Research Article

Association of serum prostate-specific antigen (PSA) level and circulating tumor cell-based PSA mRNA in prostate cancer

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ABSTRACT

Background: Prostate-specific antigen (PSA) is used for diagnosing prostate cancer, but does not reflect the characteristics of prostate cancer cells to allow assessment of cancer progression. PSA mRNA and circulating tumor cells (CTCs) could be potential biomarkers. However, the relationship between serum PSA levels and PSA mRNA in CTCs is unclear, and this study aimed to investigate this relationship.

Methods: Healthy donors (HD, n = 9), and patients with local non-metastatic stage prostate cancer (n = 30), metastatic hormone-sensitive prostate cancer (mHSPC, n = 10), and metastatic castration-resistant prostate cancer (mCRPC, n = 75), were included. The expression of PSA mRNA in CTCs was measured by droplet digital PCR. Serum PSA (ng/mL) levels and PSA mRNA (copies/μL) in CTCs were then compared using Spearman correlation coefficients.

Results: PSA mRNA expression in CTCs was observed in 30% (9/30) of patients with localized cancer, 60.0% (6/10) among patients with mHSPC, 65.3% (49/75) among patients with mCRPC, and 0% among patients with HD, indicating that the detection rate of PSA mRNA increased with cancer stage. PSA mRNA expression in CTCs also increased from localized to metastatic stages. PSA mRNA levels rapidly increased in the mHSPC and mCRPC stages. Interestingly, PSA mRNA expression in CTCs was not correlated with serum PSA levels at the localized stage (R = 0.064, P = 0.512). However, there were significant correlations between serum PSA levels and PSA mRNA expression in mHSPC (R = 0.532, P = 0.041) and mCRPC (R = 0.566, P = 0.025). The number of CTCs isolated from mHSPC and mCRPC was not proportional to serum PSA and PSA mRNA levels.

Conclusion: CTC PSA mRNA has the potential to be used as a biomarker to complement serum PSA protein analysis or replace serum PSA in metastatic stages of prostate cancer.

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1. Introduction

Prostate-specific antigen (PSA) is an essential blood test for diagnosing prostate cancer. However, it does not accurately evaluate the biological characteristics of prostate cancer cells, and there is a need to develop better biological markers to assess the progression of prostate cancer.1–5 In particular, as the treatment for metastatic PCa is diversified, there is increased interest in the development of biomarkers to predict treatment responsiveness.

With the development of technology-capable of detecting circulating tumor cells (CTCs) shed from primary tumors, many efforts to utilize CTCs to determine patients’ tumor burden and cancer progression are being actively pursued. Previous studies have shown that the number of CTCs detected in the blood of patients is closely related to survival in patients with prostate cancer.6–10

Since the discovery that androgen receptor (AR)-targeting agents are closely related to the AR splice variant expressed on CTCs...
in advanced prostate cancer, CTC-based genomic analysis has been highlighted.\textsuperscript{31–33} However, genomic analysis of CTCs remains challenging despite the development of novel techniques such as next-generation sequencing and droplet digital PCR (ddPCR) because of the presence of trace amounts of CTCs in blood. Recently, technology capable of separating CTCs more effectively has been developed and can be used for identifying biomarkers in PCa.\textsuperscript{13–15}

Previous work has shown that many CTC based molecules can be used as a marker to predict the treatment response or prognosis of patients with PCa.\textsuperscript{11–14} Among them, PSA mRNA is a very unique molecule. Since PSA mRNA is produced in epithelial cells of the prostate, it cannot be detected in the blood of men without PCa. Thus, detection of PSA mRNA in blood indicates the presence of prostate-derived cells.

Several studies have previously demonstrated the existence of CTCs in the presence of PSA transcripts in blood.\textsuperscript{16–18} Since PSA mRNA was detected in micrometastatic prostate cancer, the possibility of PSA mRNA to be used as a predictive marker for prostate cancer recurrence has been raised.\textsuperscript{19–20}

Although the role of PSA mRNA has not been identified in localized PCa, PSA mRNA has been reported as an important factor influencing survival outcomes in patients with metastatic PCa, including metastatic castration-refractory prostate cancer (mCRPC).\textsuperscript{21–23} It has also been suggested as a biomarker for the therapeutic responses of abiraterone or enzalutamide.\textsuperscript{24,25} Presently, it is unclear whether blood PSA mRNA levels are related to serum PSA levels.

Although CTCs are being researched as complementary biomarkers for clinical and molecular analysis of cancer, the relationship between serum PSA levels and PSA mRNA in CTCs has not been clearly studied. Herein, we evaluated the expression of PSA using mRNA from isolated CTCs. Ultimately, by investigating the relationship between CTC-based PSA mRNA and serum PSA levels, we assessed whether PSA mRNA could be used as a diagnostic or therapeutic marker for PCa.

2. Material and methods

2.1. Patients and sample preparation

Peripheral blood samples (5 mL) were collected from nine healthy donors (HD) and 115 prostate cancer patients (30 with localized cancer, 10 with mHSPC, and 75 with mCRPC) from May 2018 to July 2021 (Table 1). Consent was obtained from all patients who participated in the study. Our institutional review board approved the study protocol. Blood samples were collected in Vacutainer tubes (367525, BD Vacutainer) containing EDTA and stored at 4°C. Anti-pan-cytokeratin Alexa Fluor 488 antibodies (eBioscience, Waltham, MA, USA) at 4°C–8°C. To separate the CTCs, the prepared sample was injected into the sample inlet of the microchannels, and PBS with 0.2% bovine serum albumin was flushed through the buffer inlet.

Owing to the magnetic field formed around the microchannel, the CTCs labeled with magnetic beads exited the CTC outlet along the ferromagnetic wires. On the other hand, non-CTCs did not attach to the beads and were not collected (Fig. 1). 2.2. Working principle and CTC isolation

We used a previously published experimental technique to separate CTCs from blood using a disposable lateral magnetophoretic microseparator.\textsuperscript{3,14,26} The microseparator consisted of reusable substrates with embedded ferromagnetic wires and disposable superstrates equipped with microchannel networks (Fig. 1). A high-gradient magnetic field was formed around the microchannel when a constant external lateral magnetic field was applied to the reusable substrate mounted on permanent magnets.

To separate the CTCs, the prepared sample was injected into the sample inlet of the microchannels, and PBS with 0.2% bovine serum albumin was flushed through the buffer inlet.

First, the CTCs and white blood cells (WBCs) were fixed in 100 μL of 4% paraformaldehyde for 10 min. Nuclei were identified using a nucleic acid fluorescent dye (DAPI, Vector Laboratories, Burlingame, CA, USA) for fixed cell incubation. Anti-CD45 Alexa Fluor 647 antibodies (BioLegend, San Diego, CA, USA) were used to identify WBCs. To identify CTCs, fixed cells were additionally incubated with anti-pan-cytokeratin Alexa Fluor 488 antibodies (eBioscience, Waltham, MA, USA) at 4°C for 30 min.

2.3. CTC enumeration

Before injecting the sample into the magnetophoretic microseparator, the sample was diluted with 800 μL of ice-cold PBS with 0.2% bovine serum albumin.

2.4. PSA gene expression analysis using RT-ddPCR

PSA gene expression levels were analyzed by multiplex PCR pre-amplification and ddPCR after CTC lysis. Table S1 shows the pre-amplification primer sets for PSA.

Before measuring PSA gene expression using ddPCR, the pre-amplified cDNA template was diluted at a ratio of 1:10. The PSA gene detection thresholds were determined based on the maximum value measured for no-template controls as 0.12 copies/μL.

Table 1

| Localized stage | Metastatic stage |
|----------------|------------------|
| pT2-4 (N = 30) | mHSPC (N = 10) | mCRPC (N = 75) |
| Age, median, IQR, years | 71 (57–81) | 75 (64–80) | 75 (57–84) |
| PSA, median, IQR, ng/mL | 14.5 (6.5–37.8) | 125.2 (81.2–157.2) | 76.3 (29.1–162.9) |
| Gleason Score (%) | | | |
| 6 | 10 (33.3) | 1 (10.0) | 3 (4.0) |
| 7 | 9 (30.0) | 3 (30.0) | 17 (22.7) |
| ≥ 8 | 11 (36.7) | 6 (60.0) | 55 (73.3) |

mCRPC, metastatic castration-resistant prostate cancer; mHSPC, metastatic hormone-sensitive prostate cancer; N, number of patients; PSA, prostate-specific antigen.
2.5. Statistical analysis

The correlation between serum PSA and CTC-based PSA mRNA levels was analyzed using correlation coefficients. Statistical analyses were performed using R version 3.6.2.

3. Results

3.1. CTC enumeration

Fig. 2A shows the morphological forms of CTCs and WBCs following immunofluorescence staining (DAPI, CD45, pan-cytokeratin). The number of CTCs and serum PSA levels increased with the progression of prostate cancer stages. An average of 0.3 CTCs/mL and 702.3 WBCs/mL were isolated from blood of HD. In localized PCa patients, an average of 5.3 CTCs/mL and 572.3 WBCs/mL were isolated. In patients with mHSPC and mCRPC 12.3 CTCs/mL and 816.5 WBCs/mL and 710.2 WBCs/mL, respectively, were measured (Fig. 2B). Compared to localized PCa, 3–6 times more CTCs were isolated from the blood of patients with mHSPC and mCRPC. The ratio of cytokeratin-positive cells to CD45-positive cells, that is, CTCs/WBCs, was defined as the CTC purity value. With progression of PCa stage, the CTC count increased, which resulted in increased CTC purity in advanced PCa (localized PCa: 2.2%, mHSPC: 4.1%, and mCRPC: 7.1%).

3.2. PSA mRNA analysis using isolated CTCs

The PSA gene expression level was evaluated as copies per microliter, and its detection rates according to tumor stage were obtained by calculating the number of patients whose gene expression level was above the threshold for PSA mRNA. The threshold copy number was evaluated using no-template testing, which revealed values of 0.12 copies/μL for PSA. The expression of PSA mRNA in CTCs was observed in 30.0% of localized cancer, 60.0% of mHSPC, 65.3% of mCRPC patients, and 0% of HD patients. This indicated that the detection rate of PSA mRNA increased with the stage of prostate cancer (Fig. 3A). The expression level of PSA mRNA in CTCs also increased from localized to metastatic stages. PSA gene expression levels were significantly increased in mHSPC and mCRPC patients compared to those with localized PCa. Interestingly, serum PSA levels increased seven times as the tumor progressed from localized to metastatic cancer, while PSA mRNA levels increased significantly, approximately 20,000 times (Fig. 3B).

3.3. Correlation of PSA mRNA and serum PSA level

In nine of the blood samples at the localized stage, PSA mRNA expression in CTCs was detected and was not related to serum PSA levels. In the mHSPC and mCRPC stages, the expression levels of PSA mRNA in CTCs were detected in 6 and 49 samples, respectively, and they were significantly related to the levels of PSA protein in serum. Serum PSA levels and CTC-based PSA mRNA concentrations were linearly plotted. Only PSA gene detected samples were plotted in dark green, blue, and purple and used for Spearman correlation coefficients calculations (Fig. 4). Light green, blue, and purple dots are data from patients whose PSA mRNA was not detected or measured below the cut-off value. Their data were not used to calculate Spearman correlation coefficients.

In the localized stage, PSA gene and serum PSA levels were not correlated with low-risk level abundance ($R = 0.064, P = 0.512$) (Fig. 4A). There was a linear correlation between serum PSA levels and CTC-based PSA mRNA concentrations in mHSPC ($R = 0.532, P = 0.041$) and mCRPC ($R = 0.566, P = 0.025$) (Fig. 4B and C). Although PSA mRNA concentration varied, it tended to be proportional to the serum PSA level. The number of CTCs isolated from
Fig. 2. (A) Confocal microscopy of the CTCs and WBCs isolated from blood samples using the lateral magnetophoretic microseparator. CTCs were stained positive for pan-cytokeratin (green) and negative for CD45 (red), whereas WBCs were positive for CD45 (red) and negative for pan-cytokeratin (green). (B) Serum PSA levels and the number of isolated CTCs and WBCs per mL of blood according to the tumor stage.
mHSPC and mCRPC was not precisely proportional to the serum PSA level and PSA mRNA concentration (data not shown).

4. Discussion

Serum PSA is the most commonly used blood test for diagnosing prostate cancer or evaluating treatment response. However, it does not accurately represent the molecular state of the tumor cells or their biological characteristics. Serum PSA is often measured as high in men without PCa or low in advanced PCa. Many other biological markers have been studied to compensate for this and PSA mRNA has been proposed as a novel marker. PSA is produced in epithelial cells of the prostate. The detection of PSA-synthesizing cells in the blood suggests that the cells originated from prostate tissue or in the case of patients with prostate cancer, indicates the occurrence of micrometastasis or dissemination of tumor cells. The clinical use of plasma PSA transcripts in PCa has already been investigated and it was found that highly detectable PSA mRNA expression levels in preoperative samples might indicate a potential risk for the recurrence of PCa. It was also suggested that reverse transcriptase-PCR (RT-PCR)-measured PSA can be used as an effective predictor of PSA progression in men with mCRPC treated with chemotherapy. Ross et al. reported that patients who were RT-PCR PSA positive at baseline were 2.22 times more likely to progress than those who were negative for RT-PCR PSA at baseline. Although a positive RT-PCR for PSA is of unclear significance in localized PCa, RT-PCR PSA is a significant and independent prognostic factor for survival in mCRPC.

As a liquid biopsy, CTC analysis is a non-invasive method and a very useful tool for revealing the molecular properties of inaccessibible tumor cells with cancer heterogeneity. This suggests that the assessment of CTCs may provide a clue for solving the complications caused by the heterogeneity of cancer cells. The present study was conducted to clarify whether serum PSA levels were correlated with CTC-derived PSA mRNA levels in PCa.
In our study, there was no proportional relationship between PSA mRNA concentration and serum PSA level in patients with localized PCa. We cannot rule out the possibility that much smaller numbers of detected CTCs in localized PCa may have affected this result. However, in metastatic PCa, including mCRPC, PSA mRNA concentrations and serum PSA levels showed a significant proportional relationship, demonstrating the possibility that CTC-based PSA mRNA could be used as a new biomarker in advanced PCa, especially metastatic PCa.

Previously, Chung et al. demonstrated that CTC-based detection of PSA transcripts predicted the response of AR-targeting agents, such as abiraterone and enzalutamide, in patients with mCRPC.24 In line with this, Qu et al. showed that the quantity of PSA transcripts predicted the time to treatment failure and overall survival in mCRPC patients treated with abiraterone acetate and/or enzalutamide.32 These findings suggest that PSA mRNA levels could be used as an effective marker for predicting survival outcomes or drug resistance. However, most previous studies were conducted under the assumption that cancer-specific transcripts are derived from CTCs. Tumor-specific transcripts were analyzed, omitting the process of separating CTCs from the blood. Very few studies have quantitatively measured tumor-specific transcripts after isolating cancer cells from blood and confirmed that the cells were CTCs.

This study has several unique features that have not been addressed in previous studies. First, studies comparing serum PSA and PSA mRNA concentrations are scarce, and this is the first study to compare the concentrations of the two molecules according to CTCs. Tumor-specific transcripts were analyzed, omitting the process of separating CTCs from the blood. Very few studies have quantitatively measured tumor-specific transcripts after isolating cancer cells from blood and confirmed that the cells were CTCs.

Our study had certain limitations. First, although CTCs were isolated from the patients’ blood, proper isolation of mesenchymal or stem cell-like CTCs could not be guaranteed due to the limitations of the device used for isolation. Clinically relevant CTCs that have undergone EMT may have been overlooked because our study was designed to identify CTCs based only on epithelial expression. Therefore, there is a cell-type selection bias in which only specific types of CTCs were separated. Second, the number of patients with localized PCa was relatively small, and this may lead to no clear relationship between CTC-based PSA and serum PSA levels in localized PCa. We could have drawn on the clinical utility more precisely if a greater number of samples were collected. However, we found that the current approach could be applied for the identification of genetic biomarkers for prognosis.

5. Conclusions

Our study demonstrated that CTC-based PSA mRNA was correlated with serum PSA levels in metastatic PCa but not in localized PCa. CTC-based PSA mRNA could be used as a new biomarker along with serum PSA protein in the metastatic stages of PCa. This finding suggests that high-quality CTCs can be used as a complementary tool to realize precision medicine in advanced PCa.

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Conflicts of interest

All the authors declare no potential conflict of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.prnl.2022.01.002.

References

1. Broncy L, Paterlini-Brechot P. Clinical impact of circulating tumor cells in patients with localized prostate cancer. Cells 2019;8:676.

2. Nuhn P, De Bonos JS, Fizzi K, Freendland SJ, Grilli M, Kantoff PW, et al. Update on systemic prostate cancer therapies: management of metastatic castration-resistant prostate cancer in the era of precision oncology. Eur Urol 2019;75:88–99.

3. Chung JS, Morgan TM, Hong SK. Clinical implications of genomic evaluations for prostate cancer risk stratification, screening, and treatment: a narrative review. Prostate Int 2020;8:99–106.

4. Nagaya N, Rosenfield J, Lee JT, Kim YJ. RNA-seq profile of African American men with a clinically localized prostate cancer. Prostate Int 2021;9:125–31.

5. Kwan TN, Spremo S, Teh AYM, McHarg D, Thangasamy I, Woo HHT. Performance of Ga-68 PSMA PET/CT for diagnosis and grading of local prostate cancer. Prostate Int 2021;9:107–12.

6. Pantel K, Hille C, Scher HI. Circulating tumor cells in prostate cancer: from discovery to clinical utility. Clin Chem 2019;65:87–99.

7. Miyamoto DT, Sequist LV, Lee RJ. Circulating tumour cells—monitoring treatment response in prostate cancer. Nat Rev Clin Oncol 2014;11:401–12.

8. Danila DC, Fleisher M, Scher HI. Circulating tumor cells as biomarkers in prostate cancer. Clin Cancer Res 2011;17:3903–12.

9. De Bonos JS, Scher HI, Mangel GM, RB, Poon WW, Miller MC, Tissier H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008;14:6302–9.

10. Danila DC, Heller G, Gignac GA, Gonzalez-Espinoza R, Anand A, Tanaka E, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. Clin Cancer Res 2007;13:7053–8.

11. Antonarakis ES, Lu C, Wang H, Luber B, Nakazawa M, Koezer JC, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N Engl J Med 2014;371:1028–38.

12. Scher HI, Lu D, Schreiber NA, Louw J, Graf RP, Vargas HA, et al. Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. JAMA Oncol 2016;2:1441–9.

13. Cho H, Chung JS, Kim JI, Lee SI, Lee CH, Morgan TM, et al. Multigene model for predicting metastatic prostate cancer using circulating tumor cells by microfluidic magnetophoresis. Cancer Sci 2021;112:859–70.

14. Cho H, Chung JS, Han KH. A Direct Comparison between the Lateral Magneto-ophoretic Microseparator and AdnaTest for Isolating Prostate Circulating Tumor Cells. Micromachines (Basel) 2020;11:6870.

15. Ohayashi K, Akatsuka J, Endo Y, Takeda H, Hayashi T, Tomyama Y, et al. Initial detection of circulating tumor cells from metastatic prostate cancer patients with a novel small device. Prostate Int 2019;7:1311–8.

16. de la Taille A, Olsson CA, Buttyan R, Benson MC, Bagiella E, Cao Y, et al. Detection of circulating prostate carcinoma cells via an enhanced reverse transcriptase-polymerase chain reaction assay in patients with early stage prostate cancer. Independent from other pretreatment characteristics. Cancer 1997;79:2402–8.

17. Katz AE, deVries GM, Begg MD, Raffo AJ, Cama C, O’Toole K, et al. Enhanced reverse transcriptase-polymerase chain reaction for prostate specific antigen as an indicator of true pathologic stage in patients with prostate cancer. Cancer 1995;75:1642–8.
19. Mejean A, Vona G, Nalpas B, Damotte D, Brousse N, Chretien Y, et al. Detection of circulating prostate derived cells in patients with prostate adenocarcinoma is an independent risk factor for tumor recurrence. J Urol 2000;163:2022–9.

20. Seiden MV, Kantoff PW, Krithivas K, Propert K, Bryant M, Halton M, et al. Detection of circulating tumor cells in men with localized prostate cancer. J Clin Oncol 1994;12:2034–9.

21. Kantoff PW, Halabi S, Farmer DA, Hayes DF, Vogelzang NA, Small EJ. Prognostic significance of reverse transcriptase-polymerase chain reaction for prostate-specific antigen inmen with hormone-refractory prostate cancer. J Clin Oncol 2001;19:3025–8.

22. Halabi S, Small EJ, Hayes DF, Vogelzang NJ, Kantoff PW. Prognostic significance of reverse transcriptase-polymerase chain reaction for prostate-specific antigen in metastatic prostate cancer: a nested study within CALGB 9583. J Clin Oncol 2003;21:490–5.

23. Ghossein RA, Rosai J, Scher HI, Seiden M, Zhang ZF, Sun M, et al. Prognostic significance of detection of prostate-specific antigen transcripts in the peripheral blood of patients with metastatic androgen-independent prostate carcinoma. Urology 1997;50:100–5.

24. Chung JS, Wang Y, Henderson J, Singhal U, Qiao Y, Zaslavsky AB, et al. Circulating Tumor Cell-Based Molecular Classifier for Predicting Resistance to Abiraterone and Enzalutamide in Metastatic Castration-Resistant Prostate Cancer. Neoplasia 2019;21:802–9.

25. Qu F, Xie W, Nakabayashi M, Zhang H, Jeong SH, Wang X, et al. Association of AR-V7 and Prostate-Specific Antigen RNA Levels in Blood with Efficacy of Abiraterone Acetate and Enzalutamide Treatment in Men with Prostate Cancer. Clin Cancer Res 2017;23:726–34.

26. Cho H, Kim I, Jeon C-W, Han KH. A disposable microfluidic device with a reusable magnetophoretic functional substrate for isolation of circulating tumor cells. Lab Chip 2017;17:4113–23.

27. Kalfazade N, Kuskucu AM, Karadag S, Sahin S, Aras B, Midilli K, et al. Quantification of PSA mRNA levels in peripheral blood of patients with localized prostate adenocarcinoma before, during, and after radical prostatectomy by quantitative real-time PCR (qRT-PCR). Int Urol Nephrol 2009;41:273–9.

28. Ross RW, Manola J, Hennessy K, Galsky M, Scher H, Small E, et al. Prognostic significance of baseline reverse transcriptase-PCR for prostate-specific antigen in men with hormone-refractory prostate cancer treated with chemotherapy. Clin Cancer Res 2005 Jul 15;11(14):5195–8.

29. Matsusaka S, Suenaga M, Mishima Y, Kuniyoshi R, Takagi K, Terui Y, et al. Circulating tumor cells as a surrogate marker for determining response to chemotherapy in Japanese patients with metastatic colorectal cancer. Cancer Sci 2011;102:1188–92.

30. Matsusaka S, Chin K, Ogura M, Suenaga M, Shinozaki E, Mishima Y, et al. Circulating tumor cells as a surrogate marker for determining response to chemotherapy in patients with advanced gastric cancer. Cancer Sci 2010;101:1067–71.

31. Mishima Y, Matsusaka S, Chin K, Mikuniya M, Minowa S, Takayama T, et al. Detection of HER2 amplification in circulating tumor cells of HER2-negative gastric cancer patients. Target Oncol 2017;12:341–51.

32. Liu Y, Liu Q, Wang T, Bian L, Zhang S, Hu H, et al. Circulating tumor cells in HER2-positive metastatic breast cancer patients: a valuable prognostic and predictive biomarker. BMC Cancer 2013;13:202.

33. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 2011;83:8604–10.

34. McDermott GP, Do D, Litterst CM, Maar D, Hindson CM, Steenblock ER, et al. Multiplexed target detection using DNA-binding dye chemistry in droplet digital PCR. Anal Chem 2013;85:16169–27.