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Antibody detection of translocations in Ewing sarcoma

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The detection of chromosomal translocations has important implications in the diagnosis, prognosis and treatment of patients with cancer. Current approaches to translocation detection have significant shortcomings, including limited sensitivity and/or specificity, and difficulty in application to formalin-fixed paraffin-embedded (FFPE) clinical samples. We developed a new approach called antibody detection of translocations (ADOT) that avoids the shortcomings of current techniques. ADOT combines a transcriptional microarray-based approach with a novel antibody-based detection method. ADOT allows for the accurate and sensitive identification of translocations and provides exon-level information about the fusion transcript. ADOT can detect translocations in poor-quality unprocessed total ribonucleic acid (RNA). Furthermore, the technique is readily generalizable to detect any potential fusion transcript, including previously undescribed fusions. We demonstrate the feasibility of ADOT by examples in which both known and unknown Ewing sarcoma translocations are identified from cell lines, tumour xenografts and FFPE primary tumours. These results demonstrate that ADOT may be an effective approach for translocation analysis in clinical specimens with significant RNA degradation and may offer a novel diagnostic tool for translocation-based cancers.

INTRODUCTION

The identification of chromosomal translocations has important implications for understanding basic mechanisms of cancer development. In Ewing sarcoma, a pediatric solid tumour, most cases contain genomic breakpoints in the introns of Ewing sarcoma breakpoint region 1 (*EWSR1*) and Friend leukemia virus integration 1 (*FLI1*) (Delattre et al, 1992; Zucman et al, 1992). Transcription and splicing across this breakpoint results in an in-frame fusion transcript that encodes the EWS/FLI oncoprotein. EWS/FLI functions as an aberrant transcription factor to deregulate genes involved in tumorigenesis (Toomey et al, 2010). While 85% of Ewing sarcoma cases are associated with translocations that encode EWS/FLI, six additional translocations (encoding EWS/ERG (ets-related gene), EWS/ETV1 (ets variant 1), EWS/ETV4 (ets variant 4), EWS/FEV (fifth Ewing variant), (translocated in liposarcoma) TLS/ERG or TLS/FEV) have been found in the remaining cases (Sankar & Lessnick, 2011). It is widely believed that all cases of Ewing sarcoma express one of these (or yet-to-be-discovered) fusion proteins.

Ewing sarcoma fusion proteins may have multiple isoforms, depending upon which introns are involved at the translocation breakpoint. EWS/FLI has at least 10 different isoforms (Fig 1a). The most common EWS/FLI isoform is 'type 1', which fuses exon 7 of *EWSR1* to exon 6 of *FLI*. Similarly, most alternate Ewing sarcoma translocations have multiple isoforms. Although there is debate on whether different fusion isoforms portend different outcomes (Le Deley et al, 2010; van Doorninck et al, 2010), detection of translocations at the exon level has important implications for diagnosis, prognosis and treatment of patients with Ewing sarcoma. Before the classic translocation was identified, Ewing sarcoma was difficult to distinguish among small round cell tumours (Triche, 1988), leading to inaccurate diagnosis and suboptimal treatment for these patients.

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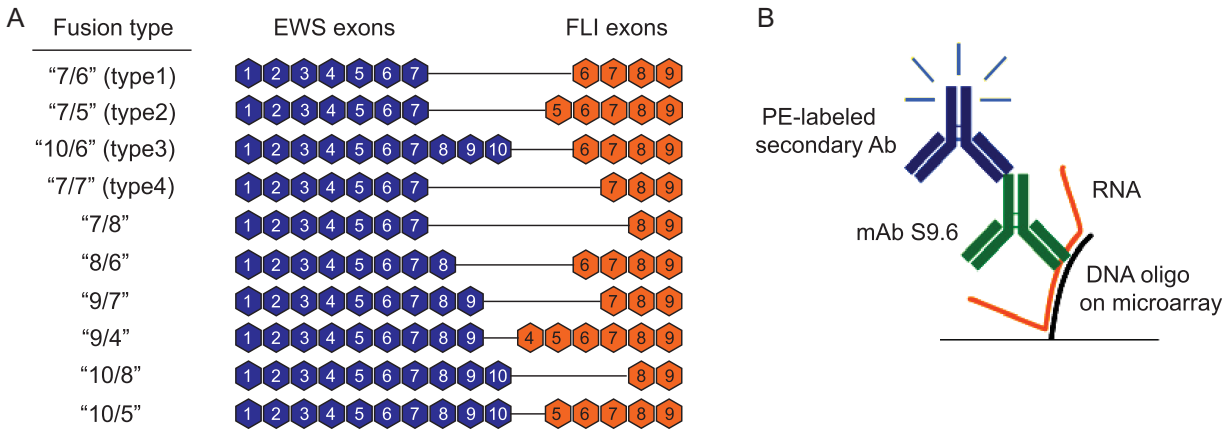

Figure 1. Schematic representation of EWS/FLI isoforms and mechanism of ADOT.

A. Ten different EWS/FLI isoforms are represented by fusions between different EWS and FLI exons.

B. The ADOT approach detects hybrids between RNA transcripts from samples and DNA oligonucleotides on the microarray. These RNA–DNA duplexes are recognized by antibody S9.6 which is then detected by PE-labelled secondary antibody.

Current approaches routinely used to identify translocations and their products in cancer have many limitations. Karyotyping can be used to detect large chromosomal abnormalities. However, this approach requires live cells to prepare metaphase chromosomes. Fluorescence *in situ* hybridization (FISH) can be conducted on fixed or embedded tissues, but will only identify rearrangements in genes to which probes are directed. Additionally, it cannot provide exon-level detail of breakpoint structure. Western blot or immunohistochemical analysis can specifically detect the translocation fusion protein but are not often used clinically because of protein quantity, antibody quality or reproducibility issues. While reverse-transcriptase polymerase chain reaction (RT-PCR) is highly specific and capable of providing details about exonic structure, it only detects fusion breakpoints for which the assay is designed, and requires high-quality ribonucleic acid (RNA) that is often not available from formalin-fixed paraffin-embedded (FFPE) specimens. Recent development in high-throughput technologies has enabled genome-wide identification of fusion transcripts. An oligonucleotide microarray screening strategy employing all combinations of exon–exon junctions for 275 oncogenic fusion genes was recently described (Skotheim et al, 2009). Again, this approach requires enrichment of messenger RNA (mRNA), which is difficult to obtain from FFPE tissues or poorly processed samples.

In the current report, we present a novel approach, antibody detection of translocations (ADOT), to utilize unprocessed total RNA to enable specific and sensitive detection of translocations in poor-quality RNA from clinical samples. This technique may be easily generalized to detect any translocation, both known and putative, in a wide variety of cancers.

RESULTS

We developed a novel technique, ADOT, to detect chromosomal translocations in cancer. We designed oligonucleotide probes for each possible exon–exon combination between potential fusion partners and printed the deoxyribonucleic acid (DNA) oligonucleotides on custom-designed microarrays. Total RNA from tumour cells or tissues was hybridized on the array. Bound RNA was detected with the S9.6 monoclonal antibody that recognizes RNA–DNA duplexes in a sequence-independent fashion (Boguslawski et al, 1986), and detected with Cy3-labelled anti-mouse IgG (Fig 1b).

Optimization of ADOT using overexpressed fusion transcripts

To test the feasibility of this technique, we synthesized a pilot microarray that included oligonucleotides for all possible fusion transcripts between *EWSR1* and either *FLI* or *ERG*, and tested this microarray with overexpressed EWS/FLI or EWS/ERG fusion transcripts. HEK293 cells were engineered to express either EWS/FLI 7/6 or EWS/ERG 7/8 transcripts (see Fig 1a for nomenclature). Total RNA was extracted from the cells and hybridized to the microarray. RNA from parental 293 cells was used as a negative control. Microarray data were processed and analyzed as described in the Supporting Information.

In RNA from engineered 293 cells, the signal intensity of positive fusion oligonucleotide (for EWS/FLI 7/6 or EWS/ERG 7/8 fusion) was much higher than most negative fusion oligonucleotides. However, some fusion oligonucleotide probes gave high signal from parental 293 cells (data not shown). Comparison with the human gene database revealed that one or more human genes contained identical sequences to each of these oligonucleotides. Subsequent experiments using cross-hybridization control oligonucleotides for these genes revealed that almost all of these are transcribed in 293 cells (data not shown), suggesting that false positive signals were primarily due to cross-reactive transcripts.

To reduce the influence of false positive signals, we normalized the data by dividing signal intensity data from samples (using RNA from engineered cells) with that from negative control parental cells (see Supporting Information for

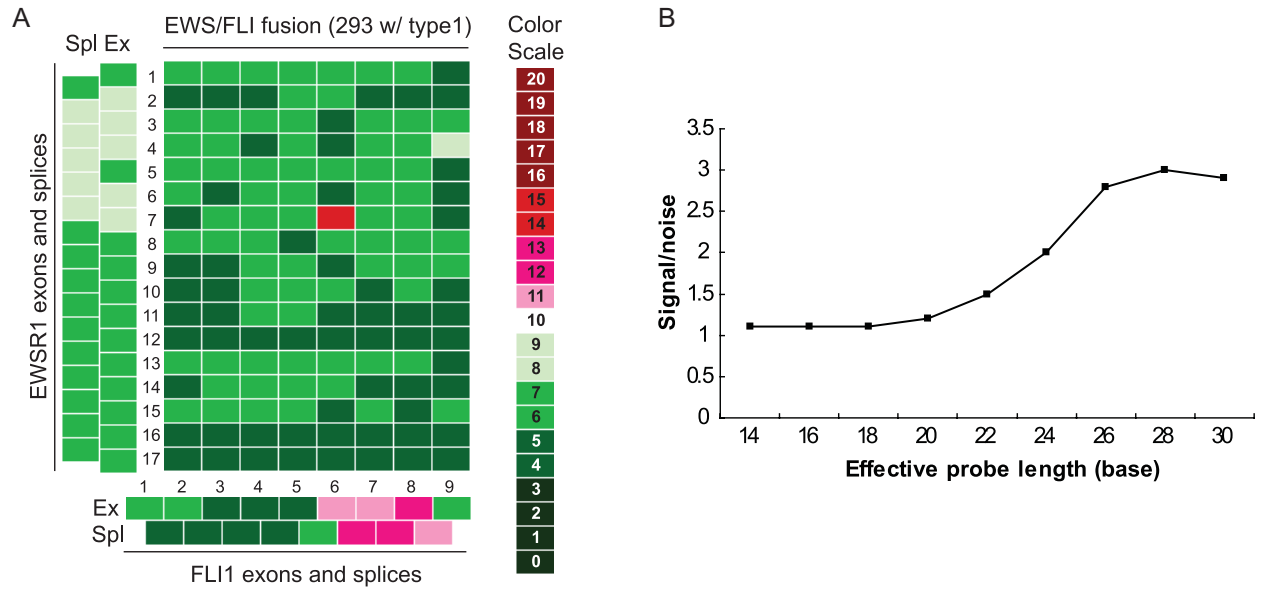

Figure 2. Proof of principle of ADOT.

A. ADOT detects translocation transcripts in RNA from 293 cells overexpressing the EWS/FLI 7/6 fusion. The central heatmap shows signal intensity of fusion probes, while the left and bottom heatmaps show those of *EWSR1* and *FLI* exon (Ex) and splice (Spl) probes, respectively. Signal intensities are shown in colour scale. In this case, high signal intensity at row 7, column 6, indicates a fusion between *EWSR1* exon 7 and *FLI1* exon 6.

B. Optimization of probe length and signal-to-noise ratio for ADOT. Fusion probes of increasing length as indicated were printed on microarray and hybridized to total RNA from EWS/FLI 7/6 overexpressing 293 cells. Signal-to-noise ratio was then calculated and plotted as a function of probe length.

details of the normalization procedure). Following normalization, the EWS/FLI 7/6 (Fig 2a) as well as EWS/ERG 7/8 (data not shown) fusion signal were clearly identified.

In addition to fusion oligonucleotides for translocations, we also designed oligonucleotides for wild-type exons and exon–exon junctions of each fusion gene partner. In contrast to the EWS/FLI fusion, neither the reciprocal FLI/EWS fusion, nor the wild-type FLI transcript, is expressed in Ewing sarcoma (Smith et al, 2006). For each fusion partner gene, we reasoned that the exons present in the fusion gene should be more highly expressed than the absent exons: *EWSR1* exons upstream of the breakpoint should show higher signal than downstream exons, and *FLI1* or *ERG* exons downstream of the breakpoint should give higher signal than upstream exons (see Supporting Information for additional details). In support of this hypothesis, overexpressed EWS/FLI 7/6 fusion transcript gave differential signal intensities from wild-type exon and exon–exon junction oligonucleotides on either side of the breakpoint (Fig 2a). These data provide a second independent confirmation of fusion point identification. Taken together, these data demonstrate that the ADOT technique can recognize overexpressed fusion transcripts in heterologous cells.

To further optimize the ADOT technique, we next sought to identify the optimal length for fusion oligonucleotides that provide the highest signal-to-noise ratio. Antibody S9.6 requires at least 15 bp of RNA–DNA duplex for binding. We therefore chose a lower limit of 14 bases. To avoid the binding of wild-type EWS, FLI or ERG transcript to the fusion oligonucleotide probes, we set an upper limit of 30 bases (*i.e.* 15 bases from each side of the fusion). Oligonucleotides were then printed with increasing lengths (from 14 to 30 bases) for each fusion probe on the array. Total RNA from EWS/FLI 7/6 overexpressing 293 cells was used for hybridization. The data were normalized by dividing signal intensity of each fusion oligonucleotide for EWS/FLI 7/6 by the average signal intensities of all other fusion oligonucleotides. This ratio was then plotted as a function of oligonucleotide length. We found that a length of 28 bases was optimal (Fig 2b). Similar results were observed with EWS/ERG fusion (data not shown). Based on these data, oligonucleotides containing 28 bases (14 bases from each side of each exon–exon boundary) were used for all subsequent experiments.

Detection of endogenous translocation transcripts by ADOT

We next asked whether ADOT could detect translocations expressed at endogenous levels, such as those found in patient-derived Ewing sarcoma cell lines. Because the cell of origin of Ewing sarcoma is unknown, a negative-control cell line to use as a normalization control was not obvious. As an alternate approach to generate a negative-control data set, we carried out ADOT experiments for a series of Ewing sarcoma cell lines in which translocation type has been previously identified. The average signal intensity was then calculated for each negative fusion oligonucleotide and used as the negative control for normalization (see Supporting Information for details). After normalization, each cell line tested demonstrated a high signal from a single probe that corresponded to the previously identified fusion for each cell line (*e.g.* A673, EWS/FLI 7/6; RDES, EWS/FLI 7/5; TC466, EWS/ERG 7/8; Fig 3a and data not shown).

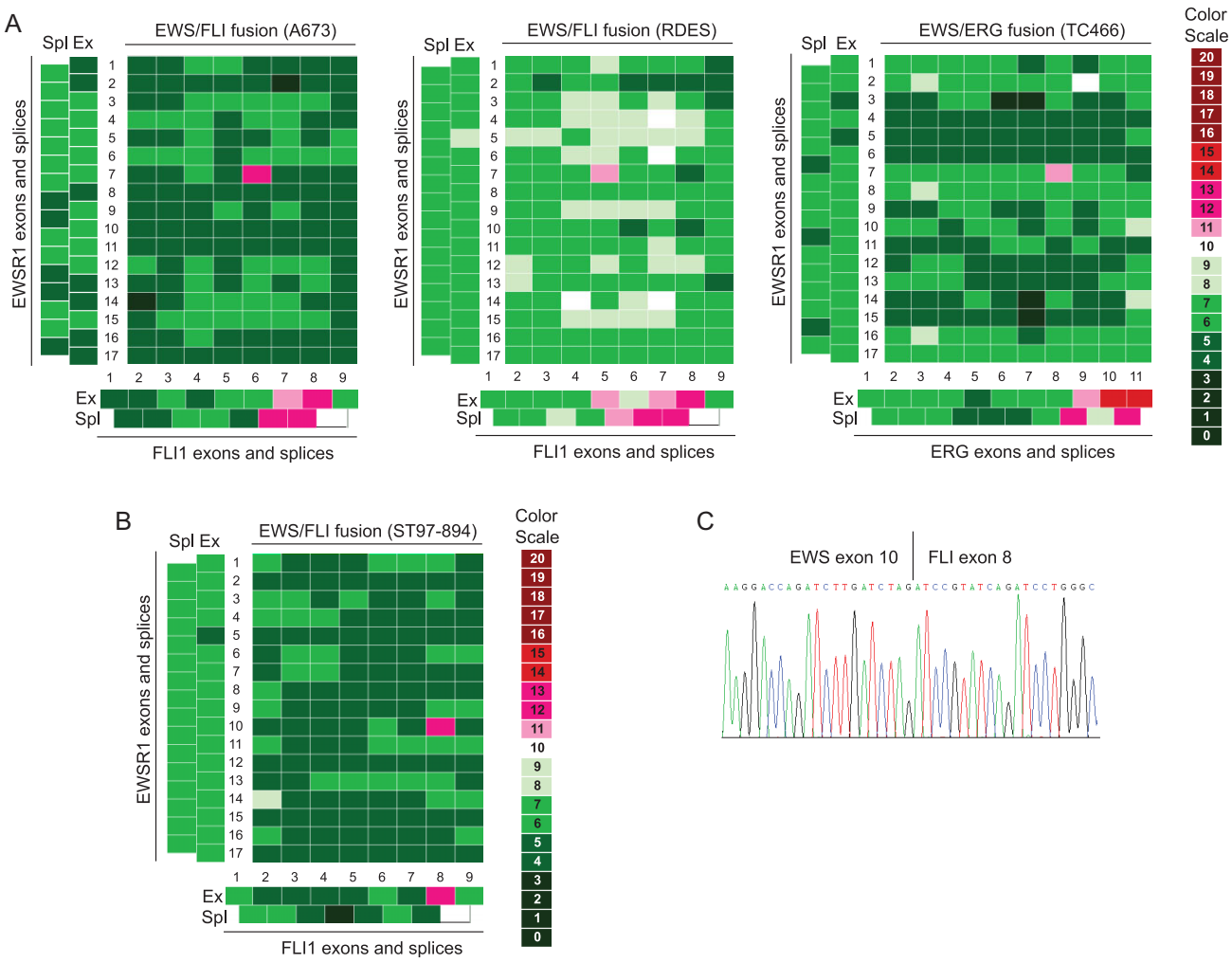

Figure 3. ADOT detects known (A) and unknown (B) translocations at endogenous levels in patient-derived Ewing sarcoma cells.

A. Heatmaps indicate that A673 cells contain EWS/FLI 7/6 translocation (left); RDES cells harbour EWS/FLI 7/5 translocation (middle); TC466 cells contain EWS/ERG 7/8 translocation (right).

B. ST97-894 cells were identified by ADOT to contain an EWS/FLI 10/8 translocation.

C. RT-PCR and sequencing analysis confirming the translocation detected in ST97-894 cells.

Importantly, the *FLI1* or *ERG* exons and splice junctions downstream of the breakpoint gave much higher signals than those upstream (Fig 3a), consistent with the fact that wild-type *FLI1* is not transcribed in Ewing sarcoma, and further confirmed the translocation types identified in these cells by ADOT. There were no significant differences in signal intensities of *EWSR1* exon and splice junction oligonucleotides up- or downstream of the breakpoint. This is likely due to abundant expression of wild-type *EWSR1* in Ewing sarcoma cells that masked the difference in exon expression level due to the translocation event. Taken together, these data demonstrate that ADOT is capable of detecting known translocations expressed at endogenous levels.

We next asked whether ADOT could accurately identify previously unknown translocations. We used ST 97-894 Ewing sarcoma cells in which the specific translocation type had not yet been identified. ADOT indicated that ST 97-894 cells contain an EWS/FLI 10/8 translocation (Fig 3b). Subsequent RT-PCR and sequencing confirmed this result (Fig 3c). These data demonstrate that ADOT can be used to identify unknown translocations expressed at endogenous levels.

Given the success of ADOT at detecting translocations expressed at endogenous levels, we next determined the sensitivity of the technique. We hybridized decreasing amounts (5, 0.5, 0.2 and 0.05 μg) of total RNA from A673 cells (EWS/FLI 7/6) to the array. As the amount of RNA decreased, the signal intensity also decreased. However, we could readily detect the fusion from as little as 0.2 μg of A673 total RNA (Fig 4). Because each A673 cell only has about 1000 expressed copies of fusion transcript (data not shown), 0.2 μg of A673 RNA contains approximately 3.3×10^{-5} fmol of EWS/FLI 7/6 fusion transcript. Thus, ADOT detects translocations with a high sensitivity.

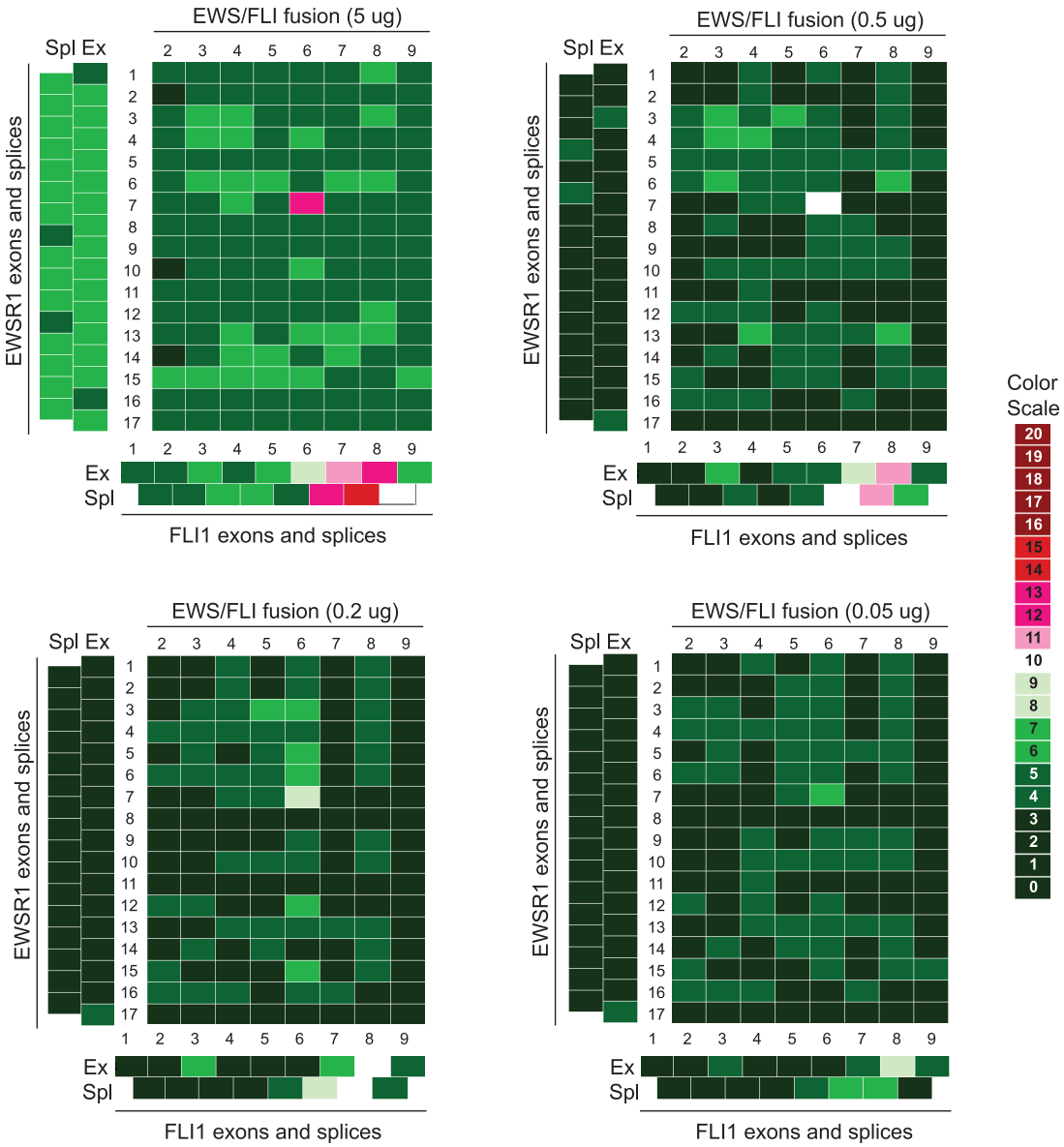

Figure 4. Sensitivity of ADOT. Heatmaps were generated by using decreasing amounts (5, 0.5, 0.2 and 0.05 μg) of total RNA from A673 cells to hybridize with microarray.

ADOT detects translocations from frozen or FFPE clinical samples

To determine whether ADOT might be applicable to the clinical realm, we analyzed diagnostic tumour specimens from patients. We first tested several frozen tumour tissues by extracting and hybridizing total RNA to the array. As shown in Fig 5a, we detected an EWS/FLI 7/5 and an EWS/ERG 7/8 translocation in tumour 1 and 2, respectively. These results were validated by RT-PCR and sequencing (Fig 5b).

Ribonucleic acid from snap-frozen tumours is generally of good quality. However, most tumours from patients are only available in FFPE format, which have low quality RNA due to cross-linking and degradation that occurs during processing. Many RNA-based approaches cannot be successfully performed from FFPE material. To test whether ADOT might be robust enough to detect translocations from these samples, we first generated a xenograft tumour by injecting A673 cells into immunodeficient mice. The tumour was recovered and fixed with formalin and embedded in paraffin. Total RNA was extracted from this FFPE xenograft and subjected to agarose gel electrophoresis. As expected, the quality of RNA was poor, consisting of low molecular weight material with loss of ribosomal RNA bands (data not shown). Furthermore, fusion transcripts could not be detected using RT-PCR (data not shown). However, using ADOT, we successfully detected the expected EWS/FLI 7/6 translocation from this FFPE xenograft tumour (Fig 5c).

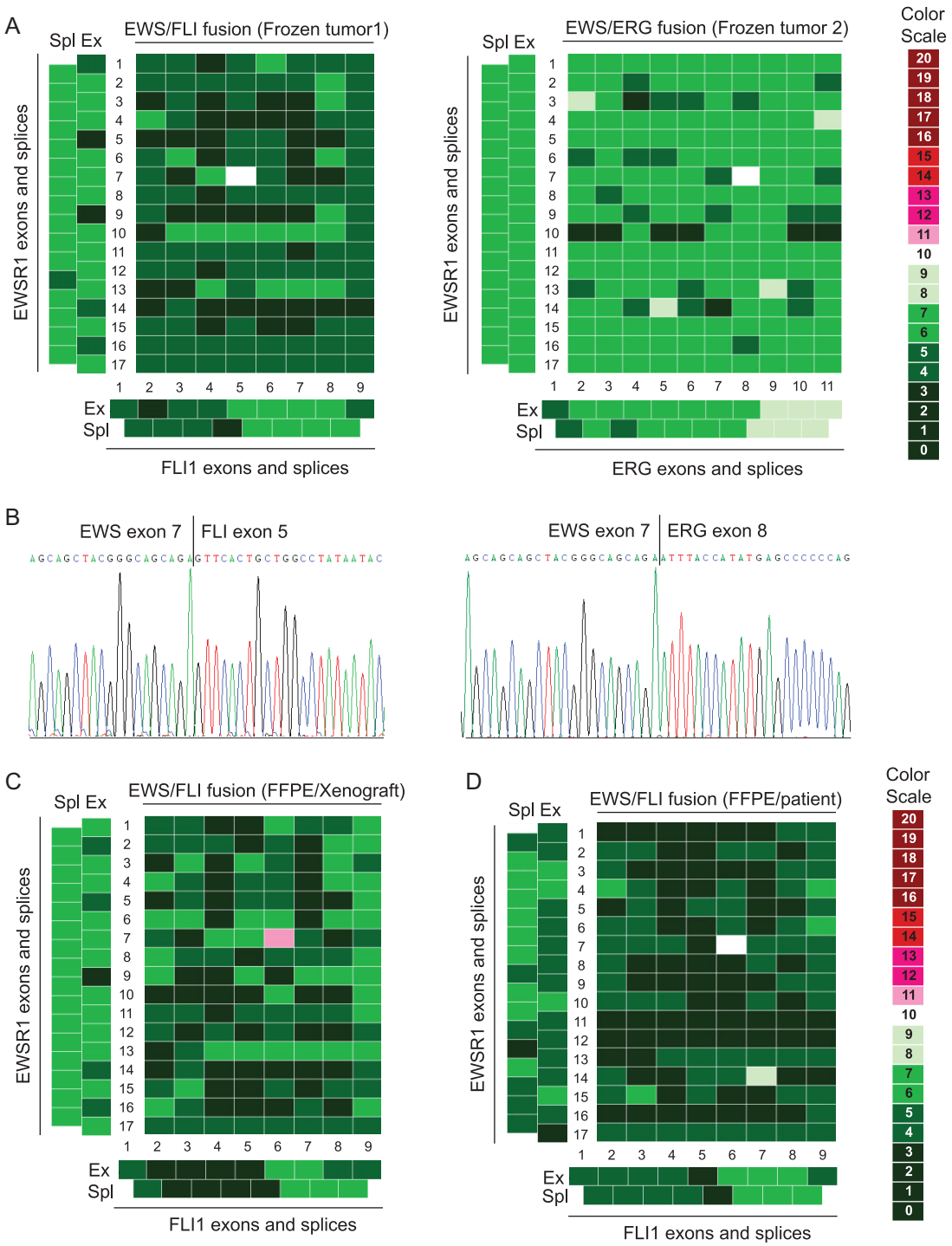

Figure 5. ADOT detects translocations in frozen (A) or FFPE (C and D) tumour samples.

A. Heatmaps showing two frozen tumours that contains an EWS/FLI 7/5 and an EWS/ERG 7/8 translocation, respectively.

B. RT-PCR and sequencing analysis confirming translocation types in frozen tumours tested in **A**.

C, D. Heatmaps showing EWS/FLI 7/6 translocation in FFPE xenograft (**C**) and patient tumour (**D**).

The paper explained

PROBLEM:

Identification of chromosomal translocations has important implications in the diagnosis, prognosis and treatment of patients with cancer. Traditional approaches to translocation detection have significant limitations, including low sensitivity and/or specificity, and difficulty in application to FFPE clinical samples.

RESULTS:

In this report, we designed a novel technique ADOT and demonstrated its utility to accurately and sensitively identify translocations associated with cancers using poor-quality total RNA from biological specimen such as FFPE primary patient tumours.

IMPACT:

Antibody detection of translocations bears promise as a discovery tool for identifying translocations in cancers, as well as a diagnostic tool for patients with translocation-associated tumours.

We next tested whether ADOT can detect translocations in FFPE primary tumours specimens from patients. Total RNA from four FFPE primary tumours were extracted and hybridized to the array. The EWS/FLI 7/6 translocation was clearly identified in three of the four samples tested (Fig 5d). RT-PCR analysis performed as part of the diagnostic workup also detected EWS/FLI in these three samples (data not shown), which confirmed our result. Prior cytogenetic analysis of the fourth sample showed that it harboured a translocation between chromosomes 7 and 22 (data not shown), indicating the presence of EWS/ETV1, one of the rare Ewing sarcoma translocations not included in the design of the current microarray. These results demonstrate that ADOT is capable of detecting translocations from FFPE samples, even in cases where RT-PCR fails.

DISCUSSION

Cancer classification is often based on morphological appearance, which can have serious limitations. For example, tumours with similar histological appearance can follow significantly different clinical courses and show different responses to therapy. In some cases, identification of specific molecular abnormalities, such as chromosomal translocations, can provide the critical diagnostic tool to effectively classify specific tumours. A highly sensitive and specific approach towards translocation detection would allow for a more complete molecular profile that would both support the basic science discovery process, and would be of great use to the clinical care of patients.

We developed a technique called ADOT to detect chromosomal translocations, and tested the approach using Ewing sarcoma as our model. ADOT combines custom oligonucleotide microarrays with the S9.6 antibody to identify chromosomal translocations in cancer. Compared to traditional microarray techniques, ADOT utilizes total RNA without poly(A) selection, reverse transcription, RNA (or DNA) amplification, or nucleic acid labelling. The S9.6 antibody recognizes an RNA–DNA hybrid of ~15 bp, enabling detection of translocation transcripts even using poor-quality RNA. This study shows that ADOT can be used to detect translocations from cell lines, frozen tumours, and FFPE tumours. RNA extracted from FFPE samples is usually highly degraded and is thus not ideal for RT-PCR. However, degraded RNA appears sufficient for hybridization to DNA probes and recognition by the S9.6 antibody in ADOT. Antibody S9.6 has no sequence specificity and does not show a significant bias for GC content. However, even one mismatched base pair reduces signal by 80-fold, and a second mismatch ~20,000-fold (Dutrow et al, 2008). ADOT is also very sensitive: it could detect translocations from as little as 200 ng of total RNA, containing $\sim 3.3 \times 10^{-5}$ fmol of translocation transcript.

Because the S9.6 antibody binds to RNA–DNA hybrids in a length-dependent manner and the sequence of fusion point is specific, we were limited in our ability to optimize the probes for similar melting temperature and GC content during the design phase. However, the normalization procedure we used negated much of the concern resulting from higher- or lower-than anticipated hybridization characteristics. Another strength of ADOT is that translocations are identified via two independent sources of information: the signal from the fusion probes, as well as those from the wild-type exon and splice probes. These two results serve as cross-references and decrease the likelihood of false identifications. Thus, we were able to accurately identify translocations from all cases except one (which likely had a translocation not included in the current version of the array).

Additional work will be required if ADOT is to move into the clinical realm. First, the design of the array will need to be expanded to include other translocations of interest. For an array focused on Ewing sarcoma, probes designed to detect EWS/ETV1, EWS/ETV4, EWS/FEV, TLS/ERG or TLS/FEV would need to be included. Furthermore, there are additional translocations that have been identified in 'Ewing's-like tumours' that should be included (Sankar & Lessnick, 2011). Indeed, one could envision an array design that includes all known translocations in cancer.

Second, an important consideration for Clinical Laboratory Improvement Amendments (CLIA) certification of this approach will be to develop test samples that can be used to assess the performance of the system in the molecular pathology

laboratory. Most of the alternate Ewing sarcoma translocations are rare, and so it is unlikely that large stocks of tumour-derived material could be available for quality assurance and quality control purposes. One approach might be to develop a series of cell lines expressing the alternate translocations and isoforms to use for such purposes.

One consideration for implementation into the clinical realm is that of cost. Costs associated with microarray-based approaches are constantly changing (in general, becoming less expensive). Using the approach we described in this report, the absolute cost of ADOT is similar to the cost of using FISH for an *EWSR1* break-apart probe. However, ADOT is more cost-effective because it can define translocation products at the exon level and can detect a greater number of translocations for the same cost. Related to this, it is possible that next-generation sequencing might become applicable to translocation analysis in the future. At the moment, however, such sequencing techniques require higher-quality RNA than is typically available in FFPE tumour specimens, these techniques are more expensive, slower and not as readily available to the clinical pathology laboratory as microarray-based approaches.

In summary, we developed a novel technique, ADOT, for the detection and analysis of chromosomal translocations. ADOT is highly sensitive and provides detailed exonic information about the translocation. Furthermore, ADOT is capable of detecting known or unknown translocations in biological samples, including those most commonly encountered during the diagnostic work-up of a patient. ADOT bears promise as a discovery tool for identifying fusion transcripts in cancers, as well as a diagnostic tool for patients with translocation-associated tumours. With additional design, ADOT could develop into an important component of the diagnostic work-up.

MATERIALS AND METHODS

Cell lines and tissues

Ewing sarcoma cell lines were grown as previously described (Lessnick et al, 2002). A673 and TC71 cells were injected into nude mice to produce xenograft tumours. Tumours were then excised, fixed in 10% formalin and embedded in paraffin. Frozen or FFPE primary tumour samples were obtained from Department of Pathology, University of Utah with Institutional Review Board approval.

RNA extraction

RNeasy mini kit (Qiagen) and High Pure RNA Paraffin Kit (Roche) were used to extract total RNA from cells/frozen tumours and FFPE xenograft tumours, respectively.

Microarray design and hybridization

Microarray oligonucleotide probes were designed using the Python algorithm (www.python.org). Microarray hybridization and antibody detection were done as previously described (Dutrow et al, 2008) with some modifications. Details of probe design and microarray hybridization are available in the Supporting Information. Additional information is available in Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/; GEO accession number GSE35450).

RT-PCR

Reverse-transcriptase polymerase chain reaction was performed using iScript SYBR green RT-PCR kit (Bio-Rad). Primer sequences are available upon request.

Author contributions

SLL, BRC and WL designed the studies; SLL, WL and BM prepared the manuscript; WL, BD, RS and HZ performed experiments; WL, BM and ND analyzed data.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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