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·*Original Article*·

Detection of *TMPRSS2:ERG* fusion gene in circulating prostate cancer cells

Xueying Mao[1], Greg Shaw[1,2], Sharon Y. James[1], Patricia Purkis[1], Sakunthala C. Kudahetti[1], Theodora Tsigani[1], Saname Kia[1], Bryan D. Young[1], R. Tim D. Oliver[1], Dan Berney[3], David M. Prowse[2], Yong-Jie Lu[1]

[1]Medical Oncology and [2]Molecular Oncology Centres, Institute of Cancer, [3]Department of Histopathology and Morbid Anatomy, Barts and London School of Medicine and Dentistry, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, UK

Abstract

Aim: To investigate the existence of *TMPRSS2:ERG* fusion gene in circulating tumor cells (CTC) from prostate cancer patients and its potential in monitoring tumor metastasis. **Methods:** We analyzed the frequency of *TMPRSS2:ERG* and *TMPRSS2:ETV1* transcripts in 27 prostate cancer biopsies from prostatectomies, and *TMPRSS2:ERG* transcripts in CTC isolated from 15 patients with advanced androgen independent disease using reverse transcription polymerase chain reaction (RT-PCR). Fluorescence *in situ* hybridization (FISH) was applied to analyze the genomic truncation of *ERG*, which is the result of *TMPRSS2:ERG* fusion in 10 of the 15 CTC samples. **Results:** *TMPRSS2:ERG* transcripts were found in 44% of our samples, but we did not detect expression of *TMPRSS2:ETV1*. Using FISH analysis we detected chromosomal rearrangements affecting the *ERG* gene in 6 of 10 CTC samples, including 1 case with associated *TMPRSS2:ERG* fusion at the primary site. However, *TMPRSS2:ERG* transcripts were not detected in any of the 15 CTC samples, including the 10 cases analyzed by FISH. **Conclusion:** Although further study is required to address the association between *TMPRSS2:ERG* fusion and prostate cancer metastasis, detection of genomic truncation of the *ERG* gene by FISH analysis could be useful for monitoring the appearance of CTC and the potential for prostate cancer metastasis. *(Asian J Androl 2008 May; 10: –)*

Keywords: *TMPRSS2:ERG*; fusion gene; prostate cancer; metastasis; circulating tumor cells; fluorescence *in situ* hybridization; polymerase chain reaction

1 Introduction

Prostate cancer is the most common non-cutaneous malignancy in men living in developed nations [1]. Most prostate cancers diagnosed at an early stage are latent, but once having progressed they become life threatening, and, despite recent advances, late stage prostate cancer is still incurable [2]. With the general application of prostate specific antigen (PSA) testing for prostate cancer screening and diagnosis, there is also a dilemma in the clinical treatment of early stage cancers, because it is difficult to predict their progression potential [2]. Current methods either over-treat the majority of early prostate cancer patients who will not die from prostate cancer even with-

Correspondence to: Dr Yong-Jie Lu, Medical Oncology Centre, Cancer Institute, Barts and London School of Medicine and Dentistry, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, UK.
Tel: +44-20-7882-6140 Fax: +44-20-7882-6004
E-mail: yong.lu@cancer.org.uk
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out treatment, or miss the opportunity to cure early stage aggressive disease. As it is the metastatic and androgen independent disease that is responsible for cancer deaths, detecting signs of aggressive cancer in clinically early stages of the disease would be invaluable [3, 4]. However, there are currently no reliable prognostic biomarkers to predict prostate cancer progression and metastastic potential [2, 4].

Fusion genes have been studied a great deal in hematological malignancies and soft tissue sarcomas, where they frequently define a tumor subtype and are associated with prognosis. The recurrent fusion of the *TMPRSS2* and *ETS* family transcription factor genes was recently identified in prostate cancer [5, 6]. Fusion genes are *de novo* genes generated in cancer cells. They can be used as a marker to detect cancer cells in minimum residual disease [7], and also as targets for cancer cell specific treatment [5, 8]. If these fusion genes, particularly the high frequency of *TMPRSS2:ERG* fusion gene, play a role in tumor progression and, most importantly, tumor metastasis, they will be invaluable markers for treatment stratification and targets for novel forms of therapy. However, studies on the association between *TMPRSS2:ETS* fusion gene and tumor progression have generated inconsistent results, and few studies have attempted to address the role of the fusion gene in advanced metastastic prostate cancer [9–17].

Long distance tumor metastasis is caused by tumor cell migration from the primary site into the blood stream, culminating in survival and re-establishment at a new site. It has been known for 150 years that tumor cells are detectable in the blood of patients with advanced cancer [18]. Sampling circulating tumor cells (CTC) may be used to study genetic and gene expression alterations in a continual, relatively non-invasive fashion, and to monitor treatment. Using genetic and/or biomarkers to detect the CTC might also provide informed treatment planning. With the recent advances in use of specific antibodies and various sorting techniques following antibody labeling, the ratio of epithelial to blood cells can be augmented so that analysis of these cells is more feasible [19–21]. However, many genes associated with prostate carcinogenesis are also expressed in hematopoietic cells, and the existence of non-tumor epithelial cells in the peripheral blood complicates CTC analysis [22, 23]. For prostate cancer, the detection of micrometastases is also limited by the lack of specific cancer cell markers [22, 24, 25]. As the *TMPRSS2:ERG* fusion gene occurs at a high frequency in prostate cancer and is a specific genetic marker of abnormal prostate cells, we have attempted to detect its existence in CTC to monitor tumor progression and metastatic potential.

2 Materials and methods

2.1 Materials

Twenty-seven primary prostate cancer biopsies from radical prostatectomy and fifteen peripheral blood samples from patients with advanced prostate cancer (with metastasis or PSA > 40 ng/mL) were obtained from local hospitals with patients' consent and local research ethical committee's approval. A cancer biopsy and a blood sample were available from 1 patient (primary biopsy PC127 and blood sample PCB34). The clinico-pathological data are summarized in Tables 1 and 2. All blood samples were taken from patients who received second line hormone therapy, except for PCB32, who was on first line treatment. Tissue biopsies were kept snap frozen in liquid nitrogen and all samples in the present study were confirmed with cancer by a consultant histopathologist (Dan Berney), who reviewed fresh frozen sections from the collected tissue.

2.2 Cell separation and circulating tumor cells purification from blood samples

Peripheral blood samples were collected in EDTA-coated tubes and processed as follows. From a small volume (5 mL) blood collection, lymphocytes and tumor cells were separated from red cells by spinning in Ficoll gradient buffer and then used directly for RNA extraction. From a large volume (20 mL) blood collection, CTC were purified by magnetic cell sorting. Initially, the red blood cells and the majority of the white blood cells were removed from the 20 mL blood sample by spinning in an Oncoquick tube pre-filled with separation buffer (Greiner, Kremsmuenster, Austria). The aspirate was then washed twice with Oncoquick washing buffer and resuspended in MACS running buffer (Miltenyi Biotech, Surrey, UK) for CTC selection. A negative selection for cells which were labeled with CD45 (a lymphocyte marker) was performed using AutoMACS (Miltenyi Biotech). This was followed by a positive selection for cells labeled with EpCAM (Epithelial Cell Adhesion Molecule) microbeads.

2.3 Immunostaining

EpCam (Abcam, Cambridge, UK) antibodies were used at concentrations recommended by the manufacturers. CTC were air dried onto slides, fixed, incubated overnight with primary antibodies and detected using appropriate secondary antibodies. Nuclear counterstaining was performed using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Stained preparations were analyzed on a Zeiss 510 confocal microscope (Carl Zeiss Ltd., Jena, Germany).

2.4 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was extracted from snap frozen tissues and Ficoll separated cells from the small blood volume samples using the Trizol method following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RNeasy Mini kit (Qiagen, Crawley, UK) was also used according to the manufacturer's instructions to extract RNA from the CTC.

Reverse transcription of RNA was performed using the Superscript II enzyme (Invitrogen). Random primers (6pN) were used for RNA extracted from the cancer biopsies and the Poly-A primer was used for RNA from the circulating cells.

The primers (for *TMPRSS2:ERG* forward primer CAGGAGGCGGAGGCGGA and reverse primer GGCGTTGTAGCTGGGGGTGAG and for *TMPRSS2:ETV1* forward primer CAGGAGGCGGAGGCGGA and reverse primer TTGTGGTGGGAAGGGGATGTTT) described by Tomlins *et al.* [5, 8] were used for PCR amplification with an annealing temperature of 65ºC. For standard RT-PCR, 35 cycles were used, and 40 cycles were used for quantitative RT-PCR (Q-RT-PCR). β-actin with the forward primer GATGAGATT GGCATGGCTTT and reverse primer CACCTTCAC CGTTCCAGTTT was used as a positive control. The Opticon DNA Engine 2 (MJ Research, Waltham, USA) was used to perform the Q-RT-PCR thermal cycling and Opticon monitor software (MJ Research) was used for data analysis.

2.5 Sequence analysis

PCR products were cloned into the pCR 2.1-TOPO Vector using the TOPO TA Cloning Kit (Invitrogen). Single bacterial clones were picked and directly lysed in PCR buffer for insert amplification using the M13F and M13R primers and then sequenced using the ABI Prism 3700 DNA Analyser (Applied Biosystem). DNA sequences were analyzed using the 4 Peaks software (Netherlands Cancer Institute, Amsterdam, Netherlands).

2.6 Fluorescence in situ hybridization

The BAC clones (RP11-95I21 and RP11-476D17) on either side of the *ERG* gene were obtained from The Sanger Institute (Cambridge, UK) and differentially labeled in red and green fluorescent dyes, as previously described [26]. CTC cells were dropped onto glass slides and fixed *in situ* with ethanol. Before hybridization, they were pretreated in 70% acetic acid for 10 min to remove the cytoplasm. Then slides and probes were denatured separately and hybridized overnight at 37ºC following standard fluorescence *in situ* hybridization (FISH) methodology. Hybridized cells were counterstained by DAPI and images were captured using an Olympus fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) mounted with a cooled coupled device camera controlled by the computer software Mac Probe 4.3 (Applied Imaging, Newcastle, UK).

2.7 Statistics

The Fisher exact test was performed to compare the frequency of *TMPRSS2:ERG* fusion gene between the primary sample and the CTC.

3 Results

We analyzed 27 primary prostate cancer biopsies from radical prostatectomy using standard RT-PCR for both the *TMPRSS2:ERG* and *TMPRSS2:ETV1* fusion genes. Of the samples, 12 (44%) were *TMPRSS2:ERG* fusion positive and in 7 samples more than one form of fusion transcripts were detected (Table 1). The most common fusion form detected in 9 of the 12 positive cases was between exon 1 of *TMPRSS2* and exon 4 of *ERG* (T1/E4), as originally reported by Tomlins *et al* [5, 8]. Other fusion forms, such as T1/E2, T1/E5 and T2/E5, as previously reported by Clark *et al.* [14, 27–30], were also detected. The *TMPRSS2:ETV1* fusion gene was not detected in any samples.

As the *TMPRSS2:ERG* fusion gene occurred at a very high frequency, we further investigated the possibility of detecting its fusion transcripts by blood testing. We analyzed 4 peripheral blood samples (one of them, PCB34, taken from a fusion positive case, PC127) using standard RT-PCR. We took 5 mL of blood from each sample, and failed to detect any fusion products.

Because both lack of CTC in the small blood volume

Table 1. Clinical data and fusion gene status of the 27 cases of prostate cancers with biopsy samples. [a]Blood sample from the same cases is available. [b]Samples with more than one fusion transcripts coexist. ND, no data; Pos, positive; Neg, negative.

Cases	Age (years)	PSA (ng/mL)	*TMPRSS2:ERG*
PC9	83	1264.0	Pos[b]
PC10	ND	ND	Neg
PC27	ND	ND	Neg
PC28	ND	ND	Pos[b]
PC29	ND	ND	Neg
PC34	ND	ND	Neg
PC39	ND	ND	Pos
PC47	71	10.9	Neg
PC48	62	ND	Neg
PC63	ND	ND	Pos[b]
PC68	ND	ND	Neg
PC75	57	6.5	Pos[b]
PC95	ND	ND	Pos[b]
PC98	66	197.7	Pos[b]
PC105	79	27.4	Neg
PC127[a]	66	59.10	Pos
PC130	73	0.53	Pos[b]
WX3	62	ND	Neg
WX5	60	6.5	Neg
WX9	70	21.3	Neg
WX16	64	9.7	Pos
WX36	74	39.0	Neg
WX43	71	8.8	Pos
WX48	65	6.9	Neg
WX49	53	8.8	Pos
WX50	63	10.7	Neg
PCaU	ND	ND	Neg

and a large amount of lymphocyte contamination could result in the failure to detect the fusion gene, we further analyzed the separated CTC from 20 mL blood samples from patients with advanced androgen independent prostate cancer receiving second line hormone therapy. The purity of cancer cells was enriched and each sample contained more than 100 nucleated CD45 negative, cytokeratin/EpCAM positive cells (Figure 1). However, of the 15 samples analyzed using both standard RT-PCR and Q-RT-PCR, including 1 case in which the primary cancer biopsy was fusion positive, no fusion transcripts were detected in any CTC (Figure 2A, B). We confirmed the sensitivity of our RT-PCR protocol in detecting *TMPRSS2:ERG* fusion transcripts by mixing different dilutions of fusion positive VCaP cells with fusion negative LNCaP cells. We detected the fusion product in approximately 20 (100 pg RNA) but not 10 (50 pg RNA) VCaP cells mixed with 200 LNCaP cells (Figure 2C).

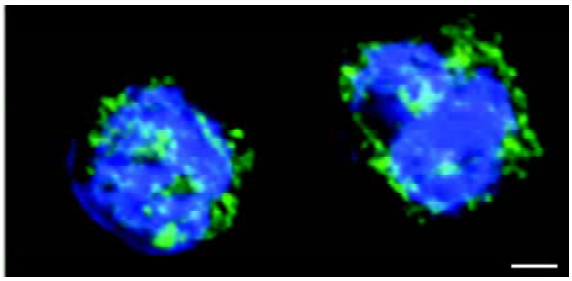

Figure 1. Representative immunofluorescence of EpCAM positive (green) circulating tumor cells (CTC) with nuclear counterstain (blue). Scale bar = 1 μm.

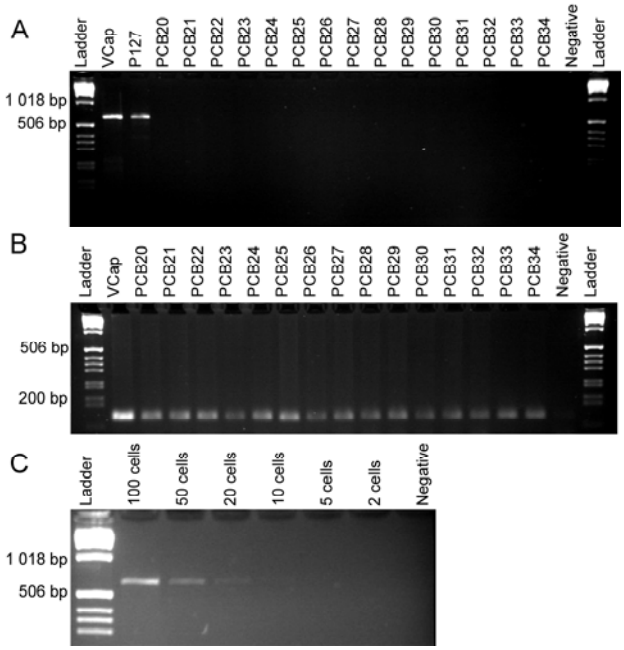

Figure 2. Reverse transcription polymerase chain reaction (RT-PCR) results of the *TMPRSS2:ERG* fusion transcript and β-actin in the isolated circulating tumor cells (CTC) and positive control samples. (A): A gel image showing the positive RT-PCR results (the bands at approximately 600 bp) from VCaP and P127 and lack of PCR products from the 15 CTC samples. (B): The β-actin RT-PCR product (121 bp band) from the VCaP and 15 CTC samples. (C): The RT-PCR result of the *TMPRSS2:ERG* fusion gene from a range of numbers of VCaP cells mixed with 200 LNCaP cells. In each experiment, molecular biology-grade water was used for negative control and a 1-Kb ladder was used for the DNA size marker.

As RT-PCR is limited to detecting the fusion transcript in single cells, we applied FISH to analyze the genomic truncation of *ERG* (which is the result of *TMPRSS2:ERG* fusion) in individual cells. Isolated CTC from 10 of the 15 blood samples were available for this type of analysis, and we found *ERG* truncation in 6 of them, including the sample accompanied by a *TMPRSS2:ERG* fusion positive primary tumor (Table 2, Figure 3). Although the frequency of *TMPRSS2:ERG* fusion was higher than that detected in our primary samples, it was not statistically significant ($P = 0.319$)

4 Discussion

There are currently no reliable markers for predicting the behavior of prostate cancer diagnosed at an early stage. Subsequently, there is a dilemma as to how to treat localized tumors, which are increasingly being detected by PSA screening of tumors. The high frequency of *TMPRSS2:ERG* fusion in prostate cancer and the detection of the fusion transcript in early stage cancers and even pre-cancerous lesions [27, 31] has promted investigations into the potential of using the fusion product to predict cancer progression [9–12, 14–17].

In the present study, we detected a high frequency of the *TMPRSS2:ERG* fusion gene in primary prostate cancer biopsies from radical prostatectomy using RT-PCR, which is comparable to previous studies [5, 14, 27–33]. We also found the co-existence of multiple forms of fusion variants of *TMPRSS2:ERG*, which correlates with several previous studies [14, 27–30]. Most importantly, we detected that *ERG* truncation generally resulted from the gene fusion at a high frequency (6/10) in CTC samples prepared from the peripheral blood, suggesting that cancer cells with *TMPRSS2:ERG* fusion frequently migrate into the blood vessel for long distance seeding. Our observation of *TMPRSS2:ERG* fusion gene positive CTC is consistent with a previous observation where fusion genes were passed on from the primary

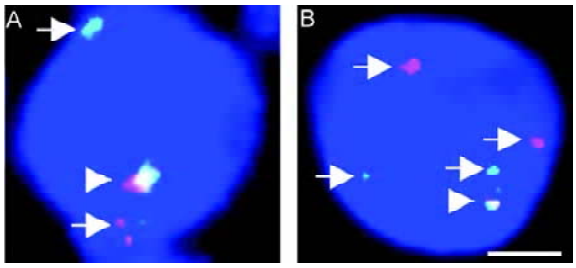

Figure 3. Representative fluorescence *in situ* hybridization (FISH) images of truncated *ERG* gene in circulating tumor cells (CTC). (A): A CTC from PCB27 with a pair of colocalized red (5') and green (3') signals (arrowhead) and one separated red and green signals (arrows). (B): A CTC from PCB29 with a pair of colocalized red (5') and green (3') signals (arrowhead) and two separated red and green signals (arrows). Images were captured at 100 × objective lens. Some green fluorescence background was seen in both cells. Scale bar = 1 μm.

Table 2. Clinical characteristics of patients from whom circulating tumor cells (CTC) were obtained and *ERG* status detected by fluorescence *in situ* hybridization (FISH). [a]*TMPRSS2:ERG* fusion positive in the primary sample. ND: no data; Neg, negative Pos, positive; PSA, prostate specific antigen.

Cases	Age (years)	PSA at sampling (ng/mL)	PSA slope (ng/mL/day)	Metastasis	*ERG* FISH results
PCB20	87	56.3	0.224	–	Pos
PCB21	90	159	0.23	+	ND
PCB22	53	19.0	ND	+	ND
PCB23	82	199.6	0.599	+	Pos
PCB24	73	51.8	0	+	Neg
PCB25	76	471.0	3.73	+	Neg
PCB26	77	152.5	0.25	+	ND
PCB27	55	43.6	0.026	–	Pos
PCB28	63	667	26.6	+	Neg
PCB29	87	164.1	ND	+	Pos
PCB30	71	153.4	3.86	+	Pos
PCB31	73	23.2	0.02	+	ND
PCB32	77	11.8	–0.11	+	ND
PCB33	78	64.0	0	ND	Neg
PCB34[a]	69	63.9	0.156	+	Pos

tumors to lymph node metastatic cells [34]. The frequency of *TMPRSS2:ERG* fusion was not significantly higher in the CTC samples when compared to that detected in our primary samples. However, the number of CTC samples analyzed in the present study is small, and further investigation of this fusion gene in a larger series of CTC samples will be required.

CTC analysis can be used to monitor tumor progression and response to therapies [35]. However, no markers can currently be used to separate cancer from normal prostate cells. Truncation of *ERG* or fusion of *TMPRSS2:ERG* is a specific marker for cancer cells. The *TMPRSS2:ERG* fusion may be used to detect tumor cells circulating in the blood in a large proportion of cases, although not all cases of CTC may be identified. This makes the detection of *ERG* truncation or *TMPRSS2:ERG* fusion in CTC a specific tool for monitoring tumor metastasis before apparent long distance metastasis occurs. In our study, we detected *ERG* alteration positive cells in two cases of prostate cancers without detected metastatic tumors. The present study has mainly established the principle in detecting *TMPRSS2:ERG* fusion in CTC using advanced prostate cancers. Further investigation is required to evaluate its application in monitoring early stage disease.

Although the *TMPRSS2:ERG* fusion was detected at the genomic level, we did not detect the fusion transcript in any of the CTC isolated from patient blood samples. RT-PCR is a sensitive method for detecting fusion gene transcripts in a small amount of cells. The protocol we used can detect a sample containing 20 *TMPRSS2:ERG* fusion positive cells from the VCaP cell line. There are more than 100 prostate epithelial cells in each of the selected populations from blood samples determined by immunostaining using EpCAM. Although we still cannot absolutely exclude the limitation of technical sensitivity as a reason for the failure to detect the fusion transcripts in the small number of CTC, it is most likely that the expression of *TMPRSS2:ERG* is switched off/down in the CTC. First, as all the patients are androgen independent, it is most likely that fusion gene expression in CTC from these patients is switched-off/down as a result of androgen ablation therapy [36]. In a previous report, the expression of *TMPRSS2:ERG* was not detectable in androgen independent cancers, including some metastatic samples, although the genomic fusion existed in some cases [14]. Second, most of the CTC in the blood stream fail to proliferate in culture and appear terminally differentiated, which might also affect fusion gene expression.

In summary, we have detected a high frequency of *TMPRSS2:ERG* fusion not just in the primary tumors but also in CTC. Although the expression of *TMPRSS2:ERG* fusion gene was not detected in CTC, genomic detection of the fusion gene by FISH in CTC could be used clinically to monitor the early signs of prostate cancer metastasis.

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