A sensitive array-based assay for identifying multiple TMPRSS2:ERG fusion gene variants

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ABSTRACT

Studies of gene fusions in solid tumors are not as extensive as in hematological malignancies due to several technical and analytical problems associated with tumor heterogeneity. Nevertheless, there is a growing interest in the role of fusion genes in common epithelial tumors after the discovery of recurrent TMPRSS2:ETS fusions in prostate cancer. Among all of the reported fusion partners in the ETS gene family, TMPRSS2:ERG is the most prevalent one. Here, we present a simple and sensitive microarray-based assay that is able to simultaneously determine multiple fusion variants with a single RT–PCR in impure RNA specimens. The assay detected TMPRSS2:ERG fusion transcripts with a detection sensitivity of \( \sim 10 \) cells in the presence of more than 3000 times excess normal RNA, and in primary prostate tumors having no \( \sim 1\% \) of cancer cells. The ability to detect multiple transcript variants in a single assay is critically dependent on both the primer and probe designs. The assay should facilitate clinical and basic studies for fusion gene screening in clinical specimens, as it can be readily adapted to include multiple gene loci.

INTRODUCTION

Chromosome rearrangements are a characteristic feature of cancer. More than 350 gene fusions, as a consequence of chromosome aberrations, have been identified (1). While gene fusions are common in hematological malignancies, their presence in solid tumors is not as well studied due to several technical and analytic problems related to tumor heterogeneity (1). Only very limited gene fusion events were discovered in solid tumors, mostly in sarcomas, until the recent discovery of TMPRSS2:ETS fusion genes in prostate cancer (2). This finding has since changed the general view that gene fusions play only a minor role in the pathogenesis of epithelial tumors. Therefore, there is renewed interest in searching for fusion genes in solid tumors, due to their potential impact on basic research and clinical application as has been demonstrated in chronic myelogenous leukemia (CML) (3,4).

The recurrent gene fusion event in prostate cancer involves an androgen controlled gene, TMPRSS2, and members (ERG, ETV1 and ETV4) of the ETS transcription factor family (2,5,6). Among these fusion genes, TMPRSS2:ERG is the most prevalent and the only member detected in the majority of reports. This fusion transcript results from \( \frac{3}{200} \text{Mb} \) interstitial deletion between these two loci at chromosome 21q22. It was found in approximately half (15–78\%) of all prostate cancers (2,6–17). As an androgen-related transcription factor controlling cell proliferation, TMPRSS2:ERG has been associated with disease pathogenesis and is a promising biomarker for prostate cancer progression, progestination and early detection (18–21). While the presence of TMPRSS2:ETS fusion genes is highly prostate cancer-specific, its significance as a prognostic biomarker is still controversial partly because many of the clinical studies have been relatively small scale. Therefore, it is important to develop a simple and robust assay for identifying various TMPRSS2:ETS and potential fusion genes in other solid tumors. However, this could be challenging due to high heterogeneity in prostate cancer and other solid tumors, compared to leukemias and lymphomas (22).

Several approaches that have been used previously for hematological malignancies have been applied to detect TMPRSS2:ERG exon fusion variants. These include fluorescent in situ hybridization (FISH) (2,12,14,17,23), RT–PCR and sequencing (2,7,9,13), quantitative PCR (qPCR) (2,8,24) and array-based comparative genome hybridization (array CGH) (10–12). FISH may be the most commonly used method, but it has relatively low resolution, and therefore, cannot accurately determine different fusion variants. Array CGH has a higher...
resolution but is costly and often fails when there is normal cell contamination.

RT–PCR and qPCR are relatively easy to perform. However, to assess multiple potential fusion variants requires multiple sets of primers and probes, and a corresponding large quantity of RNA templates. Moreover, sequencing RT–PCR products is laborious and difficult to adapt in routine clinical laboratories. Here, we describe an exon array-based detection system, combined with a RT–PCR reaction, that accurately determines multiple TMPRSS2:ERG fusion transcripts in specimens with only a minor population of tumor cells. The method adopts several features of the Virochip (25) protocol to establish a specific, sensitive and semi-quantitative assay that is very useful for analyzing highly heterogeneous solid tumors.

MATERIALS AND METHODS

RNA isolation

The cell lines described in the article were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended. Frozen unpurified prostate tissues were obtained during routine surgery, and classified pathologically by one of us. The total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The primary tumor samples were purified by Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA).

Microarrays

The exon and junction probes are 30-mer oligonucleotides synthesized by Integrated DNA Technologies (Coralville, IA, USA) or Illumina/Invitrogen (San Diego, CA, USA) and printed on poly-L-lysine slides at 50 IA, USA) or Illumina/Invitrogen (San Diego, CA, USA) synthesized by Integrated DNA Technologies (Coralville, IA, USA) or Illumina/Invitrogen (San Diego, CA, USA) following the manufacturer's instructions. The primary tumor samples were purified by Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA).

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(Qdot) against the hapten label. Conditions were optimized to use a combination of two antibodies (1:200) obtained from Invitrogen–Molecular Probes™ (Qdot 655® sheep anti-Bio primary antibody conjugate; Qdot 525® Goat anti-FITC whole IgG primary antibody conjugate). Image acquisition was done with a Zeiss Axioplan 2e microscope (Carl Zeiss, Inc.). All pictures in the corresponding three channels were deconvoluted and optical sections merged to produce 2D pictures using Axiovision 4.0 software (Carl Zeiss, Inc.) and Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2006.)

RESULTS

Microarray-based TMPRSS2:ERG exon fusion mapping

To develop a multiplexing assay that is highly sensitive in clinical samples of high complexity, we adopted our Virochip system (25). The key protocol, Round ABC, designed for unbiased amplification (29), is crucial for identifying various fusion variants in this application. Through literature review, we found that most of the TMPRSS2:ERG fusion junctions are between exons 1 or 2 of the TMPRSS2 and exons 2–5 of the ERG (2,7,9,13). Such constraints perhaps are related to whether a functional ERG protein can be made from the gene fusions (9). Therefore, we initially used a pair of primers at exon 1 of the TMPRSS2 and exon 6 of the ERG for RT–PCR. As shown in Figure 1A, PCR products were only generated when there was a gene fusion, since the two primers are located at different genes. Subsequently, the PCR products were labeled and hybridized to an exon array for mapping the exons near the fusion junction. Printed on the array are 30-mer oligonucleotide probes derived from exons 1–3 of the TMPRSS2 and exons 1–5 of the ERG (Table 1). Each selected sequence is represented by two complementary probes (F: forward and R: reverse complement) since sometimes PCR-labeled amplicons may bind to only one strand of the probe, based on empirical observations (25). We observed that probes with reverse complementary (R) orientation worked consistently with our RT–PCR labeling protocol.

A prostate cancer cell line, VCaP, (30) with a TMPRSS2 and ERG fusion (2) was used for initial feasibility testing. The total RNA was subjected to RT–PCR with a pair of primers located at exon 6 of ERG and exon 1 of TMPRSS2 (2,9). The unpurified product was labeled and hybridized on the microarray (Figure 1B). Only spots corresponding to exon 1 of TMPRSS2 and exons 4–5 of ERG developed strong signals. This result indicates the fusion junction is at the exon 1 of TMPRSS2 and exon 4 of ERG, which is consistent with a previous report (2).

To mimic a typical clinical situation, in which small population of cancer cells are present among normal host cells in a primary tumor, we spiked decreasing amounts of total RNA extracted from VCaP cells into an excess of HeLa RNA, which does not have the fusion transcripts. The detection limit is 32 pg of VCaP RNA in the presence of 100 ng of HeLa RNA (Figure 2). This translates into only 1–3 cancer cells in the presence of 3000 times normal cells. The level of sensitivity is superior to previous methods for detecting fusion transcripts (24).

TMPPRSS2:ERG fusion junction mapping in primary prostate cancer

To test the ability of the exon mapping array to detect and characterize TMPRSS2:ERG fusion transcripts in clinical samples, we isolated total RNA from frozen unpurified primary prostate tissues obtained during surgery.

Table 1. Exon probes

| Name | Sequence |
|------|----------|
| T1F  | GGCCCGGAAAGCCGCTGGAGCCGCGGAG |
| T2F  | ACATCCAGATACCATTATTACATCAGTAC |
| T3F  | GTTACACACACGATTGGACCTTACATAT |
| T1/2F| TGGACCCGGGAGGACCTGGATACGAC |
| G1F  | AGGCCATAGAGAAAGAGAC |
| G1P  | TGGAGCGGCTTGGACGAG |
| G2F  | AGGCCGAGAGAGAGAAAAAAG |
| G2P  | CCCGATAGAGAGAGAG |
| G3F  | TGGAGGGAGAAAAAGAGAGAG |
| G3P  | CAGCAGAGAGAGAGAGAGAG |
| G4F  | CGCAGGAGAGAGAGAGAGAG |
| G4P  | CAGCAGAGAGAGAGAGAGAG |
| G5F  | CGCAGGAGAGAGAGAGAGAG |
| G5P  | CAGCAGAGAGAGAGAGAGAG |
| T1R  | CTCAGAGAGAGAGAGAGAG |
| T2R  | CAGCAGAGAGAGAGAGAGAG |
| T3R  | AGCTGAGGAGAGAGAGAGAGAG |
| T1/2R| GAGCTGAGGAGAGAGAGAGAGAG |
| G1R  | TGACAGGAGAGAGAGAGAGAG |
| G1C  | AGCTGAGGAGAGAGAGAGAG |
| G2R  | TGACAGGAGAGAGAGAGAGAG |
| G2C  | AGCTGAGGAGAGAGAGAGAG |
| G3R  | TGACAGGAGAGAGAGAGAGAG |
| G4R  | AGCTGAGGAGAGAGAGAGAG |
| G5R  | AGCTGAGGAGAGAGAGAGAG |

T, TMPRSS2; G, ERG. F, forward probe; R, reverse complement probe.
Many of these tumors had a substantial fraction of normal stromal cells. Total RNA (5–50 ng) from prostate cancers (n = 20) and nonmalignant hyperplastic prostate tissues (n = 10) were subjected to RT–PCR labeling and array hybridization. The results showed that 7/20 cancers but 0/10 nonmalignant samples had TMPRSS2:ERG fusion genes. To confirm the presence of the gene fusions, direct sequencing was performed for the seven samples. The sequencing data validated the exon fusion findings revealed by the array assays. Similar to other reports (7,12,13), some samples clearly showed two or more bands on the agarose gel when the PCR products were subjected to electrophoresis, corresponding to two or more fusion transcripts in the same specimens.

The multiple fusion transcripts in a single prostate cancer sample may reflect tumor heterogeneity or alternative splicing events. In order to map multiple fusion junctions in a single assay, we redesigned the exon array to include junction probes between exons 1 and 2 of the TMPRSS2 gene and exons 1–6 of the ERG gene (Table 2).

The modified probe set showed very clearly that the patient sample #15 had two fusion transcripts and also revealed the relative ratios of the two fusion transcripts through their respective signal intensities (Figure 3). In this case, the two fusion transcripts are between exon 4 of the ERG fused to either exon 1 (T1G4) or exon 2 (T2G4) of the TMPRSS2. The signal intensity of T2G4 junction probe is weaker than that of the T1G4 junction probe (Figure 3A), consistent with the intensities of the probes within the exons. These two fusion transcripts are very likely due to alternative splicing. Figure 4 summarizes the cluster analysis (31) of the seven arrays; the results are shown in Table 3. Multiple fusion variants were found in 4/7 positive samples.

Table 3 also lists the percentages of cancer cells in the tumors, the Gleason tumor grades and the detected variants of TMPRSS2:ERG fusion transcripts. In this small sample set, there is no clear association between tumor

| Name   | Junction Sequence                                                                 |
|--------|----------------------------------------------------------------------------------|
| T1G1F  | CCTGGAGGCCGGCAGCCGCGAGGGAGCATG                                                   |
| T1G2F  | CCTGGAGGCCGGCAGGTTATTTCCAGGATCT                                                  |
| T1G3F  | CCTGGAGGCCGGCAGGTTATTTCCAGGATCT                                                  |
| T1G4F  | CCTGGAGGCCGGCAGGTTATTTCCAGGATCT                                                  |
| T1G5F  | CCTGGAGGCCGGCAGGTTATTTCCAGGATCT                                                  |
| T1G6F  | CCTGGAGGCCGGCAGGTTATTTCCAGGATCT                                                  |
| T2G1F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T2G2F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T2G3F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T2G4F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T2G5F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T2G6F  | ATGGCTTTGAACTCACCCGAGGGACATG                                                     |
| T1G1R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G2R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G3R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G4R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G5R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G6R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G1R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G2R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G3R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G4R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G5R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G6R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G1R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G2R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G3R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G4R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G5R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T1G6R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G1R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G2R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G3R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G4R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G5R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |
| T2G6R  | CATGTCCCTCGGGGGCTGCCGAGGGACATG                                                   |

T. TMPRSS2; G. ERG. F, forward probe; R, reverse complement probe.

![Figure 2](image1.png)  
**Figure 2.** Assay sensitivity. The VCaP total RNA was serially diluted in a solution containing HeLa RNA to mimic the heterogeneous cell population in primary tumors or human body fluids. The total amount of RNA for each reaction is 100 ng. The laser power (PMT 600, 100% output) was adjusted to maximize the sensitivity of detection. Therefore, the intensity of each expected feature (T1, G4, G5) is at the saturated level. The signal disappeared when the VCaP RNA was diluted from 1:3125 (32 pg) to 1:15625 (6.4 pg).

![Figure 3](image2.png)  
**Figure 3.** Detecting multiple fusion transcripts with junction probes. (A) The exon probes alone show the sum of the signal derived from individual transcripts. The junction probes reveal the species of the transcripts. These two sets of data together are very useful to distinguish weak but true signals from otherwise random background signals. (B) A scheme is presented to assist with data interpretation for (A), which shows coexistence of T1G4 and T2G4 transcripts.
grade and the presence of fusion transcripts. A relatively larger study also showed that the presence of fusion transcript was associated with tumor stage but not tumor grade (12). Consistent with the VCaP titration study (Figure 2), the clinical assay can detect the fusion transcript when only 1% tumor cells is present in the prostate tissue (sample 10).

**DISCUSSION**

We have established a simple assay that can concurrently profile variants of TMPRSS2:ERG fusion transcripts by combining a single RT–PCR with an exon array. The modified ‘Round ABC’ protocol, which was...
originally designed for genomic amplification (29) and has been widely adopted for chromatin immunoprecipitation (ChIP) and whole-genome DNA microarrays (ChIP-chip) (32,33) and Virochip (25,34) experiments, is a simple and relatively unbiased amplification procedure to semiquantitatively measure the fusion variants in a complex sample. Previously, the same simple procedure was used to obtain 83% (25 kb/30 kb) of the SARS coronavirus genome with total nucleic acids isolated from a viral culture (25).

The inclusion within the array of probes derived from individual exons and potential fusion junctions simplifies the breakpoint mapping and increases the confidence of data interpretation (Figures 3 and 4). In contrast to some reports that used multiple primers targeted to every potential fusion junction in hematological malignancies (35–38), we used a single set of primers for target amplification (Figure 1). The fusion junctions were subsequently decoded by array. This design significantly reduces the problems associated with primer dimers in the multiplex PCR reaction, and creates more room for future assays to include additional fusion genes. Furthermore, most searches for fusion genes have focused on blood cancers, because the cells can be purified before analysis. The application of the previous methodologies is less useful for highly complex solid tumors that are inevitably admixed with normal cells. For example, a previously reported MLLFusionChip could not be applied to samples with cancer cells of <5–10% in 1 μg of total RNA (39).

The current assay should facilitate a thorough compilation of the gene fusion variants in primary prostate specimens, which may be useful for stratifying the aggressiveness of prostate cancer (13). In this regard, fusion variants of EWS with another member of the ETS family, FLI1, have been shown to be an independent predictor of disease progression in Ewing’s sarcoma (40,41). It will be of interest to compare in transplanted cells the biological activities of the different TMPRSS2:ERG variants from patients with contrasting clinical outcomes (41).

While some studies have suggested that the presence of TMPRSS2:ERG fusions is associated with more aggressive disease or higher Gleason tumor grade, other investigators did not reach the same conclusion (12,14,17,20,23,42). We also did not find such an association in a small series of samples. However, all of these results are defective due to small sample size. The technology described here should make possible a larger scale investigation to find whether there is a correlation between the aggressiveness of the disease and the presence of specific fusion genes.

It is crucial to have true cancer-specific biomarkers for early cancer detection as well as for minimal residual disease monitoring, which has been extensively demonstrated in hematologic malignancies (43). Such biomarkers could help to avoid under- or over-treatment. Thus, there is past interest (24,44) in applying TMPRSS2:ERG fusion assays for such application since PSA and many other markers in development are not truly prostate cancer-specific (45). A recent study reported a TMPRSS2:ERG assay with a sensitivity of detecting 1600 VCaP cells (24). However, this level of sensitivity might not be sufficient for broad clinical application, especially with small biopsy specimens or urine samples. We were able to achieve an assay sensitivity of <32 pg of total RNA derived from VCaP cells, an equivalent to 1–3 cells (Figure 2). Because our assay is simple and amenable to automation, it is readily adaptable to clinical studies. While it has been challenging to adapt microarray-based technology to the clinic, some tests (e.g. AmpliChip CYP450 and MammaPrint) have been approved by FDA (46).

The same strategy can be applied to detect other less prevalent fusion transcripts (TMPRSS2:ETV1 and TMPRSS2:ETV4) in prostate cancer (2,5,6). In addition, the exon array approach can also be applied to other fusion genes, such as BCR-ABL in CML and clonal Ig/TCR rearrangements in lymphocytic malignancies. While this methodology development was motivated by the clinical need, it is generally applicable to other research requirements that are analogous to the situation for detecting fusion genes in the single cell level when a large excess of normal cells are present. For example, a developmental biologist may use a similar approach to screen mutants that have a desirable gene fusion when direct gene targeting is not feasible.

There are some shortcomings of using RNA transcripts as prostate cancer biomarkers, despite our ability to achieve very sensitive detection of TMPRSS2:ERG fusion variants. First, RNA is unstable and difficult to process in routine clinical assays. Second, commonly used drugs that inhibit androgen growth pathways, including GnRH agonists and testosterone antagonists, may diminish the production of the TMPRSS2:ERG mRNA fusion transcript, thereby producing false-negative results in patients on hormonal therapy with evolving androgen-independent tumors. Indeed, it has been reported that TMPRSS2:ERG mRNA fusion transcripts are not expressed in androgen-independent tumors in spite of the presence of interstitial deletions in between TMPRSS2 and ERG at chromosome 21q22 (10). While FISH is useful for identifying genomic rearrangements, it has relatively lower resolution and is difficult to use in highly heterogeneous samples with small percentages of tumor cells. We have recently developed a technology, designated Primer Approximation Multiplex PCR (PAMP) for identifying breakpoints in genomic DNA without the need to purify cancer cells from normal tissues (26). We are optimizing this assay for detecting the breakpoints between TMPRSS2 and ERG loci for primary prostate tumors to overcome any potential problems associated with RNA based biomarkers. In addition, the DNA-based assay will provide information about whether multiple fusion transcripts in a sample are derived from alternative splicing or tumor heterogeneity. The best approach may ultimately be to combine DNA and RNA based assays in a common format.

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