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Dual-targeted nucleic acid probe detection system for circulating tumor cells to enhance cancer diagnosis

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ABSTRACT: Circulating tumor cell (CTC)-based diagnostics represent a vital strategy for reducing cancer mortality; however, current detection methods face significant limitations including complex blood composition, the scarcity of CTCs in the bloodstream, and inconsistent expression of surface biomarkers, thereby restricting their clinical utility. Here, we present a dual-targeted nucleic acid probe detection system (DPDS) designed for the simultaneous recognition of EpCAM and EGFR surface biomarkers on CTCs. Systematic evaluation revealed that this DPDS exhibits concentration-dependent fluorescence enhancement, achieving a detection limit of 5–10 cells/ml in standard samples. When applied to blood samples from A549, MCF-7, and MDA-MB-231 tumor-bearing mice (tumor volume ∼900 mm^3), the DPDS demonstrated significantly improved diagnostic accuracy over single-target assays. Receiver operating characteristic (ROC) analysis yielded area under the curve (AUC) values of 0.820 (EpCAM-Cy5) and 0.829 (EGFR-FAM) for individual channels, compared to a markedly superior AUC of 0.981 for the DPDS. This study develops an innovative dual-marker liquid biopsy platform that overcomes key challenges in conventional CTC detection, including low abundance and phenotypic heterogeneity. The platform demonstrates robust performance in identifying rare CTCs (5–10 cells/ml) in peripheral blood while eliminating false negatives associated with single-marker approaches, ultimately establishing a clinically viable solution for sensitive and specific cancer diagnosis.

KEYWORDS: cancer detection, circulating tumor cells, dual-targeted nucleic acid probe, liquid biopsy

INTRODUCTION

Cancer diagnosis constitutes a pivotal challenge in contemporary medical practice, directly impacting patient survival outcomes [1–3]. Serving as the primary defense against this lethal disease, timely detection plays a decisive role in therapeutic efficacy. Early-stage diagnosis elevates the five-year survival rate to over 95% in melanoma [4]. Circulating tumor cells (CTCs) act as seeds disseminated by the primary tumor, while distant organs with a permissive microenvironment serve as the soil enabling metastatic colonization [5]. Studies have shown that CTCs may enter the patient's peripheral blood circulation early in the course of tumor development and persist throughout the entire cycle of tumor proliferation, metastasis, and dissemination [6, 7]. CTC detection has significant clinical implications for early tumor diagnosis, efficacy evaluation, prognosis assessment, recurrence and metastasis monitoring, medication guidance, and drug resistance monitoring [8, 9]. Their presence strongly correlates with metastatic potential, as CTCs evade immune surveillance, survive shear stress in circulation, and colonize distant organs to form secondary tumors [10]. CTCs harbor surface-specific proteins that serve as critical biomarkers for cancer diagnosis and monitoring [8]. Among these, epithelial cell adhesion molecule (EpCAM) and epidermal growth factor receptor (EGFR) exhibit significant clinical utility due to their roles in tumor biology and metastatic progression [11].

While CTCs have significant clinical potential as liquid biopsy biomarkers, their detection faces critical limitations. CTCs are exceptionally rare in peripheral blood, necessitating ultra-sensitive capture platforms. Furthermore, surface-specific proteins such as EpCAM and EGFR exhibit substantial heterogeneity due to tumor mutation or epithelial-mesenchymal transition (EMT), leading to false-negative outcomes in single-target detection systems [12–15].

These advancements address the dual challenges of low abundance and biomarker variability, ultimately enhancing diagnostic reliability for metastatic risk assessment. In this study, we developed a dual-targeted nucleic acid probe detection system (DPDS) for cancer detection. This system enables the identification of various cancer cell types, thereby overcoming the limitations of single-target detection (Fig. 1a,b). Moreover, it is capable of detecting trace amounts of tumor cells within blood sample, offering a novel approach for cancer diagnosis.

MATERIALS AND METHODS

Cell culture

Human pulmonary adenocarcinoma cell line A549 cells, human breast cancer cell line MCF-7 Cells, MDA-MB-231 cells and RAW 264.7 cells were cultured in

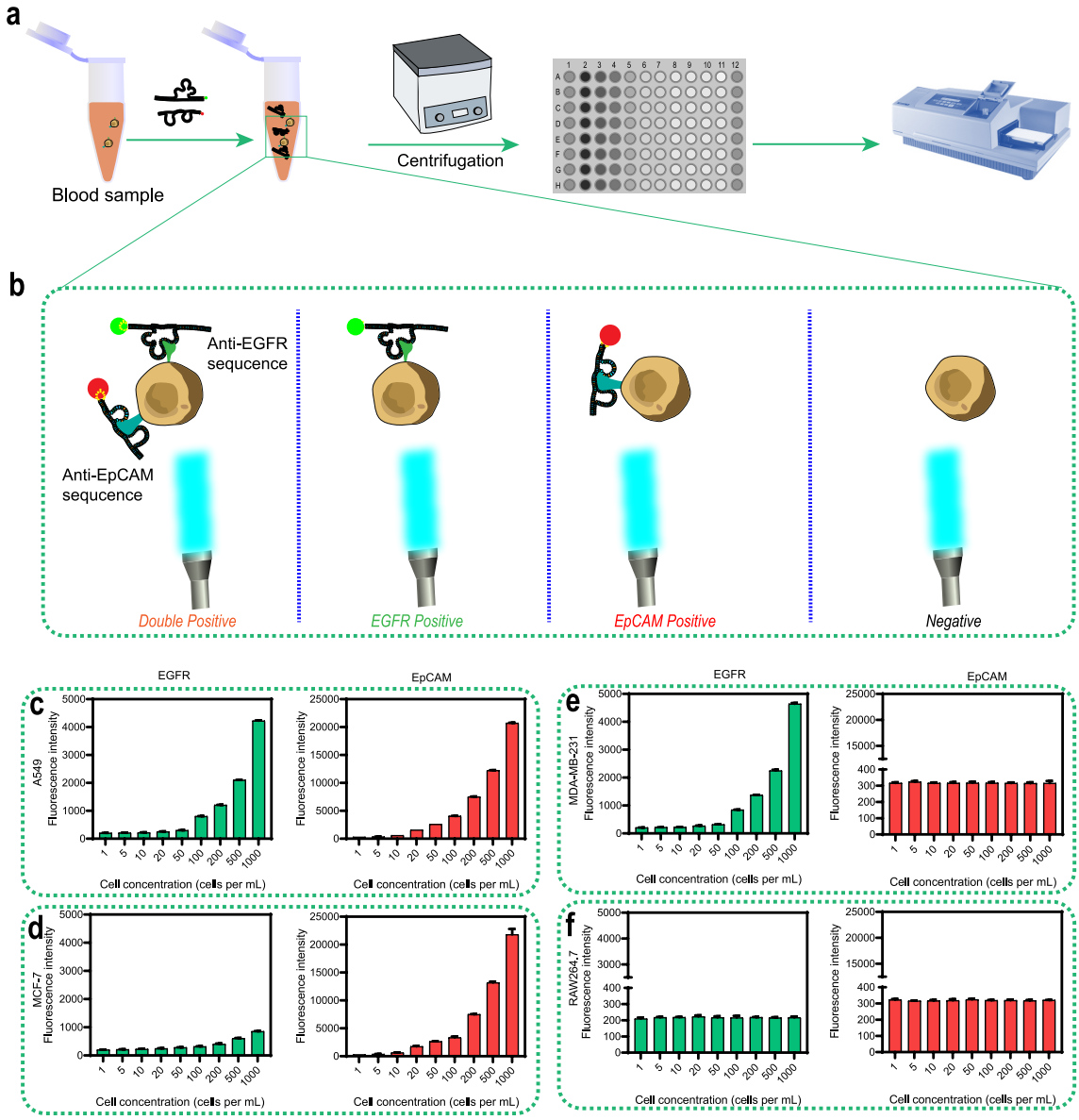

Fig. 1 Detection of samples using dual nucleic acid probes. (a) Schematic illustration of the treatment of CTCs detection via dual-targeting nucleic acid probes. (b) Schematic diagram of the possible results of the detection. (c–f) Detection of cells at various concentrations using dual nucleic acid probes: A549 cells (c), MCF-7 cells (d), MDA-MB-231 cells (e), RAW 264.7 cells (f).

DMEM medium. All media contained 10% FBS and 1% penicillin-streptomycin. All the cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Nucleic acid probes

The FAM-conjugated and Cy5-conjugated DNA Nucleic acid probes used in this study were synthesized by Sangon Biotech, Shanghai, China. The sequences of the probes used are as follows: Anti-EGFR probe: FAM-5′-TAC CAG TGC GAT GCT CAG TGC CGT TTC TTC TCT TTC GCT TTT TTT GCT TTT GAG CAT GCT GAC GCA TTC GGT TGA C-3′ and anti-EpCAM probe: Cy5-5′-CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG-3′ [16, 17].

Detection in standard samples

To evaluate the tumor cell-targeting capability of DPDS, serial dilutions of tumor cells (1, 5, 10, 20, 50, 100, 200, 500, 1000 cells/ml) were prepared and incubated with the probes (final concentration = 2.5 μM) at 4 °C for 1 h. The mixtures were then centrifuged at 200×*g* for 10 min, followed by two washes with

PBS to remove unbound probes. The final pellets were resuspended and analyzed using a microplate reader to quantify probe-cell binding efficiency. The expression of EGFR and EpCAM on A549, MCF-7, MDA-MB-231, and RAW 264.7 cells were determined with flow cytometry. In brief, A549, MCF-7, MDA-MB-231, and RAW 264.7 cells were incubated with anti-EGFR (Aladdin, Chicago, USA, Ab215365) or anti-EpCAM (eBioscience, California, USA, 11-5791-82) for 1 h, then the cells were centrifuged at 1,500 rpm for 10 min. The cells were washed with PBS for 3 times and measured with flow cytometry.

In vitro detection of aptamers to tumor cells

To evaluate the tumor cell recognition capability of the nucleic acid probes, A549, MCF-7, MDA-MB-231, and RAW 264.7 cells were seeded in confocal dishes (Biosharp, China, BS-20-GJM, 35 mm) at a density of 2×10^5 cells per well. After 24 h of incubation to allow cell attachment, the cells were co-incubated with DPDS (each at 2.5 μM) for 1 h. The nuclei were then stained with Hoechst 33342 (3 μg/ml). Subsequently, the cells were washed 3 times with PBS and observed under a confocal microscope (Leica, SP8, Germany).

In vitro detection in blood samples

To validate the ability of DPDS to identify CTCs in blood, A549, MCF-7, MDA-MB-231, and RAW 264.7 cells were spiked into mouse blood samples at a concentration of 5 or 10 cells/ml. To remove erythrocytes from whole blood samples, a hypotonic lysis buffer (10 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) was applied. Briefly, blood samples were mixed with the lysis buffer at a 1:5 (v/v) ratio and incubated on ice for 15 min to lyse RBCs while preserving nucleated cell integrity. The mixture was then centrifuged at 300×*g* for 10 min at 4 °C. The supernatant containing lysed RBCs was carefully aspirated, and the remaining cell pellet was washed twice with ice-cold PBS, pH 7.2). After the final wash, the pellet was resuspended in 500 μl PBS. The samples were incubated with DPDS at a final concentration of 2.5 μM. The mixtures were incubated at 4 °C for 1 h. Subsequently, the samples were centrifuged at 200×*g* for 10 min, followed by 2 washes with PBS to remove unbound probes. The final pellets were resuspended and analyzed using a microplate reader to quantify probe-cell binding efficiency.

Liquid biopsy for cancer detection

All animal studies were performed in accordance with animal protocol procedures approved by the Institutional Animal Care and Use Committee (IACUC) of Fujian Medical University. The A549, MCF-7, and MDA-MB-231 tumor-bearing mice models were established on Balb/c nude mice (tumor volume ~900 mm^3, healthy mice were used as control). Fourteen days after the establishment, the blood samples were collected with EDTA tubes. After removal of blood cells, the pellet was resuspended in 500 μl PBS. The samples were incubated with DPDS at a final concentration of 2.5 μM. After 1 h incubation, the mixtures were then centrifuged at 200×*g* for 10 min, followed by 2 washes with PBS to remove unbound probes. The final pellets were resuspended and analyzed using a microplate reader.

RESULTS AND DISCUSSION

Detection in standard samples

CTCs serve as biomarkers in the blood of cancer patients and can be utilized for accurate liquid biopsy-based cancer detection [18]. However, their clinical application is often limited by low abundance and inconsistent expression levels of marker proteins [19]. Before the experiment, we determined the expression levels of EGFR and EpCAM in A549, MCF-7, MDA-MB-231, and RAW264.7 cells using flow cytometry, the results were shown in Fig. S1. To evaluate the recognition capability of the DPDS toward tumor cells at varying concentrations, A549, MCF-7, MDA- normal RAW264.7 cells were incubated with the DPDS. As demonstrated in Fig. 1(c–f), under DPDS investigation, A549 cells exhibited a concentration-dependent fluorescence intensity increase in both EpCAM-Cy5 and EGFR-FAM channels. In contrast, MCF-7 cells showed a similar trend exclusively in the EpCAM-Cy5 channel, with no significant EGFR-FAM signal modulation across concentrations. Conversely, MDA-MB-231 cells displayed concentration-responsive fluorescence enhancement solely in the EGFR-FAM channel, while EpCAM-Cy5 signals remained unaffected. RAW264.7 normal cells demonstrated negligible fluorescence changes in either channel regardless of cell density. These differential responses align with the surface expression profiles of EpCAM and EGFR across cell lines. The DPDS system not only enables the detection of tumor cells at a remarkably low concentration of 5 cells/ml, but its dual-probe design also circumvents potential false-negative results by cross-validating a secondary target in cases where a primary target mutation is present [20], thereby enhancing diagnostic robustness for heterogeneous cancer populations.

In vitro detection of aptamers to tumor cells

The *in vitro* binding ability of EGFR-FAM and EpCAM-Cy5 to A549, MCF-7, and MDA-MB-231 cells were determined with confocal microscopy. After co-incubation with the probes (final concentration = 2.5 μM) for 1 h, A549 cells showed double positivity in both EGFR and EpCAM channels, while MCF-7 cells were EGFR-negative and EpCAM-positive, MDA-MB-231 cells were EGFR-positive and EpCAM-negative, and RAW 264.7 cells were EGFR-negative and EpCAM-

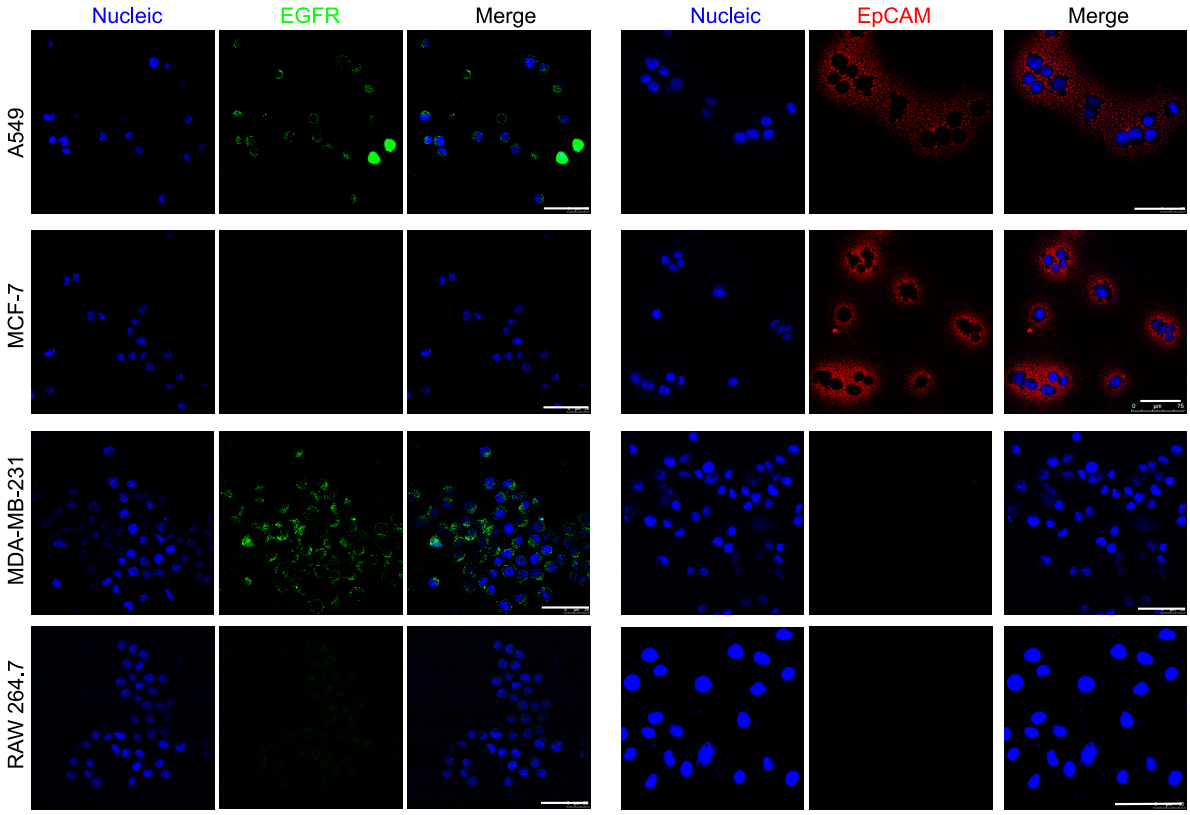

Fig. 2 Confocal microscopy analysis of EGFR-FAM and EpCAM-Cy5 binding to A549, MCF-7, MDA-MB-231, and RAW 264.7 cells. Scale bar = 50 μm.

negative (Fig. 2). Due to the heterogeneity of marker protein expression across different tumor cells, single-target diagnostic approaches are prone to failure when the target is absent [21]. The DPDS enables simultaneous recognition of tumor cells with varying EGFR and EpCAM expression levels, thereby offering potential for more accurate cancer diagnosis. These results suggest the potential utility of EGFR and EpCAM probes for cancer detection.

In vitro detection in blood samples

The complex composition of blood may interfere with CTCs detection, particularly due to the typically low CTCs concentration and interference from abundant blood cells and proteins [22]. To evaluate whether the DPDS can identify trace-level CTCs in this complex milieu, we spiked A549, MCF-7, MDA-MB-231, and RAW 264.7 cells at low concentrations (5–10 cells/ml) into healthy murine blood samples under *in vitro* conditions, simulating clinical blood samples from cancer patients. At a tumor cell concentration of 5 cells/ml, the EGFR probe detected positive signals in 44.4% of A549 samples, while the EpCAM probe achieved a detection rate of 77.7%. When the concentration increased to 10 cells/ml, the EGFR probe's detection rate improved to 66.6%, whereas the EpCAM probe demonstrated 100% detection efficacy. For MCF-7 cells at 5 cells/ml, the EGFR probe yielded a positivity rate of only 12.5%, while the EpCAM probe achieved 62.5%. At 10 cells/ml, the EGFR probe's positivity rate dropped to 0%, whereas the EpCAM probe maintained a detection rate of 87.5%. In MDA-MB-231 cells at 5 cells/ml, the EGFR probe exhibited a 100% positivity rate, while the EpCAM probe showed 0% detection. At 10 cells/ml, the EGFR probe's positivity rate decreased to 62.5%, whereas the EpCAM probe remained undetectable (0%). For RAW 264.7 cells, neither probe demonstrated any detection at either 5 or 10 cells/ml (Fig. 3a–d). These results demonstrate that DPDS can effectively identify tumor cells at low concentrations (5–10 cells/ml) in blood, and it enables precise detection of single-positive cells (e.g., MCF-7 and MDA-MB-231 cells), thereby avoiding false positives associated with single-target approaches. However, neither probe achieved 100% detection efficiency in blood samples containing low-concentration (5–10 cells/ml) tumor cells, indicating a need for further optimization to enhance detection accuracy in future studies. Our results demonstrate that EGFR and EpCAM probes effectively detected low-abundance tumor cells in blood, establishing the system's capability for subsequent CTC detection in patient blood samples.

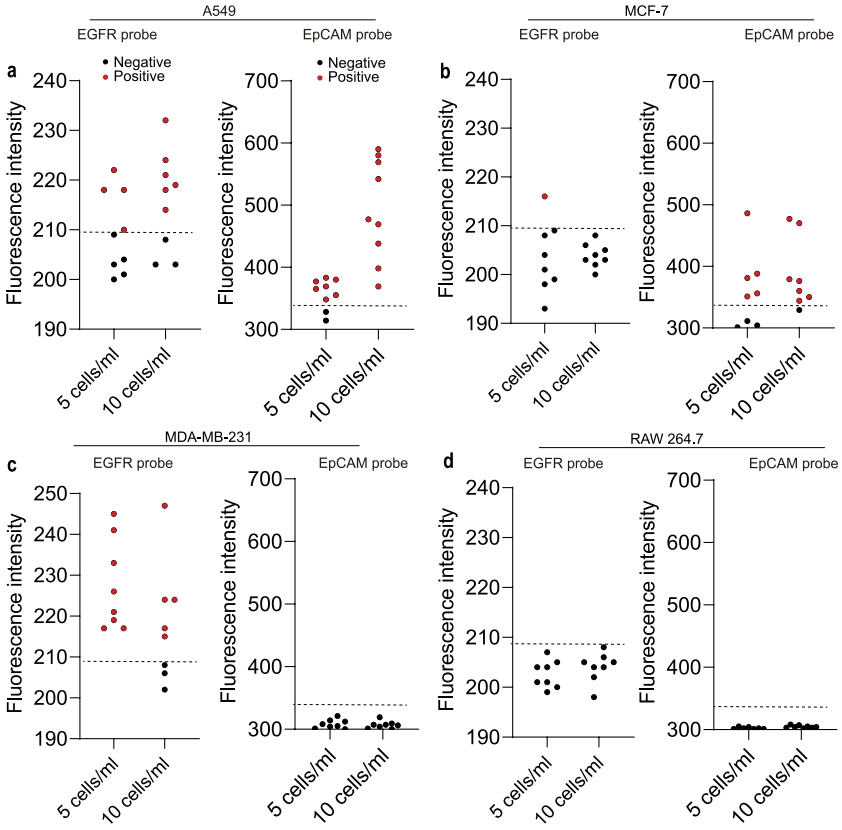

Fig. 3 *In vitro* detection of tumor cells in blood: (a) A549, (b) MCF-7, (c) MDA-MB-231, and (d) RAW 264.7 (negative control) cells.

Liquid biopsy for cancer detection

To preliminarily investigate the ability of DPDS to identify CTCs in blood, we opted to first evaluate its diagnostic performance in a murine blood tumor model before proceeding to human blood samples. This approach was chosen because mouse models allow for the customization of single-positive and double-positive tumor cell populations, providing a controlled system to assess DPDS's detection capabilities. We established A549, MCF-7, and MDA-MB-231 tumor-bearing murine xenografts to evaluate the probes' capability in detecting CTCs within blood samples. Blood samples were collected from tumor models 14 days after tumor establishment (Fig. 4a). As shown in the heatmaps of Fig. 4b,c, distinct detection patterns were observed across tumor models in the EpCAM-Cy5 and EGFR-FAM channels, with healthy control mice consistently exhibiting negative outcomes. The integrated analysis of dual-channel signals (Fig. 4d) revealed that the DPDS detected tumor-derived samples with high efficacy (capturing $>$ 75.9% of cases), while maintaining specificity against non-malignant backgrounds. We also determined the Receiver Operating Characteristic (ROC) curves to quantify the specificity and sensitivity of the DPDS (Fig. 4e). The area under the curve (AUC) was 0.820 for the EpCAM-Cy5 single channel and 0.829 for the EGFR-FAM single channel, while the dual-channel combination achieved an AUC of 0.981. In murine tumor blood samples, both single-EGFR and single-EpCAM detection approaches exhibited risks of false-negative results, whereas the DPDS demonstrated significantly improved diagnostic accuracy. These results demonstrate that DPDS enables significantly higher diagnostic accuracy compared to single-target detection, providing more reliable outcomes for cancer diagnosis. It is worth noting that these results were obtained from measurements in mouse blood, which may still differ from the more complex human blood environment.

CONCLUSION

The detection of CTCs provides clinically valuable insights across multiple domains of oncology practice, including early disease detection, therapeutic response assessment, prognostic stratification, surveillance for disease recurrence and metastatic progression, as well as guidance for treatment selection and monitoring of therapeutic resistance [23, 24]. In this study, we developed a DPDS utilizing fluorescence-labeled EpCAM and EGFR targeting for liquid biopsy analysis

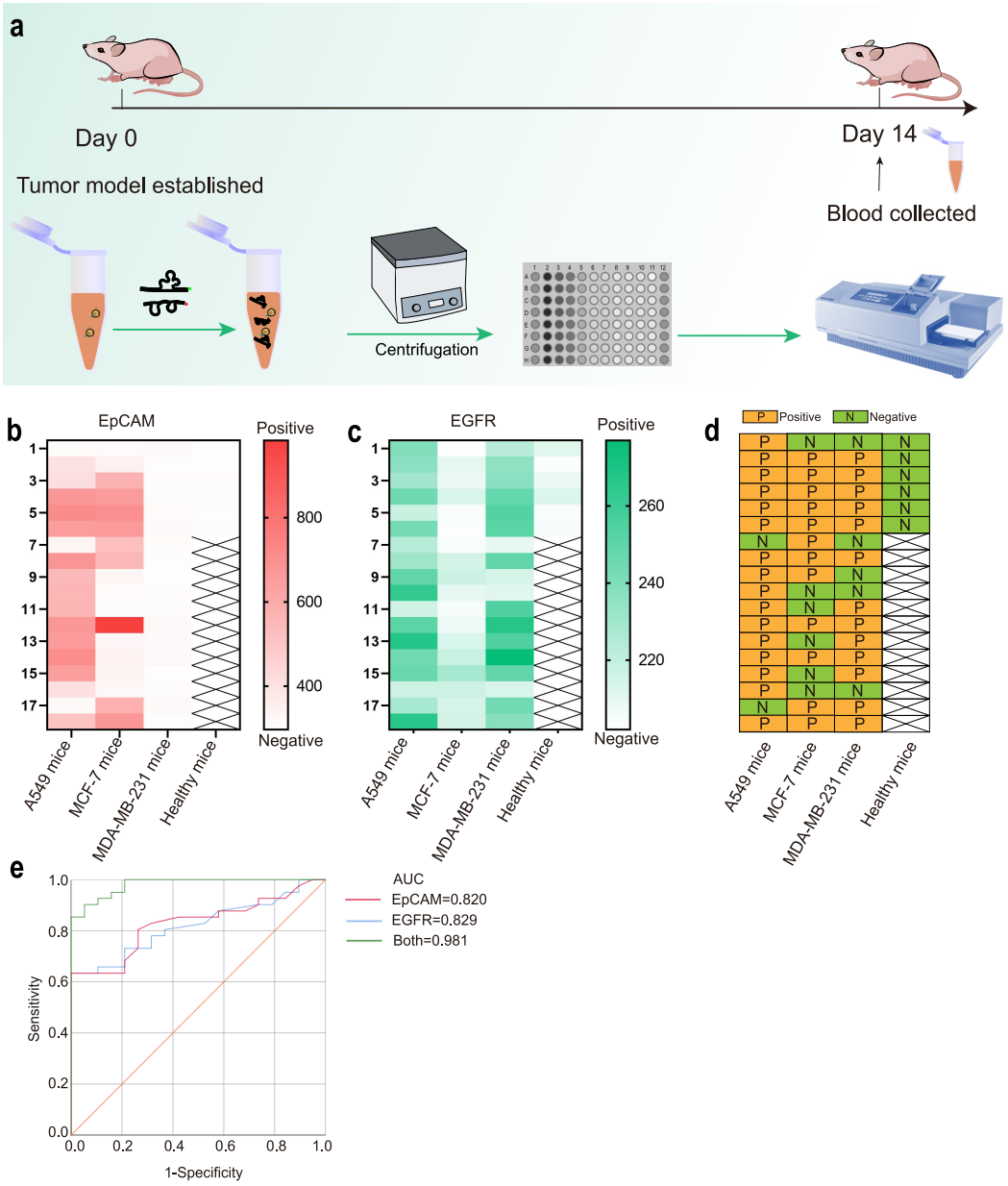

Fig. 4 Detection of blood samples from tumor-bearing mice using dual nucleic acid probes. (a) Schematic illustration showing the treatment schedule and the detection process. (b, c) Heat map showing the results of the cancer detection with anti-EpCAM nucleic acid probes (b) and anti-EGFR nucleic acid probes (c). (d) The overall detection results. (e) ROC curves showing the AUC and the specificity and sensitivity of the dual nucleic acid probes to mice xenografted with A549 cells, MCF-7 cells, MDA-MB-231 cells, and healthy mice.

in murine tumor models. The DPDS demonstrated concentration-dependent fluorescence enhancement across tumor cell gradients, achieving ultrasensitive detection of low-abundance tumor cells. Notably, this bispecific design effectively mitigated false-negative results caused by target antigen heterogeneity observed in single-probe systems. The current study did not include a scrambled probe control in the initial experiments, which may raise questions about the absolute specificity of the binding. However, the clear signal differences observed between cell lines with distinct EGFR and EpCAM expression profiles provide strong indirect evidence for probe specificity. Future studies incorporating scrambled controls will further validate this approach. In blood samples from A549-, MCF-7-, and MDA-MB-231-bearing mice, the DPDS exhibited

superior diagnostic accuracy compared to single-target detection approaches. These findings establish a probe system capable of detecting low-abundance CTCs in murine blood samples, while the dual-probe configuration effectively mitigates false-negative results caused by loss of single-biomarker expression on tumor cells, thereby providing a robust diagnostic approach for cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.098.

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Appendix A. Supplementary data

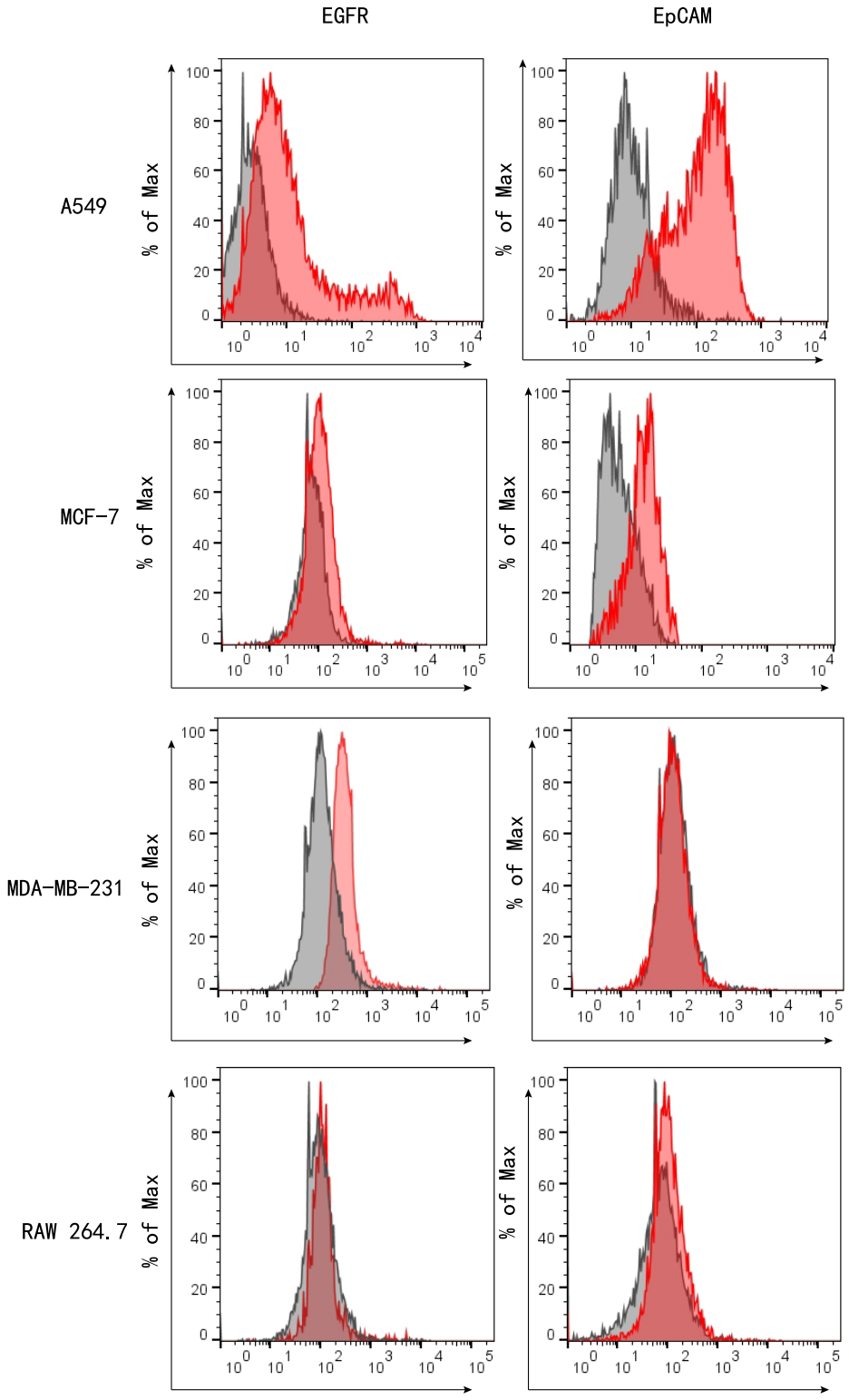

Fig. S1 Expression of EGFR and EpCAM in A549, MCF-7, MDA-MB-231, and RAW 264.7 cells monitored by Flow cytometry assays.