

# Kaposiform lymphangiomatosis effectively treated with MEK inhibition

Jessica Foster, Dong Li, Michael March, Sarah Sheppard, Denise Adams, Hakon Hakonarson, and Yoav Dori

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## Review Timeline:

|                     |             |
|---------------------|-------------|
| Submission Date:    | 24th Mar 20 |
| Editorial Decision: | 23rd Apr 20 |
| Revision Received:  | 14th May 20 |
| Editorial Decision: | 19th May 20 |
| Revision Received:  | 19th Jul 20 |
| Accepted:           | 31st Jul 20 |

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*Editor: Zeljko Durdevic*

## 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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees are positive and find the study interesting and important. However, they also have a few suggestions and some minor criticisms that I would like you to address in a revision of the current manuscript. Particular attention should be given to providing the rationale for using CD31+ cells for genetic analysis and to discussing the future approaches to large patient cohorts' trials.

Addressing the reviewers' concerns in full will be necessary for further considering the manuscript in our journal. Please note that EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. However, we realize that the current situation is exceptional on the account of the COVID-19/SARS-CoV-2 pandemic. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

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

Referee #1 (Remarks for Author):

Thank you for the opportunity to review this manuscript. The authors showed the significant improvements of the symptoms KLA patient treated with MEK inhibitor. This manuscript presents an interesting case report about novel treatment for KLA and other lymphatic anomalies having RAS gene abnormalities.

1. I wonder how the authors diagnose KLA, not other lymphatic diseases? What is the definition? The diagnoses of KLA are based on clinical presentation/features, imaging studies and/or histological data.

2. WES detected a low frequent somatic mutation in CBL gene. The mutated DNA was isolated from CD31 cells of pleural chylous effusions. Why did you use these cells? The author should indicate the association these mutated cells and the pathogenesis of the KLA patient. And, do you have samples from the thoracic or other affected lesions?

3. How do you consider the possibility that the CBL gene mutation might be associated with chemotherapy? Although there's a very low probability of this problem occurring.

I hope that my comment is very useful for the improvement of the article.

Referee #2 (Remarks for Author):

It was a great pleasure reading this very interesting report. Lymphangiomatosis is an ultrarare medical condition existing as Kaposiform "variant" in children but also in adulthood.

As therapeutic options are very limited, the findings reported here may offer new therapeutic options to some patients with (Kaposiform) lymphangiomatosis.

Main limitation of this casuistic lies in the nature of a report on a single patient and authors should at least discuss approaches for larger future patient cohorts. Moreover, authors are asked to include a short statement on the "adult" form of lymphangiomatosis.

the quality of the HRCT showing the interlobular septal thickening is limited and authors may try to provide a better resolution and especially a slice where the interlobular septa can be seen more pronounced.

the reporting of the lung functional parameters is quite unusual. Authors should report absolute values (in l) and %predicted and discuss that improvements seen might also be due to the decrease of pleural effusion

Referee #3 (Remarks for Author):

This is a case report of a patient with KLA who was found to have a CBL mutation and who responded in a dramatic fashion with lymphatic remodeling and resolution of chylous complication to MEK inhibition. Similar responses have been reported in KLA patients with RAS mutations treated with mTOR inhibitors and ARAF mutations treated with MEK inhibition. The novelty with this case report is that the putative causative mutation was in an effector that is upstream of RAS and known to be associated with Noonan's syndrome and leukemias. The report is well written. I have a few comments:

As acknowledged, DNA analysis performed on an expanded endothelial cell population (CD31++) from chylous fluid was limited to some extent because of low allele frequency and scarcity of sample. The pulmonary function test results published as z-scores will be unfamiliar to most clinicians--would favor a more conventional approach (such as FEV1 in liters or percent predicted). It would be interesting to know if the non-CD31+ cells in the chylous fluid were mutation positive or negative. It should be mentioned whether the patient's requirement for intermittent drainage of chylous fluids completely resolved. It bears emphasizing the low dose therapy with MEK inhibitors may be sufficient, limiting the side effects that are known to plague treatment of melanoma and other neoplasms

Referee #1 (Remarks for Author):

Thank you for the opportunity to review this manuscript. The authors showed the significant improvements of the symptoms KLA patient treated with MEK inhibitor. This manuscript presents an interesting case report about novel treatment for KLA and other lymphatic anomalies having RAS gene abnormalities.

1. I wonder how the authors diagnose KLA, not other lymphatic diseases? What is the definition? The diagnoses of KLA are based on clinical presentation/features, imaging studies and/or histological data.

*Thank you for this comment. In this case, the diagnosis was made by histology after biopsy, which is the gold standard. The diagnosis was initially made at an outside institution, and the slides were sent to our institution where we also confirmed the diagnosis of KLA based on histologic appearance with CD31 and D2-40 reactivity in the foci of spindled cells. This is updated in the manuscript on page 4 to be more clear. It should be noted the diagnosis was suspected based on her symptoms of persistent pneumonia with bloody pleural effusions and mild coagulopathy. In our experience many cases of KLA are diagnosed by clinical history and radiologic appearance, with biopsy if safe and feasible to do so. Many patients have a mild coagulopathy with low platelet count and fibrinogen, which can make biopsy challenging. The original case series that defined KLA (see reference Croteau et al 2014) reported the characteristic clinical findings and the defining histologic appearance. We have updated the discussion on page 6 to reflect this.*

2. WES detected a low frequent somatic mutation in CBL gene. The mutated DNA was isolated from CD31 cells of pleural chylous effusions. Why did you use these cells? The author should indicate the association these mutated cells and the pathogenesis of the KLA patient. And, do you have samples from the thoracic or other affected lesions?

*Thank you for pointing this out and we have clarified in the text on page 8. CD31 cell sorting was chosen to select for all endothelial cells, which would include lymphatic cells including abnormal KLA cells. No further selection was completed, which is likely why the mutant allele frequency was so low, as there was a significant number of normal endothelial cells and potentially other cells such as monocytes that also stain positive for CD31. In our experience*

*this is frequently the case for somatic mutations in vascular anomalies. We attempted to extract DNA from the primary biopsy sample, but as the sample was obtained in 2012 it was significantly degraded and did not pass quality checks. All other samples tested including saliva, blood, normal lymph node biopsy and skin biopsy were negative for the mutation as noted on page 5.*

3. How do you consider the possibility that the CBL gene mutation might be associated with chemotherapy? Although there's a very low probability of this problem occurring.

*Thank you for this comment as well. We agree it is incredibly low likelihood that chemotherapy induced the CBL mutation. The chemotherapy she received included vincristine, prednisone, and rapamycin, none of which are associated with inducing point mutations. We have updated the discussion to include this point on page 7.*

I hope that my comment is very useful for the improvement of the article.

Referee #2 (Remarks for Author):

It was a great pleasure reading this very interesting report. Lymphangiomatosis is an ultrarare medical condition existing as Kaposiform "variant" in children but also in adulthood.

As therapeutic options are very limited, the findings reported here may offer new therapeutic options to some patients with (Kaposiform) lymphangiomatosis.

Main limitation of this casuistic lies in the nature of a report on a single patient and authors should at least discuss approaches for larger future patient cohorts.

*Thank you so much for this comment, and we have added to our discussion larger clinical trials which are now starting based on this work (page 8).*

Moreover, authors are asked to include a short statement on the "adult" form of lymphangiomatosis.

*Thank you for this comment, we have added a description of adults diagnosed with KLA, which is a rare phenomenon but has been described (page 6).*

The quality of the HRCT showing the interlobular septal thickening is limited and authors may try to provide a better resolution and especially a slice where the interlobular septa can be seen more pronounced.

*Thank you, we have updated the figure.*

The reporting of the lung functional parameters is quite unusual. Authors should report absolute values (in l) and %predicted and discuss that improvements seen might also be due to the decrease of pleural effusion

*Thank you again, we have updated to the absolute values and percent predicted (page 14). We have also added a comment to discuss that the improvement may also be due to the decrease in pleural effusion (page 7-8).*

Referee #3 (Remarks for Author):

This is a case report of a patient with KLA who was found to have a CBL mutation and who responded in a dramatic fashion with lymphatic remodeling and resolution of chylous complication to MEK inhibition. Similar responses have been reported in KLA patients with RAS mutations treated with mTOR inhibitors and ARAF mutations treated with MEK inhibition. The novelty with this case report is that the putative causative mutation was in an effector that is upstream of RAS and known to be associated with Noonan's syndrome and leukemias. The report is well written. I have a few comments:  
As acknowledged, DNA analysis performed on an expanded endothelial cell population (CD31++) from chylous fluid was limited to some extent because of low allele frequency and scarcity of sample.

*Yes thank you, as noted above to reviewer #1: CD31 selection was chosen to select for all endothelial cells, which would include lymphatic cells including abnormal KLA cells. No further selection was completed, which is likely why the mutant allele frequency was so low, as there was a significant number of normal endothelial cells and potentially other cells such as monocytes that also stain positive for CD31. We attempted to extract DNA from the primary biopsy sample, but as the sample was obtained in 2012 it was significantly degraded and did not pass quality checks.*

The pulmonary function test results published as z-scores will be unfamiliar to most clinicians--would favor a more conventional approach (such as FEV1 in liters or percent predicted).

*Thank you for this comment, we have updated the table to absolute values and percent predicted (page 14).*

It would be interesting to know if the non-CD31+ cells in the chylous fluid were mutation positive or negative.

*Unfortunately we did not save non-CD31+ cells from the chylous fluid; we only selected CD31+ cells for expansion. However we are able to comment that all other types of cells tested including saliva, blood, normal lymph node biopsy and skin biopsy were negative for the mutation as noted on page 5.*

It should be mentioned whether the patient's requirement for intermittent drainage of chylous fluids completely resolved.

*Yes, thank you for pointing this out, the patient's requirement for intermittent drainage completely resolved and she remains without this requirement to this day. We have updated on page 6.*

It bears emphasizing the low dose therapy with MEK inhibitors may be sufficient, limiting the side effects that are known to plague treatment of melanoma and other neoplasms

*Thank you again, this is a very important point and we have added this to the discussion on page 8.*

19th May 2020

Dear Dr. Foster,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

The authors performed the requested editorial changes.

31st Jul 2020

Dear Dr. Foster,

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine pending the submission of the URL for your WES data. Please send us the URL as soon as you receive it from the NHGRI.

**YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND** ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jessica Foster

Journal Submitted to: EMBO Molecular Medicine

Manuscript Number: EMM-2020-12324

### Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

#### A- Figures

##### 1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if  $n < 5$ , the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

##### 2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/ varied/ perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g.,  $P$  values =  $x$  but not  $P$  values  $< x$ ;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

#### B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

|                                                                                                                                                                                         |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?                                                                                     | NA |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.                                                                       | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?                                                      | NA |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.                | NA |
| For animal studies, include a statement about randomization even if no randomization was used.                                                                                          | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done                                                                                                | NA |
| 5. For every figure, are statistical tests justified as appropriate?                                                                                                                    | NA |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.                                                                      | NA |
| Is there an estimate of variation within each group of data?                                                                                                                            | NA |

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<http://www.selectagents.gov/>

|                                                                                   |    |
|-----------------------------------------------------------------------------------|----|
| Is the variance similar between the groups that are being statistically compared? | NA |
|-----------------------------------------------------------------------------------|----|

### C- Reagents

|                                                                                                                                                                                                                                                                                                                                                                  |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia ( <a href="#">see link list at top right</a> ), 1DegreeBio ( <a href="#">see link list at top right</a> ). | NA |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.                                                                                                                                                                                                            | NA |

\* for all hyperlinks, please see the table at the top right of the document

### D- Animal Models

|                                                                                                                                                                                                                                                                                                                                                                                                                                          |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.                                                                                                                                                                                                                                                            | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.                                                                                                                                                                                                                                                                       | NA |
| 10. We recommend consulting the ARRIVE guidelines ( <a href="#">see link list at top right</a> ) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH ( <a href="#">see link list at top right</a> ) and MRC ( <a href="#">see link list at top right</a> ) recommendations. Please confirm compliance. | NA |

### E- Human Subjects

|                                                                                                                                                                                                                                                                                                                                                        |                                                                                                  |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| 11. Identify the committee(s) approving the study protocol.                                                                                                                                                                                                                                                                                            | "All studies were completed on an IRB approved protocol, and the patient gave informed consent." |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.                                                                                                | "All studies were completed on an IRB approved protocol, and the patient gave informed consent." |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained.                                                                                                                                                                                                                                            | NA                                                                                               |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples.                                                                                                                                                                                                                                                          | "The DNA sample from the CD31 cells was exhausted during the experimental process"               |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.                                                                                                                                                                                                                                             | NA                                                                                               |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram ( <a href="#">see link list at top right</a> ) and submit the CONSORT checklist ( <a href="#">see link list at top right</a> ) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA                                                                                               |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines ( <a href="#">see link list at top right</a> ). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.                                                                                          | NA                                                                                               |

### F- Data Accessibility

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                            |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| 18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions                                                                                                                                              | "Data will be hosted on dbGAP at NHGRI, Accession number: phs001802.v2.p1" |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad ( <a href="#">see link list at top right</a> ) or Figshare ( <a href="#">see link list at top right</a> ).                                                                                                                                                                                                                                              | NA                                                                         |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP ( <a href="#">see link list at top right</a> ) or EGA ( <a href="#">see link list at top right</a> ).                                                                                                                                                                                                                                       | "Data will be hosted on dbGAP at NHGRI, Accession number: phs001802.v2.p1" |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines ( <a href="#">see link list at top right</a> ) and deposit their model in a public database such as Biocompare ( <a href="#">see link list at top right</a> ) or JWS Online ( <a href="#">see link list at top right</a> ). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | "Data will be hosted on dbGAP at NHGRI, Accession number: phs001802.v2.p1" |

### G- Dual use research of concern

|                                                                                                                                                                                                                                                                                                                                   |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents ( <a href="#">see link list at top right</a> ) and list of select agents and toxins (APHIS/CDC ( <a href="#">see link list at top right</a> )). According to our biosecurity guidelines, provide a statement only if it could. | NA |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|