EMBO Molecular Medicine

Dysregulated mesenchymal PDGFR-β drives kidney fibrosis

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(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 29 July 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine, and please accept my apologies for the delay in replying due to my recent traveling. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. As you will see from the reports below, the referees acknowledge the interest of the study and are overall supporting publication of your work pending appropriate revisions.

Addressing the reviewers' concerns in full will be necessary for further considering the manuscript in our journal, and acceptance of the manuscript will entail a second round of review. 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.

I look forward to receiving your revised manuscript.

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

Referee #1 (Remarks for Author):

Buhl et al demonstrated that constitutively active form of PDGFR-β signalling (Foxd1Cre::Pdgfrb+/J::tdTomato mice) drives development and progression of kidney fibrosis in different mouse disease models. They used sophisticated mouse models and techniques to provide direct evidence that fibrosis per se can drive chronic organ damage and establish a model of primary fibrosis allowing specific studies targeting fibrosis progression and regression. This is the revised manuscript. I have following questions.
(1) Figure 1 title: PDGFR-β expression is upregulated in fibrotic human kidneys. It actually includes mouse kidneys.

(2) Figure 2, they need to show the evidence of activation of downstream of PDGFR-β signalling. They also need to use mesenchymal marker+/Ki67+ to calculate proliferation of mesenchymal cells in vivo.
(3) Have they quantified time-course inflammation? Inflammation usually precedes fibrosis or co-exists. If fibrogenesis precedes inflammation, it would strengthen their hypothesis that fibrosis per se can drive chronic organ damage.
(4) Figure 6, they need to use mesenchymal marker(+)/EPO(+) to show the origin of EPO.
(5) Figures 10 and 11 give confusing information.

Referee #2 (Remarks for Author):

Buhl et al report an original approach and new model to explore the role and contribution of fibrosis itself in CKD progression. This is clinically relevant since fibrosis-targeting drugs are dismissed by critics that argue that they target a late process that is secondary to kidney injury rather than causative. A wide arrange of methods and models have been used to characterize the role of PDGFRbeta and set u the fibrosis-induced CKD model.

I have only minor comments
1.figure 5. Ccr and BUN data do not match well. Ccr here and in fig 8 d, e suggests early hyperfiltration that is surprisingly associated to higher BUN in pdgfrb overactivity mice. Do the authors have serum creatinine data? is there any reason for this apparent discrepancy? Did all mice groups eat the same amount?

2. fig 7. what was the impact of imatinib on renal function?

3. fig 9. please provide full array results as suppl data

Referee #3 (Remarks for Author):

In this manuscript, Buhl and colleagues evaluated the role of Pdgfr-beta in kidney mesenchymal cells. They show that activation of the receptor is enough to drive kidney fibrosis. Importantly pharmacological inhibition of the receptor by imatinib could reverse fibrosis in the tubulointerstitium but not in the glomeruli. This model will be very useful to test new experimental treatments, which are much needed for this disease.

The study also unravels a key role for mesenchymal (but not tubular) Pdgfrb expression in kidney development. Although the data is not essential to the paper (and not mentioned in the abstract), it is a nice addition.

Overall, the manuscript includes a large amount of novel, interesting high-quality data, profoundly changing our understanding of the role of Pdgfrb in kidney development and fibrosis.

Minor details:
The amino-acid changes corresponding to "J" and "K" mutations should be mentioned.
In figure 1B, there is confusion between panels (e) and (a) in the legend.
In figure 5A, please show the data points.
In figure 10, the end of the caption "and suggested a role for STAT1" is confusing, since the text concludes that the process is STAT1-independent.
Supplementary figure 12: please correct the spelling of "Makrophages"

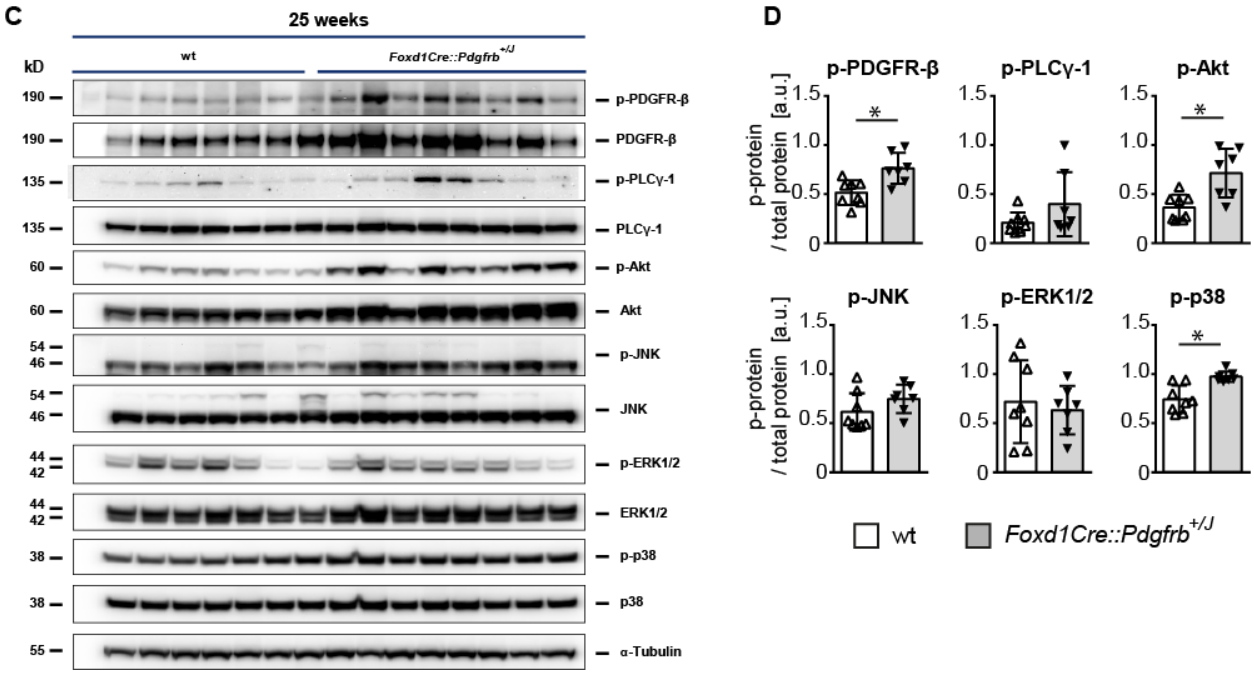
1st Revision - authors' response 23 October 2019

Referee #1 (Remarks for Author):
(1) Figure 1 title: PDGFR-β expression is upregulated in fibrotic human kidneys. It actually includes mouse kidneys.

Authors' reply: Thanks for pointing this out, we have now corrected this ("..in fibrotic human and murine kidneys").

(2) Figure 2, they need to show the evidence of activation of downstream of PDGFR-β signalling. They also need to use mesenchymal marker+/Ki67+ to calculate proliferation of mesenchymal cells in vivo.

Authors' reply: As suggested, we have performed WB analyses, showing activation of pathways downstream of PDGFRb, particularly of Akt and p38 (added as Figure EV2 in manuscript and show here).



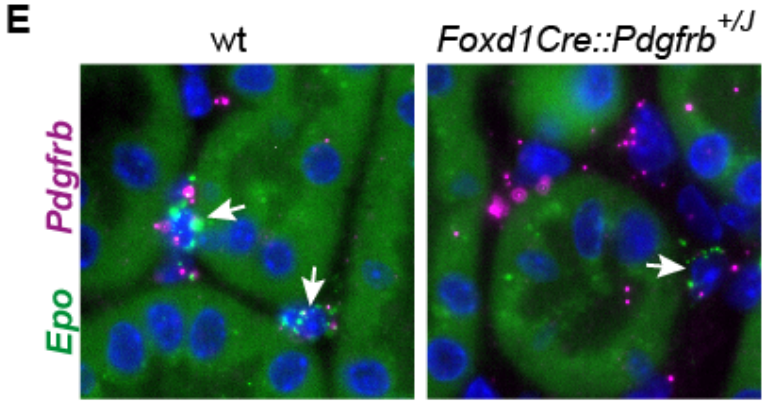
Unfortunately, even after multiple attempts using various approaches, we were not able to establish Ki67 co-staining with mesenchymal markers of sufficient specificity, which would allow rigorous enumeration.
We hope that our data on FoxD1-Tomato+ cells, which specifically mark the interstitial mesenchymal cells and show clear expansion of these cells in the mutant mice (Figure 2 F and G), and our in vitro data on proliferation of primary isolated mesangial cells and fibroblasts (Figure 2 H) are convincing enough to prove mesenchymal proliferation.

(3) Have they quantified time-course inflammation? Inflammation usually precedes fibrosis or co-exists. If fibrogenesis precedes inflammation, it would strengthen their hypothesis that fibrosis per se can drive chronic organ damage.

Authors' reply: We analyzed inflammation in the time-course in both glomeruli and interstitium (see Figures 4, Supp. Fig. 4 and 6). These data showed only a slight increase in influx of inflammatory cells (microinflammation at best), which was only observed at late time-points, coincident with tubular injury. On the contrary, fibrosis was already obvious at earliest analyzed time-point, i.e. week 6. Similarly to the reviewer, we also believe that this strengthens our hypothesis that fibrosis per se can drive organ damage.

(4) Figure 6, they need to use mesenchymal marker(+)/EPO(+) to show the origin of EPO.

Authors' reply: We now used Pdgfrb and Epo ISH to further confirm that EPO is produced by renal fibroblasts, well in line with the current dogma (see Figure 6E in the manuscript and below)

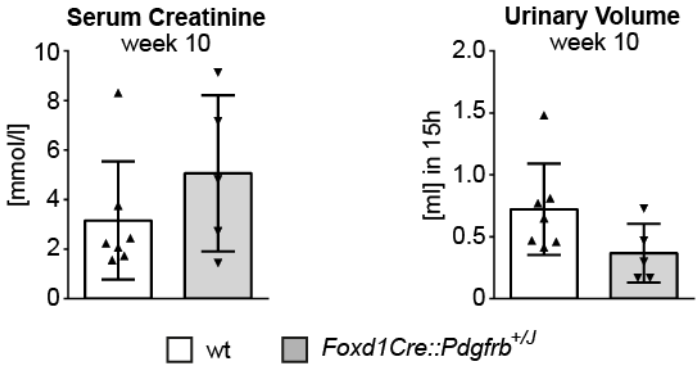


(5) Figures 10 and 11 give confusing information.

Authors' reply: Figure 10 shows the data on the experiments using an additional mouse line, with another promotor (Sox2) and another PDGFRb mutation (D849V), i.e. the Sox2Cre::Pdgfrb$^{+/K}$ mice. This line exhibits a similar phenotype as Foxd1Cre::Pdgfrb$^{+/J}$ mice, supporting our findings in two independent mice models. The figure also shows data on the role of Stat1 by using Sox2Cre::Pdgfrb$^{+/K}$::Stat1$^{-/-}$ mice. The data shown in this figure are discussed on page 15. We now also rephrased the figure legend.
Figure 11 shows the effect of Pdgfrb deletion in Foxd1Cre::Pdgfrb$^{fl/fl}$ mice, which resulted in failed glomerulogenesis and associated early postnatal death due to kidney failure. These data confirm the essential role of Pdgfrb signaling in normal embryonic development.
The data shown in this figure are discussed on page 16.
We hope that now, together with the discussion of the data, the information of the figures is less confusing.

Referee #2 (Remarks for Author):
I have only minor comments
1.figure 5. Ccr and BUN data do not match well. Ccr here and in fig 8 d, e suggests early hyperfiltration that is surprisingly associated to higher BUN in pdgfrb overactivity mice. Do the authors have serum creatinine data? Is there any reason for this apparent discrepancy? Did all mice groups eat the same amount?

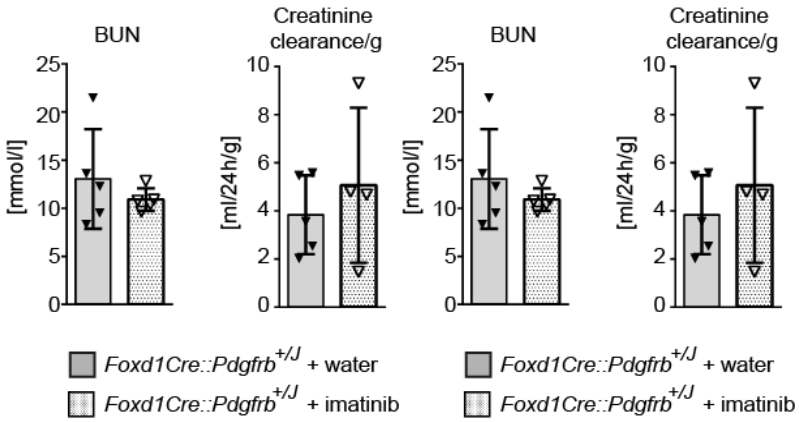
Authors' reply: The data on differences in creatinine clearance at early time-points might seem somewhat higher in the mutant mice, but were not statistically significant. Similarly, the serum creatinine values were not significantly different between the groups at this age (see figure below). At the same time the urinary volume was somewhat smaller in the Foxd1Cre::Pdgfrb$^{+/J}$ mice (not statistically significant). We therefore do not think that these mice might have an early hyperfiltration.



Unfortunately, we have not measured the food intake in these mice.

2. fig 7. what was the impact of imatinib on renal function?

Authors' reply: We haven't seen any significant impact on renal function (see figure below), most likely due to relatively short treatment duration combined with rather mild renal function decline in the Foxd1Cre::Pdgfrb$^{+/J}$ at this age.



3. fig 9. please provide full array results as suppl data

Authors' reply: To comply with the reviewer's suggestion and the journal regulations, we have submitted our data to the GEO repository. The reference to this database is now also included in the data availability section (Page 31).

Referee #3 (Remarks for Author):
Minor details:
The amino-acid changes corresponding to "J" and "K" mutations should be mentioned.

Authors' reply: PDGFRβ J mutant isoform has a V536A mutation in the juxtamembrane domain, whereas the PDGFRβ K mutant isoform is a D849V mutation, which is located to the kinase domain. Both amino acid changes are now mentioned in the main text on page 7 and 15:

Page 7: "To generate mice with a renal mesenchymal cell-specific, constitutively active PDGFR-β, we used mice in which one wt Pdgfrb allele was substituted by a conditional knock-in of Pdgfrb with an activating point mutation (V536A) in the juxtamembrane domain of PDGFR-β, denoted as "J" (Pdgfrb$^{+/J}$), behind a floxed STOP cassette 33."

Pare 15: "We also investigated Sox2Cre::Pdgfrb$^{+/K}$ mice, which bear a different activating PDGFR-β point mutation (D849V) in the kinase domain (Pdgfrb$^{+/K}$) compared to the juxtamembrane mutation in PDGFRb$^{+/J}$ mice."

In figure 1B, there is confusion between panels (e) and (a) in the legend.

Authors' reply: We are sorry for this, now corrected.

In figure 5A, please show the data points.

Authors' reply: As requested, we now show the data points in this figure.

In figure 10, the end of the caption "and suggested a role for STAT1" is confusing, since the text concludes that the process is STAT1-independent.

Authors' reply: Thanks, we have now corrected and rephrased it:

Figure 10: "Glomerular pathology in Sox2Cre::Pdgfrb+/K mice resembled the findings in Foxd1Cre::Pdgfrb+/J but suggested no role for STAT1"

Supplementary figure 12: please correct the spelling of "Makrophages"

Authors' reply: Thanks for pointing this out, now corrected.

2nd Editorial Decision 7 November 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have received the referees' reports, and as you will see they are now supportive of publication of your study. I am therefore pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments.

I look forward to reading a new revised version of your manuscript as soon as possible.

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

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

No more questions.

Referee #1 (Remarks for Author):

It is an original research manuscript but it is not a research report in that it doesn't contain a specific finding, model or methodology with a high impact on the field of molecular medicine.

Referee #2 (Remarks for Author):

My concerns have been addressed

2nd Revision - authors' response 8 December 2019

Authors made the requested editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Peter Boor
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-11021

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.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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?	Sample size was kept small regarding the 3R guidelines. Sample sizes are based on previous similar and preliminary experiments that resulted in statistical significant results.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	As in point 1a, the sample sizes were based on previous similar and preliminary experiments that resulted in statistical significant results.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the study. Only single urine samples were excluded if they were contaminated with feces.
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.	Whenever possible, particularly in most analyses like histomorphological or immunofluorescence etc, investigator was blinded to e experimental groups to reduce bias. Animal randomization is described below.
For animal studies, include a statement about randomization even if no randomization was used.	We took consecutive available transgenic mice and littermates. For treatment studies, such as imatinib or UUO and angiotensin infusion, mice were randomly assigned to treatment vs. placebo or model vs. No-model.
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.	Please see above point 3.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Please see above point 3.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was assumed (see dot blotswithin the manuscript).

Is there an estimate of variation within each group of data?	From previous studies we used this to calculate sample size as described above.
Is the variance similar between the groups that are being statistically compared?	Variances between groups can be recognized by standard deviation bars given for all analyzes and was comparable in majority of the datasets.

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 (see link list at top right), 1DegreeBio (see link list at top right).	Companies and catalog numbers or clone numbers are listed for every antibody used in this study in a separate table. All are well established and assessed for specificity in our lab using a number of negative and positive controls.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Only primary isolated cells were used in this study. We perfrom mycoplasma testing regularly in our lab, and no contamination was detected in the last years.

* 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.	Mouse strains: FoxD1GC, Pax8Cre, tdTomato-reporter mice (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J), 129S4/SvJae-Pdgfrbtm11Sor/J (017622), Sox2-Cre, STAT1flox, Pdgfrb-eGFP mice (Tg(Pdgfrb-EGFP)JN169Gsat/Mmucd), Pdgfrb(S)J and Pdgfrb(S)K. Male and female mice of an age between 8 days and 35 weeks were used for the experiments. Animals were held in SFP1 conditions in rooms with constant temperature and humidity, 12h / 12h light cycle and had ad libidum access to food and water.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the local review boards and federal authorities of North Rhine-Westphalia, state of Germany.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (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 (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Animal studies were performed in line with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The study was approved by the local review boards of Aachen
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.	Statement is included in the method section.
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.	NA
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 (see link list at top right) and submit the CONSORT checklist (see link list at top right) 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 (see link list at top right). 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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All used datasets are available and accession codes are provided in the "Data Availability" section.
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 (see link list at top right) or Figshare (see link list at top right).	Source data are provided for all main figures.
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 (see link list at top right) or EGA (see link list at top right).	Data of human microarrays are available at GEO database, see "Data Availability" section
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 (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No