Supplementary Data for

The Novel Association of Circulating Tumor Cells and Circulating Megakaryocytes with Prostate Cancer Prognosis

Lei Xu,[1,2]* Xueying Mao,[1]* Tianyu Guo,[1] Pui Ying Chan,[3] Greg Shaw,[4] John Hines,[4] Elzbieta Stankiewicz,[1] Yuqin Wang,[1] Tim Oliver,[1] Amar Ahmad,[5] Daniel Berney,[1] Jonathan Shamash,[3] Yong-Jie Lu[1]

*These authors contributed equally to this work.

Corresponding Author: Yong-Jie Lu. Email: y.j.lu@qmul.ac.uk

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Supplementary Data

Experimental Procedures

Patients and blood samples

A total of 81 patients with written consent were recruited from December 2014 in St Bartholomew's Hospital, Barts Health NHS, London, UK, comprising 38 with untreated localized prostate cancer, and 43 with progressive CRPC ready to commence an alternative treatment. Metastases were investigated by radionuclide bone scan and computed tomography (CT). Progressive diseases were defined with a minimum of two increasing PSA levels at least two weeks apart or by radiographic criteria new lesions by bone scan or as new or enlarged soft tissue by CT or magnetic resonance imaging. Blood specimens from 24 healthy donors were collected with signed Ethics committee approved consent form. 7.5mL of whole blood was donated from each participant for CTC enumeration. Blood samples were drawn into EDTA vacutainer tubes (Becton Dickinson and Company, Polymouth, UK) and acquired at the middle of phlebotomy after the collection for routine clinical blood test to avoid contamination with epithelial cells from the skin. All blood samples were stored at room temperature and processed within 24 hours of blood draw. Use of blood samples from patients and healthy donors in this study was approved by National Research Ethics Service committee London City & East with a Research Ethics Committee reference of 09/H0704/4+5. The clinical characteristics of the above 81 patients are summarized in **Table 1 and** detailed in **Table S1**.

Cell culture

The human prostate cancer cell line, PC3, was used to simulate cancer samples as a validation test for repeated FISH after immunofluorescence before performing in clinical samples. Megakaryocyte cell lines were obtained by treating human hematopoietic precursor

cells K562 (a patient-derived leukemia cell line which expresses both erythroid and megakaryocyte markers and can be induced to differentiate). K562 cells were cultured in Iscove's Modified Dulbecco's Media (Sigma Aldrich) + 10% fetal bovine serum (Gibco, Life technologies) and induced with 50 nM of phorbol myristate acetate (PMA) (Sigma Aldrich) to differentiate into mature megakaryocytes. This was used as validation for CD34 and CD41 immunostaining. All the cell lines are from ATCC.

CTC harvest and fixation on slides

200 µL of CTC cell suspension was harvested into 1.5 mL Low-Retention microcentrifuge tube (Fisherbrand™), centrifuge at 1,000 g for 3 minutes, re-suspended in 10 µL buffer (0.075M KCl) and transfer onto slide using Superslik™ surface and wide orifice pipette tips (VWR). The tube was rinsed with another 10 µL of buffer and added to the drop on the slide. The slide was air-dried followed by fixation with acetone on ice for 20 minutes.

Immunofluorescence staining

After blocking by 10% normal donkey serum for 10 minutes, cells for CTC analysis were incubated with PE-conjugated anti-CD45 (Clone: 5B1, Miltenyi Biotec) for 15 minutes. Cells were then permeabilized with 0.1% Triton X-100 for 5 minutes, stained with FITC-conjugated anti-CK (Clone: CK3-6H5, Miltenyi Biotec) and Alexa Fluor®647-conjugated anti-VIM (Clone: EPR3776, abcam) for 30 minutes.

Cells for megakaryocytes identification were incubated with anti-CD41 (Clone: M148, abcam) and anti-CD34 (Clone: H-140, Santa-cruz) for 1 h after permeabilized with 0.1% Triton X-100 for 5 minutes. Alexa Fluor®488 donkey anti-mouse and Alexa Fluor®546 donkey anti-rabbit (Life technologies) secondary antibodies were then incubated with the cells for 20 minutes. After the application of antibodies, slides were mounted in SlowFade® gold antifade mountant with DAPI. Enumeration was performed after the slide was scanned

by Ariol image analysis system (Leica Microsystems (Gateshead) Ltd, UK), equipped with an Olympus BX61 microscope.

Multiple FISH procedure

FISH analysis was performed for *AR* (RP11-479J1), *PTEN* (CTD-846G17), *ERG* 3' (RP11-476D17) and *ERG* 5' (RP11-95I21), *NXK3.1* (RP11-213G6), *C-MYC* (RP11-349C2), *RB1* (RP11-305D15 and RP11-174I10), *CCND1* (RP11-278A17, RP11-599F23, RP11-681H17 and CTD-2009H2), 6q16 (RP11-639P13, RP11-258I9, CTD-2281M23 and CTD-2073M5), 16q22.1 (RP11-510M2), *TMPRSS2* (RP11-535H11), chromosome 1 centromere, *RAF1* 3' (RP11-64E16, RP11-136B7 and RP11-738A2) and *RAF1* 5' (RP11-715I4, RP11-764F12 and RP11-449E21) in CTCs and the spike experiment. Plasmid clone for chromosome1 centromere and BAC clones for *RAF1* 3' and *RAF1* 5' were from Institute of Cancer Research. All other BAC clones for FISH probes were purchased from Life Technologies (UK). Probes were prepared as previously described (1). Probes for *PTEN*, *ERG* 5', *NKX3.1*, 6q16, 16q22.1, *TMPRSS2*, chromosome 1 centromere and *RAF1* 5' were labeled by Fluorescein-12-dUTP (Roche, IN, USA). Probes for *AR*, *ERG* 3', *C-MYC*, *RB1*, *CCND1,* 6q16, and *RAF1* 3' were labeled by Tetramethyl-rhodamine-5-dUTP (Roche, IN, USA). Before hybridization, slides were fixed in methanol: acetic acid (3:1) for 10 minutes, and then pretreated in 70% acetic acid in 10 minutes. Approximately 100 ng of each of the labeled clones were resuspended in hybridization buffer in probe pairs. The mixture was applied to the slide, denatured at 95°C for 10 minutes and incubated at 37°C overnight in a wet box. The slide was washed following the standard FISH method.

Two FISH probes were hybridized with the slide in each round of FISH. To apply new FISH probes, the FISH signals were removed in 70% formamide/2XSSC solution at 68°C for 4 minutes, followed by rinsing in 2XSSC and water. After being air dried, the slide was ready to hybridize with the new pair of FISH probes.

After mounting with antifade DAPI, the FISH signals were scanned by Ariol.

Reference:

1. Mao X, Shaw G, James SY, Purkis P, Kudahetti SC, Tsigani T*, et al.* Detection of TMPRSS2:ERG fusion gene in circulating prostate cancer cells. Asian J Androl **2008**;10(3):467-73.