Centrosome amplification is a frequent event in circulating tumor cells from subjects with metastatic breast cancer

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Centrosome amplification (CA) is a common phenomenon in cancer, promotes genomic stability and cancer evolution, and has been reported to promote metastasis. CA promotes a stochastic gain/loss of chromosomes during cell division, known as chromosomal instability (CIN). However, it is unclear whether CA is present in circulating tumor cells (CTCs), the seeds for metastasis. Here, we surveyed CA in CTCs from human subjects with metastatic breast cancer. CTCs were captured by CD45 exclusion and selection of EpCAM-positive cells using an exclusion-based sample preparation technology platform known as VERSA (versatile exclusion-based rare sample analysis). Centriole amplification (centrin foci > 4) is the definitive assay for CA. However, determination of centrin foci is technically challenging and incompatible with automated analysis. To test if the more technically accessible centrosome marker pericentrin could serve as a surrogate for centriole amplification in CTCs, cells were stained with pericentrin and centrin antibodies to evaluate CA. This assay was first validated using breast cancer cell lines and a nontransformed epithelial cell line model of inducible CA, then translated to CTCs. Pericentrin area and pericentrin area x intensity correlate well with centrin foci, validating pericentrin as a surrogate marker of CA. CA is found in CTCs from 75% of subjects, with variability in the percentage and extent of CA in individual circulating cells in a given subject, similar to the variability previously seen in primary tumors and cell lines. In summary, we created, validated, and implemented a novel method to assess CA in CTCs from subjects with metastatic breast cancer. Such an assay will be useful for longitudinal monitoring of CA in cancer patients and in prospective clinical trials for assessing the impact of CA on response to therapy.

Abbreviations
CA, centrosome amplification; CTC, circulating tumor cell; PBMC, peripheral blood mononuclear cell; PLK4, polo-like kinase 4; PMP, paramagnetic particles; VERSA, versatile exclusion-based rare sample analysis.
1. Introduction

Despite major advances in understanding the genetic, molecular, and cellular underpinnings of human cancer, metastatic disease remains incurable and accounts for the majority of cancer deaths. Genomic instability is a hallmark of human cancers (Gordon et al., 2012) and, when it occurs at the level of whole chromosomes, yields an alteration of cellular chromosomal content. One-time errors in chromosome segregation during cell division result in aneuploidy, an abnormal chromosomal content in daughter cells. When errors in chromosome segregation persist in multiple divisions, aneuploidy varies between cells and over time, a phenomenon known as chromosomal instability (CIN). CIN is a common feature of cancer, which led to the hypothesis that it can promote tumorigenesis. However, it has been found that aneuploidy can promote tumors, suppress tumors, or do neither, depending on the rate of CIN (Denu et al., 2016; Funk et al., 2016; Silk et al., 2013; Weaver et al., 2007; Zasadil et al., 2016). Whereas low rates of CIN can weakly promote tumor growth, high rates cause cell death and suppress tumors (de Cárcer et al., 2018; Funk et al., 2016; Janssen et al., 2009; Maia et al., 2015; Rowald et al., 2016). Preliminary evidence suggests that CIN may confer enhanced sensitivity to taxanes, widely used chemotherapy agents that interfere with microtubule dynamics (Janssen et al., 2009; Maia et al., 2015; Zasadil et al., 2014). Although the mechanistic underpinnings of CIN are incompletely known, centrosome amplification (CA) is a common and readily detectable cause of CIN (Denu et al., 2016; Ganem et al., 2009; Silkworth et al., 2009).

The centrosome is the primary microtubule-organizing center of the cell and helps control cellular polarity, migration, and division (Meres and Cleveland, 1997). The potential mechanisms leading to CA include cell doubling (e.g., cytokinesis failure and cell-cell fusion) or over duplication of centrioles (Denu et al., 2018; Godinho et al., 2009; Holland et al., 2010). CA occurs in multiple cancer types (Chan, 2011), including breast (Lingle et al., 1998), where CA is associated with higher tumor stage, worse outcomes, and invasive features (Denu et al., 2016). Further, CA increases cellular invasiveness (Godinho et al., 2014), suggesting that tumors with CA could have greater metastatic potential; however, this has not been demonstrated in human subjects.

Circulating tumor cells (CTCs) are a rare population of tumor cells released into peripheral circulation from primary and metastatic tumor sites that may both contribute to the development of metastatic disease and reflect the heterogeneity that likely exists between various tumor deposits (Mundy, 2002). Further, the number of CTCs in peripheral blood has been shown to have prognostic information in both early and advanced disease, (Bidard et al., 2014; Janni et al., 2016) and these cells are thought to be at least in part responsible for metastasis and resistance to chemotherapy (de Bono et al., 2008; Pukazhendhi and Glück, 2014). Using CTCs as a biomarker affords the advantage of capturing cells that are biologically relevant to the metastatic process.

To date, we can find no reports of CA in CTCs. In the present study, we developed and validated an automated method to assess CA in CTCs from patients with metastatic breast cancer. This method may serve as a minimally invasive tool to measure CA, which occurs in CTCs from a substantial fraction of subjects with metastatic breast cancer. Importantly, this assay for CA may serve as a predictive biomarker in the future for therapeutic response to agents whose efficacy is affected by CA or CIN.

2. Materials and Methods

2.1. Cell culture

CAL-51 cells were obtained from DSMZ, and MCF7 and MDA-MB-231 cells were obtained from ATCC. Cell lines were validated by short tandem repeat (STR) analysis in 2015 with the University of Wisconsin Translational Initiatives in Pathology (TRIP) laboratory. Cell lines were grown in DMEM with 10% fetal bovine serum FBS and 1% pen/strep solution at 37°C and 5% CO2. Doxycycline-inducible RPE1 cells were obtained from Dr. David Pellman (Harvard) and validated by assessing centrioles before/after addition of doxycycline (Godinho et al., 2014). These cells were grown in DMEM:F12 media with 10% FBS and 1% pen/strep solution. To induce PLK4 overexpression and CA, RPE1 cells were treated with doxycycline (10 µg·mL⁻¹) for 48 h. Mycoplasma testing was performed on all cell lines using R&D Systems Mycoprobe Mycoplasma Detection Kit with help from the University of Wisconsin Small Molecule Screening and Synthesis Facility.

2.2. Collection and processing of subject blood samples

The Institutional Review Board (IRB) at the University of Wisconsin–Madison approved this study (IRB...
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MCF7s were identified and allowed to dry. Cells were then fixed with 100% ice-cold methanol for 10 min at –20 °C and stained with cytokeratin-790 and primary antibodies against pericentrin overnight in a humidified chamber at 4 °C. The following antibodies were used: DSBX-labeled anti-EpCAM/TROP1 (R&D systems, AF960); pan-cytokeratin (Abcam, Cambridge, UK, ab7753); Alexa Fluor 647-conjugated anti-CD11b (BioLegend, San Diego, CA, USA, 101218); Alexa Fluor 647-conjugated anti-CD14 (BioLegend, San Diego, CA, USA, 101218); Alexa Fluor 647-conjugated anti-CD34 (BioLegend, 343618); anti-CD45 (Abcam, ab4448, 1:1000); and Alexa Fluor 647-conjugated anti-CD34 (BioLegend, 343618). Background subtraction was performed for each channel using rolling ball diameter of 10 Nikon NIS-Elements version 4.51.01, and spot detection module was utilized to identify and detect the nuclei (Hoechst). Binary editor was used to review and edit the cell identification binary layer. A permanent binary layer was developed for CTC analysis. Cytokeratin+/Hoechst+/CD45− cells were identified as CTCs. Pericentrin area and intensity were assessed by manually drawing a binary layer representing pericentrin foci on identified cells. The area and intensity were assessed for the encircled area of pericentrin for all CTCs and peripheral blood mononuclear cells (PBMCs) using NIS-Elements.

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cold methanol for 15 min. Fixed cells were blocked for 30 min in 3% BSA and 0.1% Triton X-100 in PBS (PBSTx + BSA). Primary antibodies were incubated in PBSTx + BSA for 1 h at RT and washed three times in PBSTx, followed by secondary antibody incubation in PBSTx + BSA for 30 min at RT and two washes with PBSTx. Cells were counterstained with DAPI and mounted on glass slides with Prolong Gold antifade medium (Invitrogen). Cells were stained for centrin (Millipore, 04-1624), pan-cytokeratin (Abcam, ab7753), and exclusion markers (see above). Pictures were taken using the NIS-ELEMENTS AR MICROSCOPE IMAGING Software (Nikon, Melville, NY, USA) in Nikon Eclipse Ti-E with ORCA-Flash 4.0 Digital CMOS camera (Hamamatsu) florescent microscope with 10-20X objectives in all the channels. Centrin foci were manually counted under 100X magnification and scored in CTCs and PBMCs.

### 2.6. Statistical analysis

Data were analyzed using GraphPad Prism software (version 6). Standard t-tests were performed to

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**Fig. 1.** Centrosome size is the best predictor of centriole number in breast cancer cell lines. (A) Representative immunofluorescence images of CAL-51 and MDA-MB-231 breast cancer cell lines. Scale bars = 10 µm. The smaller images on the right of the panel are enlargements of the centrosome. (B) Quantification of centrin foci. (C) Quantification of pericentrin area. (D) Quantification of pericentrin intensity. (E) A composite variable was created using the product of pericentrin area and intensity. (F–H) Correlation of centrin foci with pericentrin area (F), pericentrin intensity (G), or pericentrin area x intensity (H). (I) This table summarizes the correlation coefficients and statistical significance of these correlations. Throughout the figure, each dot represents one cell and bars represent mean ± SD.
compare CA in CTCs versus PBMCs. Differences with P-values ≤ 0.05 are considered significant.

3. Results

3.1. Characteristics of pericentrin reliably estimate centriole amplification in breast cancer cell lines

The gold standard for assessing CA is by centriole enumeration using centrin staining (Denu et al., 2018); however, this is technically challenging and cumbersome, particularly on patient samples, because it requires manual identification of CTCs and enumeration of centrioles. To develop a high-throughput, objective, and repeatable method to assess CA, we correlated centrioles (manual enumeration of centrin foci) with more readily attainable centrosome parameters, namely pericentrin size and intensity, as these have been previously shown to correlate with CA (Lingle and Salisbury, 1999). The CAL-51 and MDA-MB-231 cell lines were used as models of low and high CIN, respectively. We performed both manual counting of centrin foci (gold standard) and automated analysis of pericentrin features. As expected, MDA-MB-231 cells exhibited a significantly greater degree of CA than CAL-51 cells (Fig. 1A,B). MDA-MB-231 cells also showed increases

Fig. 2. Centrosome size is the best predictor of centriole number in PLK4-overexpression model of CA. (A) Representative immunofluorescence images of RPE1 cells with doxycycline-inducible PLK4. The smaller images on the right of the panel are enlargements of the centrosome. Scale bars = 5 μm. (B) Quantification of centrin foci. (C) Quantification of pericentrin area. (D) Quantification of pericentrin intensity. (E) A composite variable was created using the product of pericentrin area and intensity. (F–H) Correlation of centrin foci with pericentrin area (F), pericentrin intensity (G), or pericentrin area x intensity (H). (I) This table summarizes the strength and statistical significance of these correlations. Throughout the figure, each dot represents one cell and bars represent mean ± SD.

| Centriole correlation with: | $R^2$ | $P$ value |
|----------------------------|-------|-----------|
| Pericentrin Area           | 0.22  | < 0.01    |
| Pericentrin Intensity      | 0.04  | < 0.01    |
| Pericentrin Area x Intensity| 0.15 | < 0.01    |
in pericentrin intensity, area, and area x intensity (Fig. 1C–E). All three measures of pericentrin were significantly correlated with centriole number (Fig. 1F–I), with pericentrin area showing the best correlation in this paired cell analysis. We conclude that high-throughput assessment of pericentrin correlates well with centriole number.

3.2. Reliable detection of CA in VERSA-captured cells

To further validate our assay for assessing CA in CTCs, we used a doxycycline-inducible PLK4 RPE1 cell line as an *in vitro* model system (Fig. 2A). In these cells, PLK4 overexpression induces CA (Godinho *et al.*, 2014). We induced PLK4 overexpression with doxycycline treatment and analyzed pericentrin intensity, pericentrin area, and centrin foci in fixed cells. In this RPE1 cell model, doxycycline-inducible overexpression of PLK4 significantly increases centrin foci, pericentrin intensity, pericentrin area, and the composite variable (product of pericentrin area and intensity) (Fig. 2B–E). Similarly to breast cancer cell lines, centriole number correlates with all three measurements of pericentrin (Fig. 2F–I), with pericentrin area showing the best correlation.

3.3. VERSA reliably detects CA in inducible PLK4 RPE1 model

We previously described the VERSA microfluidic platform, which reliably captures circulating tumor cells (CTCs) from blood in subjects with metastatic cancer.

![Image of VERSA platform](image)

Fig. 3. Assessment of CA using the VERSA platform. (A) Doxycycline-inducible PLK4 RPE1 cells were run through the VERSA platform and analyzed. Representative images of pericentrin and centrin staining and imaging are shown. Scale bars = 10 μm. The smaller images on the right of the panel are enlargements of the centrosome. (B–D) Correlation of centrin foci with pericentrin area (B), intensity (C), and area x intensity (D). (E) This table summarizes the strength and statistical significance of these correlations. Throughout the figure, each dot represents one cell and bars represent mean ± SD.
CD45, CD14, CD34). EpCAM, Hoechst, cytokeratin, and exclusion markers (CD11b, CD45, CD14, CD34). EpCAM+ cells were then stained with additional antibodies. In CTCs, pericentrin staining was done in the VERSA using half the cells, whereas the remaining half of cells were cytopspun and stained on coverslips to assess centrin. CTCs were defined as positive for cytokeratin and negative for the exclusion markers. To verify that CA could be reliably detected using this platform before proceeding with analyzing human subjects samples, we induced PLK4 overexpression with doxycycline treatment, then captured cells by CD45 exclusion and EpCAM selection using the VERSA platform, and stained for pericentrin and centrin (Fig. 3A). While there remains a significant positive correlation between all pericentrin measurements with the number of centrin foci (Fig. 3B–E), after processing through the VERSA, pericentrin area x intensity and pericentrin area show the strongest correlations with centrin foci ($R^2 = 0.59$ and 0.57, respectively).

We then piloted our method with a small number of subjects to ensure that we could capture CTCs and detect pericentrin (Fig. S1). We also established objective numerical cutoffs for expression of pan-cytokeratins and exclusion channels that would define CTCs versus PBMCs (Fig. S1). Furthermore, we performed the same assay with blood from three healthy individuals and were unable to detect CTCs.

To assess the sensitivity of our assay, we spiked MCF7 breast cancer cells into blood from healthy donors. The VERSA method was able to capture approximately 95% of MCF7 cells, and after device fixation, permeabilization, staining, and washing, we identified approximately 30% of MCF7 cells (Fig. S2).

### 3.4. Centrosome amplification is a frequent event in metastatic breast cancer CTCs

To measure CA in breast cancer, we collected blood samples from 12 female subjects with metastatic breast cancer. Subjects characteristics, such as age, time since diagnosis, tumor features (e.g., subtype, stage, grade), and lines of therapy are summarized in Table 1. The VERSA platform was used to capture EpCAM-positive metastatic breast cancer CTCs and assess centrin and pericentrin using the workflow shown in Fig. 4A. Matched PBMCs served as a control. Manually scored centrin foci were used as a direct measure of CA (Fig. 4B–C, Fig. S3). The number of centrin foci per CTC ranged from 0 to 16, with 9 of 11 samples showing evidence of CA (centrin foci> 4; Fig. S3). The percentage of CTCs with CA varied from 0% to 50% with an average of 28.8% (Fig. 5A). PBMCs are not expected to exhibit CA, and the greatest percentage of PBMCs with> 4 centrin foci was 21% (subject 8). Therefore, samples in which> 21% of cells had> 4 centrin foci were considered to exhibit CA. Based on this threshold, CA was observed in subjects 1, 2, 3, 4, 5, 6, 8, 10, and 12 (Fig. 5A).

Because CA has been reported to promote invasive features (Denu et al., 2016; Godinho et al., 2014), we hypothesized that the degree of CA would correlate with the absolute number of CTCs detected for each subject. However, a strong correlation between CA and number of CTCs was not identified (Fig. S4).

In many subjects, CTCs showed significantly greater pericentrin area (Fig. 5B), pericentrin intensity (Fig. 5C), and pericentrin area x intensity (Fig. 5D) compared to their matched PBMCs, consistent with CA. While both pericentrin area and pericentrin area x intensity positively correlated with CA, pericentrin area x intensity correlated particularly well with

**Table 1. Characteristics of breast cancer subjects**

| Subject characteristics | N = 12 |
|-------------------------|-------|
| Age, median (range), years | 62 (51–75) |
| Time since diagnosis (range), years | 8 (3–18) |
| Primary disease | | |
| Histology type, % (N) | | |
| IDC | 75 (9) |
| ILC | 25 (3) |
| Tumor stage, % (N) | | |
| n/a | 8 (1) |
| I | 8 (1) |
| II | 42 (5) |
| III | 17 (2) |
| IV | 25 (3) |
| Tumor grade, % (N) | | |
| n/a | 8 (1) |
| 1 | 8 (1) |
| 2 | 42 (5) |
| 3 | 42 (5) |
| Tumor size, median (range), cm | 2 (1.1–11) |
| Lymph node status positive, % (N) | 58 (7) |
| ER positive, % (N) | 83 (10) |
| PR positive, % (N) | 67 (8) |
| HER-2 positive, % (N) | 25 (3) |
| Metastatic disease | | |
| Metastatic sites, % (N) | | |
| Bone | 67 (8) |
| Liver | 33 (4) |
| Brain | 8 (1) |
| Lung | 42 (5) |
| Other | 42 (5) |
| Lines of therapy, median (range) | 7 (2–9) |
Fig. 4. Method for assessing CA in CTCs. (A) Schematic diagram of the VERSA platform. Briefly, the blood samples were processed with Ficoll gradient to isolate all the nucleated cells. Nucleated cells were further depleted of CD45-positive cells using a magnetic column and transferred to a VERSA microfluidic device for EpCAM capture. Samples were then divided in half for pericentrin staining in VERSA or centrin staining after cells are cytospun onto coverslips. For pericentrin quantification in CTCs, the entire sample was imaged, and CTCs were detected automatically using preset thresholds for the expression of cytokeratin and exclusion channel (CD45/CD34/CD11b/CD14). For quantification of centrin foci, the other half of the sample was cytospun onto coverslips, fixed in methanol, and stained. CTCs were manually identified, and centrioles were manually counted. (B,C) Representative images of pericentrin (B) and centrin (C) staining in CTCs and PBMCs isolated from subjects with metastatic breast cancer. Scale bars = 10 μm.
number of centrin foci ($R^2 = 0.81$, Fig. 5E–G). In fact, pericentrin area \times intensity was able to predict an increase in centrin foci accurately in 100% (11/11) of subjects for which more than one CTC could be scored; this excludes subject 7, for which only a single CTC was evaluable. We conclude that automated imaging of pericentrin centrin area \times intensity is a valid surrogate measure of CA in metastatic breast cancer.

4. Discussion

CA has been observed in wide variety of human cancers (Denu et al., 2016; Giehl et al., 2005; Kramer et al., 2005; Lingle et al., 1998; Pihan et al., 1998) and can promote CIN and aneuploidy (Ganem et al., 2009; Silkworth et al., 2009) and metastasis (Godinho et al., 2014). In the present report, we directly measure CA...
in CTCs from subjects with metastatic breast cancer and find that it is strikingly common, occurring in 75% of subjects with metastatic breast cancer and up to 50% of CTCs per subject. To the best of our knowledge, this is the first report demonstrating CA in cancer CTCs.

Though number of centrin foci is the gold standard measure of CA, measurements of centrin are technically challenging under optimal experimental conditions and were incompatible with automated analysis of CTCs, making centrin foci impractical as a clinical assay. However, pericentrin staining is robust. We therefore validated pericentrin as a surrogate marker of the number of centrin foci and optimized pericentrin staining in our VERSA-based, automated analysis. We find that pericentrin area \( \times \) intensity accurately predicts centriole amplification in 100% of subjects for which greater than one CTC was evaluable. As this high-throughput assay can readily analyze patient samples, this permits widespread testing of CA.

As CA is known to promote invasive features (Denu et al., 2016; Godinho et al., 2014), we hypothesized that the degree of CA would correlate with the absolute number of CTCs detected for each subject. Furthermore, in a study where SUIT-2 human pancreatic cancer cells were xenografted into nude mice, CA was more prevalent in metastatic foci than the original xenograft (Shono et al., 2001). However, we did not observe a strong correlation between CA and the number of CTCs. One potential explanation is the small number of subjects in our study, which may have limited the power to detect such a correlation. A second explanation is that many other factors may contribute more to invasiveness than CA alone. Alternatively, CA may be more prevalent in the primary tumor from which the CTCs are derived, so it may not be expected that the CTCs themselves would have more CA. Evidence for this explanation comes from a previous study showing that CA promotes tumor cell invasiveness in a nonautonomous fashion (Ganier et al., 2018). A final possibility is that CA is not as strong at promoting metastatic dissemination as predicted by preclinical models.

One limitation of this study is the small number of recruited subjects, requiring validation in a larger sample size. However, this study provides proof of principle that CA is detectable in CTCs, and pericentrin area \( \times \) intensity can be used as an automated, quantitative biomarker in future studies.

In the future, our reported assay may be useful as a predictive biomarker. For example, since CIN may predict response to taxane chemotherapy (Funk et al., 2016; Lee et al., 2013; Rajagopalan and Lengauer, 2004; Tanaka and Hirota, 2016; Weaver, 2014), measuring CA in CTCs may be useful in predicting taxane sensitivity in patients with metastatic breast cancer.

5. Conclusion

In conclusion, we report the presence of frequent CA events in EpCAM-captured CTCs from subjects with metastatic breast cancer. This assay will be useful for longitudinal monitoring of CA in cancer patients and in prospective clinical trials for assessing therapeutic response to agents whose efficacy is affected by CA or CIN.

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Conflict of interest

JM Lang has ownership interest (including patents) in Salus Discovery. Other authors disclosed no potential conflicts of interest.

Author contributions

JML, MEB, and BAW conceived the project and secured funding. AS and RAD conceived the project and acquired the data. SKW, JMS, JS, and TW acquired the data. In addition, all authors interpreted the data and contributed to the manuscript drafting, revision, and approval.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Capture of EpCAM-positive CTCs from subjects with metastatic breast cancer.

**Figure S2.** MCF7 spike-in to quantify the assay sensitivity.

**Figure S3.** Quantification of centrin foci in CTCs.

**Figure S4.** CA does not correlate well with absolute number of CTCs captured.