ANTIBODY DETECTION OF TRANSLOCATIONS IN EWING SARCOMA

Wen Luo, Brett Milash, Brian Dalley, Richard Smith, Holly Zhou, Natalie Dutrow, Bradley R. Cairns and Stephen L. Lessnick

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 December 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from two of the three referees whom we asked to evaluate your manuscript. The last report is quite late, but the two already in are very concordant in their recommendations. We will provide the missing report when it will become available.

As you will see both referees are positive about the study but suggest some rewriting and refocusing as indicated. Therefore, we would be happy to welcome a revised version of your manuscript if you can address the concerns that have been raised within the space and time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript may depend on another round of review, your responses should be as complete as possible.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 27,500 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally seven pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,
Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The identification of chromosomal translocations has important clinical and prognostic implications in cancer. Luo et al report a new approach to detect chromosomal translocation in cancer. This procedure combines microarray-based approach with an antibody-based detection method and called antibody detection of translocations (ADOT). The technical merit of the paper is high and the genetic analysis thorough. ADOT avoids the shortcomings of current techniques such as karyotyping, FISH, and RT-PCR. In this report, authors used Ewing sarcoma and its associated chromosomal translocations as the model for developing a new technique for detection of tumour translocation types.

Referee #1 (Other Remarks):

The Ewing Sarcoma, the most common tumour of bone and soft tissue in children, are characterized by a unique chromosomal translocation t(11;22)(q24;q12). The majority of cases are associated with more common molecular type translocation, but the additional translocations have been found in the remaining cases. Luo et al report a new approach to detect chromosomal translocation. This procedure combines microarray-based approach with an antibody-based detection method and was called antibody detection of translocations (ADOT). Using RT-PCR and sequencing analysis the authors confirmed translocation types. The technical merit of the paper is high and the genetic analysis thorough. The authors used Ewing sarcoma cell lines and frozen primary tumour samples as the model for developing a new technique.

Minor points:

The authors used only Ewing sarcoma cells for estimating new method. They wrote about genetic aberrations in Ewing sarcoma in the Introduction, Results and Discussion. But the name of manuscript is very common - Detection translocation in cancer. That would be grade to precise the field of investigation in the name of paper or write more about possibility of new methods to detect chromosomal aberrations in different type of cancer.

Referee #2 (Comments on Novelty/Model System):

This paper presents a technical approach to detecting tumour-specific translocations using small amounts of RNA extracted from FFPE tissues. The technique is novel and appears to be highly sensitive and may provide significant advantages for classifying specific tumour translocations from archived tissues. I better description of how the data were "normalized" to background would be helpful, as well as some additional discussion regarding the potential to make this approach "CLIA" validated and cost considerations would improve the overall presentation.

Referee #2 (Other Remarks):

This is a well written manuscript describing a novel technique that allows for the detection of fusion transcripts from small amounts of total RNA obtained from FFPE tissue. The data are quite impressive and suggest a potential novel diagnostic test that could be adapted to a variety of tumours with suspected tumour-specific translocation derived fusion proteins. It would help if the authors provide a bit more detail regarding the "normalization" process as this seems to be critical to the interpretation of results, as well as further detail regarding the use of the exon/splice probes to confirm results. Finally, it would be beneficial for the authors to comment in their discussion section about their thoughts on what it would entail to move this to a CLIA type test including costs. It would also be nice to include in this discussion thoughts regarding this approach versus ultimate adoption of RNA-seq at some future time.
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Authors’ response:
We are pleased that the referee found the technical merit of the paper to be high and the genetic analysis thorough.

(Other Remarks):
The Ewing Sarcoma, the most common tumour of bone and soft tissue in children, are characterized by a unique chromosomal translocation t(11;22)(q24;q12). The majority of cases are associated with more common molecular type translocation, but the additional translocations have been found in the remaining cases. Luo et al report a new approach to detect chromosomal translocation. This procedure combines microarray-based approach with an antibody-based detection method and was called antibody detection of translocations (ADOT). Using RT-PCR and sequencing analysis the authors confirmed translocation types. The technical merit of the paper is high and the genetic analysis thorough. The authors used Ewing sarcoma cell lines and frozen primary tumour samples as the model for developing a new technique.

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Authors’ response:
Per the referee’s suggestion, we have changed the title of the manuscript to “Antibody Detection of Translocations in Ewing Sarcoma” to more accurately reflect the focus of the work.

Referee #2

(Comments on Novelty/Model System):
This paper presents a technical approach to detecting tumour-specific translocations using small amounts of RNA extracted from FFPE tissues. The technique is novel and appears to be highly sensitive and may provide significant advantages for classifying specific tumour translocations from
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Authors’ response:
We appreciate that the referee found the manuscript to be well-written and that the data were “quite impressive.”

Per the referee’s suggestion, we have expanded the description of the normalization process in the Supporting Information, and provided additional references to the Supporting Information in the body of the main manuscript where appropriate.

Also per the referee’s suggestion, we have provided a figure in the Supporting Information that provides greater clarity regarding the use of exon and splice junction probes to cross-confirm the results obtained from the translocation splice junction probes. This new information is referred to in the body of the main manuscript.

Finally, we have also expanded the discussion section of the manuscript to include our thoughts on additional work that would be required to meet Clinical Laboratory Improvement Amendments (CLIA) requirements, our thoughts on cost, and our thoughts on ADOT versus the possible use of RNA-seq as a translocation-focused diagnostic test.

Please note that additional minor alterations were made for grammatical and space considerations.

2nd Editorial Decision
25 January 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to re-assess it (and I also added the missing report from the 1st review). As you will see both reviewers are supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- provide a reply commenting Referee #3 concerns (text only)
- submit your microarray experiments to either GEO or ArrayExpress and incorporate the accession number into your manuscript (in the material and methods section, please see below for details)
- include a conflict of interest statement in the main manuscript, before the references section
- provide a general title for figures 1 and 2 and correct the legends accordingly (please refer to any article online for an example)

Data of gene expression experiments described in submitted manuscripts should be deposited in a MIAME-compliant format with one of the public databases. We would therefore ask you to submit your microarray data to the ArrayExpress database maintained by the European Bioinformatics
Institute for example. ArrayExpress allows authors to submit their data to a confidential section of the database, where they can be put on hold until the time of publication of the corresponding manuscript. Please see http://www.ebi.ac.uk/arrayexpress/Submissions/ or contact the support team at arrayexpress@ebi.ac.uk for further information.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2:

Authors have adequately addressed previous issues raised

Referee #3

I have reviewed with much interest the article entitled 'Antibody detection of translocation (ADOT) in cancer', in which Luo and colleagues describe a novel technique that combines custom Agilent oligonucleotide microarrays with the S9/6 antibody to identify potential fusion transcripts in cancer. This is a novel technique which differs from the conventional microarray techniques by using total RNA without poly(a) selection, reverse transcription, RNA (or DNA) amplification, or nucleic acid labeling. ADOT takes advantage of S9.6 antibody ability of recognizing strips of 15bp of RNA-DNA duplexes in a sequence independent fashion.

I have no major objections with the study design or the method execution. The experiments presented quite clearly make the case for the utility of ADOT for fusion detection in both frozen and archival tissue. However, the reader would benefit from a more detailed description of the custom Agilent array used here, particularly highlighting further the differences with the commercially available microarray (used for gene expression, etc): to include:
- reproducibility among chips,
- certain build-in controls in-printed on each chip other than the exons of interest (i.e. house-keeping genes, etc); The authors mention the normalization between signal intensity from the sample with that of negative control cells.
- the scanning method of the Cy3 labeled signal (what type of scanner, is it similar with scanning the microarray signal, etc).

It will be also useful for the authors to comment on the potential limitation of this technique in detecting fusions in tumours where break occurs within exons (rather than the common intronic break); which may provide false negative results.

There is no debate that ADOT may find its applicability among research tools to identify novel fusion variants or to explore rare fusion transcripts that lack preexisting RT-PCR assays. However, the potential use of ADOT in the routine clinical work-up of Ewing sarcoma-type tumours is not self-explanatory. With the wide applicability of FISH analysis in FFPE tissues, most Ewing sarcoma diagnoses are now being confirmed by the presence of EWSR1 gene rearrangement, rather than RT-PCR from archival material. One would argue that at least from the point of view of cost effectiveness and turn-around time, designing FISH probes for Fli1, ETV1 and ERG may in fact be more readily applied in paraffin material in these cases, if one questions the exact fusion partner. And since the fusion transcript variability has not translated into different molecular or clinically relevant subsets, it remains debatable how ADOT specific information on the fusion transcript exonic composition would fit into the clinical practice.
Referee #3

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- the scanning method of the Cy3 labelled signal (what type of scanner, is it similar with scanning the microarray signal, etc).

Authors’ response:

We are pleased that the referee was generally positive about the manuscript. The array is scanned using a standard Agilent G2505B Microarray Scanner using the same protocols developed for standard Agilent microarray scans.

We have uploaded information on the microarray design (including the probes used as built-in controls) in our submission to the Gene Expression Omnibus repository. This will allow any interested investigator to obtain the identical arrays that we used for our experiments. Details of the scanning procedure are also included in the GEO upload.

While we have not carefully evaluated the chip-to-chip reproducibility of the custom ordered microarray, we note that although the probe sequences are custom-ordered, the printing/manufacturing processes used are identical to the standard commercially-available microarrays from the same company (Agilent). Hence, we expect the arrays to be as reproducible as any off-the-shelf Agilent array.

It will be also useful for the authors to comment on the potential limitation of this technique in detecting fusions in tumours where break occurs within exons (rather than the common intronic break); which may provide false negative results.

Authors’ response:

This is an excellent point. However, in the case of Ewing sarcoma, every chromosomal break ever described has been intronic, rather than exonic. Thus, for Ewing sarcoma this is
unlikely to be an issue. However, for other tumours, this could be of concern, and any probes designed for chromosomal translocations in other tumours would have to take this issue into account.

There is no debate that ADOT may find its applicability among research tools to identify novel fusion variants or to explore rare fusion transcripts that lack pre-existing RT-PCR assays. However, the potential use of ADOT in the routine clinical work-up of Ewing sarcoma-type tumours is not self-explanatory. With the wide applicability of FISH analysis in FFPE tissues, most Ewing sarcoma diagnoses are now being confirmed by the presence of EWSR1 gene rearrangement, rather than RT-PCR from archival material. One would argue that at least from the point of view of cost effectiveness and turn-around time, designing FISH probes for Fli1, ETV1 and ERG may in fact be more readily applied in paraffin material in these cases, if one questions the exact fusion partner. And since the fusion transcript variability has not translated into different molecular or clinically relevant subsets, it remains debatable how ADOT specific information on the fusion transcript exotic composition would fit into the clinical practice.

Authors’ response:

This is also an excellent point. Currently, FISH for a “split” EWSR1 signal is often used to confirm a diagnosis of Ewing sarcoma. However, it should be noted that such a FISH-based test will also score positive for a series of non-Ewing tumours that also contain EWSR1-based translocations, such as Clear Cell Sarcoma (harboring EWS/ATF translocations), Desmoplastic Small Round Cell Tumours (harboring EWS/WT1 translocations), Extraskeletal Myxoid Chondrosarcoma (EWS/NR4A3 translocations), Myxoid Liposarcoma (EWS/DDIT3 translocations), and even Acute Leukemias (EWS/ZNF384 translocations).

One could certainly work towards developing FISH probes for other translocations found in Ewing sarcoma (and Ewing-sarcoma like tumours). These would have to detect EWS/FLI, EWS/ERG, EWS/ETV1, EWS/ETV4, EWS/FEV, FUS/ERG, FUS/FEV, EWS/NFATC2, EWS/POU5F1, EWS/SMARCA5, EWS/ZSG, EWS/SP3, and CIC/DUX4. With appropriate development, a single microarray could detect every one of these. Furthermore, the same array could detect nearly every translocation found in every tumour. A cost/benefit analysis is outside of the scope of the current work, but we imagine that taking a single sample and analyzing it for every possible translocation in one assay would have some time-related benefits in addition to potential cost benefits, as well.

Finally, we agree that initial work comparing the most common translocation variant (the 7/6 EWS/FLI translocation) to all others did not demonstrate a prognostic advantage for the 7/6 translocation. The availability of a rapid cost-effective assay to collect exon-level translocation data would make it possible to continue to ask whether exon-level data could be used for prognostic information in the future.