. (Papadopoulou and Bell, 2011; Sabatte et al., 2008; Ye et al., 2017; Zhai et al., 2012; Zheng et al., 2012). Particularly, SERS has shown signals with low background noise in biological samples including those drawn from blood by avoiding autofluorescent signals (Premasiri et al., 2012; Wang et al., 2011). These comprehensive advantages render SERS as highly competitive in meeting the needs of multiplex quantification of molecules in living cells. However, to the best of our knowledge, a Raman imaging-based detection and analysis method of CCSC has not yet been reported. To detect and analyze CCSCs, three requirements must be satisfied for CTC research: a high detection yield of target cells in the blood, non-invasive isolation of captured cells, and the simultaneous detection of surface markers with different expression levels.

To address the aforementioned challenges for the detection and downstream analysis of CCSCs and CTCs, we report a novel combinatorial Raman-Active Nanoprobe (RAN)-based chip platform with the capability of simultaneous detection, isolation, and further analysis of CCSCs and various CTC subtypes though Raman imaging (Fig. 1). The RAN is a multifunctional probe designed with four tunable components including i) a Raman reporter as a barcoding component, ii) an antibody as a cancer cell detecting component, iii) a biotinylated double-stranded DNA (dsDNA) as a non-invasive isolating component, and iv) a gold nanoparticle as a Raman signal-enhancing component. In this demonstration, individually conjugated RANs are employed to detect both CCSCs and several major breast cancer CTC subtypes using five different surface markers: CD133, EpCAM, EGFR, HER2, and MUC1 (Fig. 1**a**). The CCSCs and CTCs are detected and isolated in an effective, selective, and noninvasive manner on the microfluidic chip *via* avidin-biotin reactions followed by restriction enzyme digestion of the dsDNA linker from the RANs (Fig. 1**b**). We have shown that selectively isolated CCSCs and CTCs successfully exhibit tumorigenicity and secondary tumor subtypes in both *in vitro and in vivo* model systems.

2. Materials and methods

2.1. Raman-active nanoprobe synthesis and characterization

In this work, we prepared five different combinations of RANs. Each type of RAN was conjugated with modification of a previously reported method by Qian et al. (2008). Fig. S1a shows the illustrations of the step-by-step conjugation process of the AuNP/Raman reporter/PEG/antibody/DNA conjugate. Briefly, RANs were prepared by adding a freshly prepared 1–5 μM Raman reporter solution dropwise to a rapidly mixing gold colloid at a 1:6 reporter solution and 60 nm AuNP colloid (BBI solutions EM.GC60, Cardiff, UK) volume ratio. Considering that each Raman reporter shows different signal enhancement effects with AuNPs, the concentration of reporters was determined from the signal to noise (S/N) ratio of the representative peak for each RAN. Concentrations of different Raman reporters (Thiophenol (TP, Sigma-Aldrich 240249), Nile Blue A (NBA, Sigma-Aldrich N0766), 1-naphthalenethiol (NPT, Sigma-Aldrich 724742), 4-mercaptopyridine (MPD, Sigma-Aldrich 148202), 2-quinolinethiol (QNT, Sigma-Aldrich

Table 1
Classification of breast cancer based on pathological features. Depends on the surface marker expression profiles, the subtype of breast cancer was categorized.

Subtype	Surface marker expression profile					Reference
	CD133	HER2	EGFR	EpCAM	MUC1	
Cancer Stem Cells	+					(Jordan et al., 2006; Medema, 2013; Visvader and Lindeman, 2008)
Luminal type	–			+		(Carey et al., 2007; Kao et al., 2009; Neve et al., 2006)
HER2 positive type		+			–	(Carey et al., 2007; Kao et al., 2009; Neve et al., 2006)
Basal-A type		–	+	–	+	(Carey et al., 2007; Kao et al., 2009; Neve et al., 2006)
Basal-B type	–	–	+	–	–	(Carey et al., 2007; Kao et al., 2009; Neve et al., 2006)

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