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Neighboring mutation-mediated enhancement of dengue virus infectivity and spread

Lu Chen, Xianwen Zhang, Xuan Guo, Wenyu Peng, Yibin Zhu, Zhaoyang Wang, Xi Yu, Huicheng Shi, Yuhan Li, Liming Zhang, Lei Wang, Penghua Wang, and Gong Cheng
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Corresponding author(s): Gong Cheng (gongcheng@mail.tsinghua.edu.cn)

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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. 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.)

Dear Prof. Cheng

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, all three referees consider the findings of general interest and overall conclusive and support publication after some minor issues have been addressed.

Given these positive evaluations and constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (October 19th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Your manuscript will be published in our Reports section and therefore the results and discussion sections must be combined.

*****IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.*****

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends

in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

9) Figure legends and data quantification:

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

10) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This work investigates the association between envelop protein mutation and prevalence of dengue virus infection. PI collected 18715 E protein sequences and identify four sites 27, 32, 34, and 43 which appear in dengue variants. Authors further identify 10 mutations in effective variants which are low infectivity in 1995 become prevalent in 2016-2020. Among these mutations, dengue variants with double mutations (226K and 228E) have higher infectivity in vitro and in vivo. Compared to wild type strain, the double mutants displayed higher binding affinity to mosquito C-type lectins (mosGCTLs) and human C-type lectin DC-SIGN. As these C-type lectins are critical in dengue virus infectivity to mosquito and human cells, these observations nicely explain the mechanism of increased infectivity of double mutants in mouse model and human population. The approach is straightforward and the data are convincing. This work may be helpful to predict dengue infectivity in the future.

Referee #2:

The authors describe, in my view, a detailed study of broad interest regarding the molecular forecasting of future DENV epidemics. I do not have expert knowledge of the experimental methods and will restrict my comments to the phylogenomic analysis and presentation of the main text. While I hope the authors will consider my comments below, which I believe will increase the impact of the work, it is my opinion that the work is suitable for publication as written.

The authors begin by selecting residues of interest based on temporal trends in the frequency among samples collected, "we chose the effective variants, in which the occurrence frequency progressively increased from less than 20% (<20%) before 1995 to more than 80% (>80%) in 2016-2020, as the stable substitutions representing a dominant amino acid fixed or almost fixed in the DENV contemporary isolates". I believe this approach is reasonable for the current work; however, the authors may consider emphasizing in the main text that this approach does not distinguish the fixation of "founder" or "passenger" mutations from adaptive mutations which have played an important role in flavivirus epidemics, in particular ZIKA https://www.nature.com/articles/s41467-020-20747-3. The latter are difficult to predict from phylogenomics alone given the (low) number and distribution of early samples https://www.pnas.org/doi/10.1073/pnas.2121335119 (my own work, so I am biased).

Much of the remainder of the text is focused on the impact of two particular residues, envelope 226K and 228E. While I acknowledge the impact of each substitution is genotype specific as the authors discuss, for the sake of simplicity, I will not make subtype distinctions below. The authors describe these residues as "cooperative", in summary, describing the following dynamics: 226K emerged first in circulating variants and decreases infectivity in mammalian hosts, 228E emerged later and increases infectivity, and the double substitution also increases infectivity relative to the ancestral variant. If DENV were not vector-borne, I believe 228E would be adequately described in this case as a "compensatory" mutation which may either act additively or epistatically in relation to 228E. However, DENV is vector born and the stated finding that "The DENV load was higher in mosquitoes infected by the DENV2 mutants with a T226K substitution compared with that of the parental 16681 WT strain (Fig 3H)" complicates this landscape. I encourage the authors to devote additional material in the main text describing the range of potential epistatic relationships between sites 228 and 226, which may be supportive or as this finding suggests contrasting, among mammals and mosquitos. For example, one potential explanation for the pattern observed is that 226K emerged first due to a vector population bottleneck and increased selective pressures for vector infectivity at the cost of host infectivity (one vector may make contact with many hosts). Later, the compensatory mutation 228E emerged as a result. Alternatively, 226K may have simply been the result of a viral population bottleneck as described above.

More generally, I feel the text could be reshaped to put a greater emphasize on context and motivation and less on the description of specific experimental results. The authors do an admirable job corroborating their claims of functional importance in multiple model systems (cell line, host, and vector) as well as structural analysis; however, I believe some of this material could be moved to the supplement. In the introduction, the authors state, "Furthermore, our study forecasted that a potential clade turnover mediated by the DENV2 Cosmopolitan genotype might lead to a potential forthcoming dengue epidemic". I think it would be great if the authors expanded their discussion of this generalizable trend in the main text. As the authors already suggest, shifting social and environmental factors are reshaping the ways virus subtypes interact, which can generate new selective pressures and provide the impetus for new outbreaks (again, I am biased because it is my own work, but this is something discussed here for many human RNA viruses including DENV: https://www.pnas.org/doi/10.1073/pnas.2121335119). Similarly, the authors may consider expanding the discussion to incorporate related "cooperative" or epistatically linked mutations which played a key role in facilitating prior epidemics (e.g. in ebola: https://pubmed.ncbi.nlm.nih.gov/27814505/ and preliminary investigations of epistasis within SARS-CoV-2, again my work so I am biased, https://journals.asm.org/doi/10.1128/mbio.00135-22).

I restate that in my view this is a detailed study of broad interest and suitable for publication as written.
Sincerely,
Nash Rochman (received and reviewed 6/30/22)

Minor comments:

I find this statement vague:
Thus, natural selection is a force to drive the increase in DENV fitness in their native hosts, thereby promoting disease circulation and dynamics (Vasilakis and Weaver, 2008).

I find this statement unclear as written. Does this refer to the experimental preparation for this work or a lack of sequencing information in general?
Nonetheless, the infectious clone of DENV1 Hawaii strain was not successfully rescued.

I find this statement unclear as written. The authors state this residue increases vector load so while it may decrease the probability of vector uptake due to reduced host load, I am not sure that it follows this residue confers reduced vector transmissibility.

potentially because the T226K mutant was less infectious in mammalian hosts and thus less transmissible to mosquitoes

I do not understand this rationale (apart from, an understandable, minimization of experimental cost). In other systems the authors assess the impact of both single substitutions and the double substitution to determine the apparent "cooperative" and perhaps epistatic relationship. Whether each single substitution occurs naturally is not strictly relevant and revealing the characteristics of 228E would be informative here.

Since the cluster with single G228E substitution did not exist in native isolates of the DENV2 Asian I genotype (Fig 3A), we infected AG6mice with 2,500 p.f.u. of the mutants existing in nature (16681-T226K and 16681-T226K/G228E) and the parental 16681 strain via a subcutaneous injection into one footpad.

I do not have any technical comments regarding the validity of the modelling approaches used; however, I do believe the text would be improved if the authors stated in the main text the rational for using these three distinct approaches. I would personally prefer to read in greater detail in the main text how one method is applied and move the supporting findings of the other two to the supplement.

we modeled the annual occurrence frequency (AOF) of the T226K and G228E substitutions in the DENV2 Cosmopolitan genotype through 3 independent mathematic approaches, including the numerical kinetics model, support vector machine regression (SVR), and Gaussian process regression (GPR-1)

Referee #3:

Chen et al. assessed the fitness advantage of substitutions in the E protein in mosquitoes and in mice. This study further shows that the substitutions T226K and G228E in combination enhanced infectivity in mosquitoes and mammalian hosts. This study proposes that epistatic interaction exists between the two positions, acquisition of T to K at amino acid position 226 could drive the acquisition of G to E at position 228. The experiments are well-designed and offers important insights to viral factors at play during dengue epidemics.

Some minor comments:
1. In discussion, need to acknowledge that like Asian genotype, the Cosmopolitan genotype especially in recent years has been responsible for many dengue epidemics. Also, what is the role of other sites of mutational frequency >5% which does not get selected for over the years in dengue evolution?
2. In Figure 2, is there a reason why oral feeding of mosquitoes were not performed?
3. Why would BIDI170T and PF89-L301S increase DENV loads in mosquitoes? Can elaborate in discussion?
4. For Figure 3, in mosquito-host-mosquito transmission cycle, why is DENV load of T226K mutant not increased compared to wild-type? Would we also see no difference in DENV load if we use an artificial blood meal to infect mosquitoes with this mutant?
5. In Fig 3L, why is there no difference for day 5 and 8 post infection?
6. Is AAEL011408 known to be an entry receptor for DENV in mosquitoes?
7. For Fig 4G, can the authors give a reason why T226K/G228E presented higher binding when G228E does not influence binding to C-type lectin?
8. Has the method used in Figure 5 been published before? Are there any references from which this method was based on?

Dear Editors,

Thank you for the comments on our manuscript (EMBOR-2022-55671V2*)* titled "*Neighboring mutation-mediated enhancement of dengue virus infectivity and spread*". We have taken all of the comments into consideration and have revised our manuscript accordingly. We believe that our revised analyses and modifications of the text have substantially improved the manuscript.

We were encouraged by the positive comments from editor and reviewers, such as "*all three referees consider the findings of general interest and overall conclusive and support publication after some minor issues have been addressed*" (Editor), "*these observations nicely explain the mechanism of increased infectivity of double mutants in mouse model and human population. The approach is straightforward and the data are convincing. This work may be helpful to predict dengue infectivity in the future.*" (Reviewer #1), "*The authors describe, in my view, a detailed study of broad interest regarding the molecular forecasting of future DENV epidemics.*" (Reviewer #2), "*This study proposes that epistatic interaction exists between the two positions, acquisition of T to K at amino acid position 226 could drive the acquisition of G to E at position 228. The experiments are well-designed and offers important insights to viral factors at play during dengue epidemics.*" (Reviewer #3). The reviewers also provided important suggestions to improve the manuscript. We have revised our manuscript per the reviewers' suggestions. Along with this letter, we provide point-by-point responses to the queries raised by the reviewers.

Responses to Editor's Comments:

#1. *Your manuscript will be published in our Reports section and therefore the results and discussion sections must be combined.*
Answer: According to the requirement, we have combined the results and discussion sections together.

#2. *A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.*
Answer: We have added the "*Data Availability*" section after the Materials & Methods (Line 704-705, Page 27).

#3. *Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.*
Answer: The revised manuscript meets the statistic requirement.

#4. *A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.*

Answer: We have uploaded a revised manuscript with a track-mode, in which the changes are highlighted to be clearly visible.

#5. *Individual production quality figure files as .eps, .tif, .jpg (one file per figure).*
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178 /authorguide for more info on how to prepare your figures.
Answer: The figures have been prepared according to the Figure Preparation Guidelines.

#6. *A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.*
Answer: We have provided a point-by-point response letter to the editorial and reviewers' comments.

#7. *A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF*.
Answer: We have provided a completed checklist according to the requirement.

#8. *Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (https://www.embopress.org/page/journal/14693178/ authorguide#authorshipguidelines)*
Answer: We have linked the ORCID ID to my account in the manuscript tracking system.

#9. *We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.*
Answer: We have provided 5 Expanded View Figures in the revised manuscript.

#10. *For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: https://www.embopress.org/page/journal/ 14693178/authorguide#expandedview*

Answer: We have provided an Appendix file with the manuscript. The Appendix figures have been referred to in the main text.

#11 *Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.*
Answer: This item is not relevant for our manuscript.

#12 *Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database. See also (https://www.embopress.org/page/journal/14693178/authorguide#dataavailability).*
Please note that the Data Availability Section is restricted to new primary data that are part of this study.
Answer: We have added the "*Data Availability*" section after the Materials & Methods in the revised manuscript (Line 704-705, Page 27).

#13 *The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests*
Answer: We have added the "*Disclosure and competing interests statement*" in the revised manuscript. The statement mentioned that the authors declare that they have no conflict of interest (Line 730-731, Page 30).

#14 Figure legends and data quantification:
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
See also the guidelines for figure legend preparation: https://www.embopress.org/page/journal/14693178/authorguide#figureformat
- Please also include scale bars in all microscopy images.
Answer: We have revised the figure legends to meet the requirements of the guidelines for figure legend preparation.

#15 *We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files areavailable https://www.embopress.org/page/journal/14693178/authorguide#source data.*
Answer: We have provided documents including the source data for figure panels (*Source Data and Source Data for Expanded View and Appendix*).

#16 Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat.
Answer: This item is not relevant for our manuscript.

#17 *As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.*
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."
Answer: We agree to publish the Review Process File with the manuscript.

Responses to Referee #1

#1A. *This work investigates the association between envelop protein mutation and prevalence of dengue virus infection. PI collected 18715 E protein sequences and identify four sites 27, 32, 34, and 43 which appear in dengue variants. Authors further identify 10 mutations in effective variants which are low infectivity in 1995 become prevalent in 2016-2020. Among these mutations, dengue variants with double mutations (226K and 228E) have higher infectivity in vitro and in vivo. Compared to wild type strain, the double mutants displayed higher binding affinity to mosquito C-*

type lectins (mosGCTLs) and human C-type lectin DC-SIGN. As these C-type lectins are critical in dengue virus infectivity to mosquito and human cells, these observations nicely explain the mechanism of increased infectivity of double mutants in mouse model and human population. The approach is straightforward and the data are convincing. This work may be helpful to predict dengue infectivity in the future.
#1A. Answer: We thank the reviewer's comments.

Responses to Referee #2:

#2A. *The authors begin by selecting residues of interest based on temporal trends in the frequency among samples collected, "we chose the effective variants, in which the occurrence frequency progressively increased from less than 20% (<20%) before 1995 to more than 80% (>80%) in 2016-2020, as the stable substitutions representing a dominant amino acid fixed or almost fixed in the DENV contemporary isolates". I believe this approach is reasonable for the current work; however, the authors may consider emphasizing in the main text that this approach does not distinguish the fixation of "founder" or "passenger" mutations from adaptive mutations which have played an important role in flavivirus epidemics, in particular ZIKA https://www.nature.com/articles/s41467-020-20747-3. The latter are difficult to predict from phylogenomics alone given the (low) number and distribution of early samples https://www.pnas.org/doi/10.1073/pnas.2121335119 (my own work, so I am biased).*
#2A Answer: Thanks for reviewer's suggestion. We have emphasized that the approach did not distinguish the fixation of "founder" or "passenger" mutations from adaptive mutations (Liu et al., 2021, Nat Commun 12, 595; Rochman et al., 2022, EMBO Rep 23, e55393; Weaver et al., 2021, Nat Rev Microbiol 19, 184-195) due to the inadequate number and distribution of early sequences (Mutz et al., 2022, Proc Natl Acad Sci U S A 119, e2121335119) in the revised manuscript (Line 98-101, Page 5).

#2B. *Much of the remainder of the text is focused on the impact of two particular residues, envelope 226K and 228E. While I acknowledge the impact of each substitution is genotype specific as the authors discuss, for the sake of simplicity, I will not make subtype distinctions below. The authors describe these residues as "cooperative", in summary, describing the following dynamics: 226K emerged first in circulating variants and decreases infectivity in mammalian hosts, 228E emerged later and increases infectivity, and the double substitution also increases infectivity relative to the ancestral variant. If DENV were not vector-borne, I believe 228E would be adequately described in this case as a "compensatory" mutation which may either act additively or epistatically in relation to 228E. However, DENV is vector born and the stated finding that "The DENV load was higher in mosquitoes infected by the DENV2 mutants with a T226K substitution compared with that of the parental 16681 WT strain (Fig 3H)" complicates this landscape. I encourage the authors to devote additional*

material in the main text describing the range of potential epistatic relationships between sites 228 and 226, which may be supportive or as this finding suggests contrasting, among mammals and mosquitos. For example, one potential explanation for the pattern observed is that 226K emerged first due to a vector population bottleneck and increased selective pressures for vector infectivity at the cost of host infectivity (one vector may make contact with many hosts). Later, the compensatory mutation 228E emerged as a result. Alternatively, 226K may have simply been the result of a viral population bottleneck as described above.

#2B Answer: According to the reviewer's suggestion, we have replaced "cooperative" to "compensatory" in the revised manuscript (Line 143, Page 7; Line 165, Page 8; Line 181, Page 8; Line 190, Page 8; Line 198, Page 9, Line 201, Page 9; Line 207, Page 9; Line 229, Page 10; Line 272, Page 11; Line 283, Page 12, Line 301, Page 12; Line 320, Page 13; Line 378, Page 15; Line 958, Page 38; Line 986, Page 39; Line 1013, Page 40).

Our results suggest a potential epistatic relationship between the substitutions at the 226th and 228th sites. Indeed, the process of viral evolution is usually dominated by effective natural selection when viral populations are large. However, DENVs are mosquito-borne RNA viruses prone to mutation. A viral population bottleneck in mosquitoes could enable DENVs vulnerable to genetic drift and fitness loss (Weaver et al., 2021, Nat Rev Microbiol 19, 184-195). Our results indicate that the T226K substitution rendered DENV2 more transmissible by mosquitoes, but less infective in mammals. A potential explanation for this kind of compensatory substitutions is that the T226K mutation could emerge first due to a DENV2 population bottleneck in mosquitoes at the cost of host infectivity in mammalian hosts. However, the sequentially compensatory G228E substitution reversed the T226K-mediated reduction of DENV2 infectivity in mammalian hosts, thereby enabling the DENV2 with the T226K and G228E double substitutions to rapidly disseminate in nature. We have added this discussion to the revised manuscript (Line 168-179, Page 8).

#2C. *More generally, I feel the text could be reshaped to put a greater emphasize on context and motivation and less on the description of specific experimental results. The authors do an admirable job corroborating their claims of functional importance in multiple model systems (cell line, host, and vector) as well as structural analysis; however, I believe some of this material could be moved to the supplement. In the introduction, the authors state, "Furthermore, our study forecasted that a potential clade turnover mediated by the DENV2 Cosmopolitan genotype might lead to a potential forthcoming dengue epidemic". I think it would be great if the authors expanded their discussion of this generalizable trend in the main text. As the authors already suggest, shifting social and environmental factors are reshaping the ways virus subtypes interact, which can generate new selective pressures and provide the impetus for new outbreaks (again, I am biased because it is my own work, but this is something discussed here for many human RNA viruses including DENV: https://www.pnas.org/doi/10.1073/pnas.2121335119). Similarly, the authors*

may consider expanding the discussion to incorporate related "cooperative" or epistatically linked mutations which played a key role in facilitating prior epidemics (e.g. in ebola: https://pubmed.ncbi.nlm.nih.gov/27814505/ and preliminary investigations of epistasis within SARS-CoV-2, again my work so I am biased, https://journals.asm.org/doi/10.1128/mbio.00135-22).
#2C Answer: According to the reviewer's suggestion, we have removed the additional description of specific experimental results and procedures in the revised manuscript (Line 118, Page 6; Line 163, Page 7; Line 182, Page 8; Line 186, Page 8).

The epidemiological surveillance discovered that the single T226K substitution had sporadically appeared in the DENV2 Cosmopolitan genotype. We therefore speculated that the G228E substitution might sequentially occur in the Cosmopolitan genotype under the evolutionary pressure of natural selection and fitness adaptation. The bioinformatic modeling further suggested that these epistatic adaptations might impel a major emergence of the DENV2 Cosmopolitan genotype in the near future. Indeed, epistatic mutations play an important role in shaping the emergence, reemergence and spread of many human viruses. For example, epistatic substitutions in the Glycoprotein of Ebola virus have increased its tropism for human cells, which may contribute to the wide geographic distribution of specific viral lineages (Urbanowicz et al., 2016, Cell 167, 1079-1087). The epistatic mutations in the receptor-binding domain (RBD) of SARS-CoV-2 variants affect the interaction between RBDs and neutralizing antibodies, leading to vaccine escape (Rochman et al., 2022, mBio 13, e0013522; Rochman et al., 2021, Proc Natl Acad Sci USA 118, e2104241118). Accumulating evidence indicates that epistasis-mediated selective evolutionary pressures may provide the impetus for new outbreaks of human viruses. We have added this discussion in the revised manuscript (Line 336-344, Page 13-14).

#2D. *I find this statement vague: Thus, natural selection is a force to drive the increase in DENV fitness in their native hosts, thereby promoting disease circulation and dynamics (Vasilakis and Weaver, 2008).*
#2D Answer: We have revised the sentence to "*A high rate of intrinsic mutation of DENV results in genetic diversity, thereby promoting disease circulation and dynamics (Vasilakis and Weaver, 2008, Adv Virus Res 72, 1-76).*" (Line 66-67, Page 4).

#2E. *I find this statement unclear as written. Does this refer to the experimental preparation for this work or a lack of sequencing information in general?*
Nonetheless, the infectious clone of DENV1 Hawaii strain was not successfully rescued.
#2E Answer: We recognize the reviewer's concern. Because of the high toxicity of flavivirus proteins to *E. coli* (the engineering bacteria for viral genome assembly), the generation of infectious flavivirus clones is always a challenge in the field (Münster et al., 2018, Viruses 10, 368; Pu et al., 2011, J Virol 85, 2927-41; Suzuki et al., 2007, Virology 362, 374-83). The stability and efficacy of a viral cDNA construction in *E. coli* are largely dependent on the specific viral genome sequences. As mentioned in the

manuscript, the genome of DENV1-I Hawaii strain was divided into four cDNA fragments for separate synthesis. However, the infectious clone was not successfully constructed in *E. coli*. We will address this technical issue in a future investigation. We have added the statement in the revised manuscript (Line 112-113, Page 6; Line 460-464, Page 18).

#2F. *I find this statement unclear as written. The authors state this residue increases vector load so while it may decrease the probability of vector uptake due to reduced host load, I am not sure that it follows this residue confers reduced vector transmissibility.*
potentially because the T226K mutant was less infectious in mammalian hosts and thus less transmissible to mosquitoes
#2F Answer: We have removed this sentence from the revised manuscript (Line 147, Page 7).

#2G. *I do not understand this rationale (apart from, an understandable, minimization of experimental cost). In other systems the authors assess the impact of both single substitutions and the double substitution to determine the apparent "cooperative" and perhaps epistatic relationship. Whether each single substitution occurs naturally is not strictly relevant and revealing the characteristics of 228E would be informative here.*
Since the cluster with single G228E substitution did not exist in native isolates of the DENV2 Asian I genotype (Fig 3A), we infected AG6mice with 2,500 p.f.u. of the mutants existing in nature (16681-T226K and 16681-T226K/G228E) and the parental 16681 strain via a subcutaneous injection into one footpad.
#2G Answer: Our results from multiple experiments have demonstrated an epistatic relationship between T226K and G228E mutations (Figure 2A, Figure 2E, Figure 2I-J, Figure 4B-E, and Figure 4G). In the above-mentioned experiments (Figure 3A-L), we aimed to assess if T226K and T226K/G228E mutations (naturally occurring) impacted DENV transmission and prevalence of DENV2 Asian I strains via both mosquito and AG6 mouse models. We did not include the single G228E mutant here since it does not exist in nature; rather it always appears together with T226K.

#2H. *I do not have any technical comments regarding the validity of the modelling approaches used; however, I do believe the text would be improved if the authors stated in the main text the rational for using these three distinct approaches. I would personally prefer to read in greater detail in the main text how one method is applied and move the supporting findings of the other two to the supplement.*
we modeled the annual occurrence frequency (AOF) of the T226K and G228E substitutions in the DENV2 Cosmopolitan genotype through 3 independent mathematic approaches, including the numerical kinetics model, support vector machine regression (SVR), and Gaussian process regression (GPR-1)
#2H Answer: To address this possibility, we modeled the annual occurrence frequency (AOF) of the T226K and G228E substitutions in the DENV2 Cosmopolitan genotype through a Gaussian process regression approach (GPR-1), which is flexible and

precise for modeling time-series data with limited sample sizes (Kong et al., 2018, Mech Syst Signal Process 104, 556-574; Cheng et al., 2019, Nat Commun 10, 1798) (Fig 5A). We trained the GPR-1 model with available sequences of the DENV2 Asian I genotype from 1995 to 2019 to predict the AOF of the T226K and G228E substitutions in the DENV2 Cosmopolitan genotype (Figure 5A and Appendix Table S2). In addition to the GPR-1 model, we employed two parallel approaches, the numerical kinetics and support vector machine regression (SVR) models, as independent verification of the predictions (Cheong et al., 2022, Nat Commun 13, 774; Thornburg et al., 2022, Cell 185, 345-360) (Figure EV5A and B). We then validated these models using the available DENV2 Asian I genotype sequences. We have revised this part according to the reviewer's comment (Figure 5A, Figure EV5A and B, and Appendix Table S2) (Line 305-314, Page 12-13).

Responses to Referee #3:

#3A. *In discussion, need to acknowledge that like Asian genotype, the especially in recent years has been responsible for many dengue epidemics.*
#3A Answer: According to the reviewer's suggestion, we emphasized the importance of DENV2 Cosmopolitan genotype to dengue epidemics in recent years. The added content is "Like the Asian I genotype, the Cosmopolitan genotype has been responsible for many dengue outbreaks especially in recent years. (Chen and Vasilakis, 2011, Viruses 3, 1562-608)." (Line 298-300, Page 12).

#3B. *Also, what is the role of other sites of mutational frequency >5% which does not get selected for over the years in dengue evolution?*
#3B Answer: In this study, the sites with a mutational frequency greater than 5% were defined as the positions of effective variants. Of note, there were 41, 27, 32, and 34 sites meeting the criteria for effective variants in the four DENV serotypes, respectively (Figure EV1A-K). We next assessed the association between the occurrence of a mutation and the time of tracking. Ten stable substitutions, which represent dominant amino acids fixed or almost fixed in the DENV contemporary isolates, were identified in 4 DENV serotypes for further investigation (Figure 2A-H). However, the remaining substitutions with a mutational frequency >5% do not stably exist in the DENV contemporary isolates. We speculate that a strong evolutionary pressure to impel the variations of these remaining sites. We would further assess the role of these remaining substitutions with a mutational frequency >5% in DENV infectivity and transmission in a future study.

#3C. *In Figure 2, is there a reason why oral feeding of mosquitoes were not performed?*
#3C Answer: We recognize the reviewer's concern. The approach of thoracic microinjection has been commonly used to assess the infectivity of arboviruses in mosquitoes (Cheng et al., 2010, Cell 142, 714-25; Goenaga et al., 2015, Viruses 7, 5801-12; Hussain et al., 2013, J Virol 87, 851-8; Liu et al., 2014, PloS Pathog 10, e1003931; Liu et al., 2017, J Virol 91, e01348-16). This approach delivers the same

number of virions to each mosquito, while the numbers of virions acquired by oral feeding vary with each mosquito. Nonetheless, we further validated the role of T226K mutation in the infectivity of DENV2-Asian I 16681 strain in mosquitoes infected by oral feeding (Figure 3H).

#3D. *Why would BIDI170T and PF89-L301S increase DENV loads in mosquitoes? Can elaborate in discussion?*

#3D Answer: Our result showed that the DENV loads in mosquitoes were significantly enhanced by three substitutions including T226K in the DENV2-Asian I 16681 strain (16681-T226K), I170T in the DENV2-Asian American BID strain (BID-I170T), and L301S in the DENV3-I PF89 strain (PF89-L301S), compared with their parental DENVs (Figure 2A-C). However, the mechanism by which the BID-I170T and PF89-L301S promote viral infectivity is unknown. Both the 170th and 301st residues are located in the envelope (E) protein that mediates virion attachment and entry into mosquito and host cells. Of note, the 301st residue is located in domain III that interacts with DENV receptors, thus the L301S mutation could enhance DENV virion binding to its attachment factors/receptors such as C-type lectins (Cheng et al., 2010, Cell 142, 714-25), prohibitin (Kuadkitkan et al., 2010, Virology 406, 149-61), heat-shock related proteins (Salas-Benito et al., 2007, Am J Trop Med Hyg 77, 283-90) and laminin-binding protein (Sakoonwatanyoo et al., 2006, Intervirology 49, 161-72). Alternatively, these mutations could regulate E fusion with host endolysosomal membrane and viral genome release. Indeed, a previous study showed that several mutations around the N153 glycosylation site (including I170T) enhanced DENV infection in mosquitoes (Figure 5 in Dolan et al., 2021, Elife 10, e61921). Intriguingly, abrogation of N153 glycosylation greatly increased the DENV infectivity in mosquito C6/36 cells by regulating virion release (Lee et al., 2010, J Virol 84, 5171-80), implicating that the I170T mutation might promote the DENV infection through modulating the N153-glycosylation-mediated interaction between DENV virions and mosquito cells. We have added the discussion in the revised manuscript (Line 346-363, Page 14).

#3E. *For Figure 3, in mosquito-host-mosquito transmission cycle, why is DENV load of T226K mutant not increased compared to wild-type? Would we also see no difference in DENV load if we use an artificial blood meal to infect mosquitoes with this mutant?*

#3E Answer: In the experimental setting of mosquito-host-mosquito transmission cycle, we thoracically microinjected an equal titer (10 p.f.u.) of various DENV2 strains into mosquitoes. Over a complete mosquito-host-mosquito transmission cycle, the infection rates of mosquitoes feeding on the infected mice were calculated to assess the adaptation advantage of stable substitutions. The viral loads of 16681-T226K were significantly higher than its parental strain in mosquitoes (Figure 2A), while its viremia was lower than its parental strain in AG6 mice (Figure 2E). The contrasting effects of T226K substitution on DENV2 infectivity in mammals and mosquitoes resulted in equal infection rate in the last step (i.e., naive mosquitoes who had a blood meal from infected mice) of a complete mosquito-host-mosquito transmission cycle (Figure 3K-I).

Furthermore, we assessed both the infectivity and prevalence of the T226K mutant in *A. aegypti* mosquitoes by an artificial blood meal. Mosquitoes were infected with 5 × 10^5 p.f.u. mL^{-1} of each mutant and parental DENVs through blood feeding (Figure 3G). Eight days postinfection, the DENV loads in mosquitoes were determined by qRT–PCR. The infection rate of 16681-T226K were higher than its parental 16681 WT strain in mosquitoes (Figure 3H).

#3F. *In Fig 3L, why is there no difference for day 5 and 8 post infection?*

#3F Answer: In this experiment, we exploited the *Fisher's exact* test for statistical analysis, in which the p value was calculated by an equation $p = \frac{\binom{a+b}{a}\binom{c+d}{c}}{\binom{n}{a+c}} = \frac{\binom{a+b}{b}\binom{c+d}{d}}{\binom{n}{b+d}} = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{a!b!c!d!n!}$ (Table R1) (Agresti, 1992, Statist Sci 7, 131-153). The p value is associated with the sample size in a data group. Herein, the infection rate of 16681-T226K/G228E mutant-infected mosquitoes trended higher than that of either 16681-T226K or WT virus-infected mosquitoes at 5 and 8 days postinfection (Figure 3K and L). However, the sample size at these 2 time points were smaller than that of Day 6 and Day 7, yielding no statistical difference.

Table R1. Infection data of mosquitoes for 16681 and mutant.

	16681	mutant	Row Total
Infected	a	b	a+b
Uninfected	c	d	c+d
Column Total	a+c	b+d	a+b+c+d(n)

#3G. *Is AAEL011408 known to be an entry receptor for DENV in mosquitoes?*

#3G Answer: C-type lectins (CTLs) are essential host factors for flavivirus infection in both mammals and mosquitoes (Osorio and Sousa, 2011, Immunity 34, 651-64). Indeed, a variety of CTLs have been reported to facilitate multiple flavivirus infection in mosquitoes (Cheng et al., 2010, Cell 142, 714-25; Liu et al., 2017, J Virol 91, e01348-16). A previous study showed that knockdown of *AAEL011408* significantly reduced DENV2 viral load in *Aedes aegypti* (Liu et al., 2014, PloS Pathog 10, e1003931). Herein, we further found that mosGCTL-AAEL011408 directly interacted with DENV2 purified virions (Figure 4G), indicating a role of this *A. aegypti* C-type lectin in DENV2 infection. In the future, we will further study the mechanism of mosGCTL-AAEL011408-mediated DENV entry into mosquito cells.

#3H. *For Fig 4G, can the authors give a reason why T226K/G228E presented higher binding when G228E does not influence binding to C-type lectin?*

#3H Answer: We modeled the structure of carbohydrate-recognition domain (CRD) of *A. aegypti* mosGCTL-AAEL011408 by trRosetta (transform-restrained Rosetta). The interfacing residues between DENV2 E protein and CRD of mosGCTL-AAEL011408 have been predicted by Rosetta and PISA. We discovered that the 226th residue in DENV2 E protein, rather than the 228th residue, was located on the binding interface

(Figure 4F), suggesting that the 228th substitution is dispensable for the interactions between the DENV E protein and the C-type lectin. Consistent with this, the G228E substitution did not influence the infectivity of the DENV2 16681 strain in *A. aegypti* mosquitoes (Figure 2A).

We next assessed the binding affinity of these DENV mutants for mosGCTL-AAEL011408 by an SPR assay. The T226K, but not the G228E substitution, strengthened the binding affinity between mosGCTL-AAEL011408 and purified DENV2 virions. The 16681-T226K/G228E virions also presented higher binding affinity for mosGCTL-AAEL011408 than did the WT virions (Figure 4G), further validating that the dispensable role of 228th residue in DENV binding to this C-type lectin.

#3l. *Has the method used in Figure 5 been published before? Are there any references from which this method was based on?*

#3l Answer: The Gaussian Process Regression (GPR) method is effective in modeling both small sample and complex nonlinear regression systems (Cheng et al., 2019, Nat Commun 10, 1798; Mena et al., 2021, Science 372, eabg5298). The SVR method is a nonparametric machine learning tool that is widely used in small sample regression systems (Cheong et al., 2022, Nat Commun 13, 774; Zhang et al., 2022, Nat Commun 13, 617; Zhou and Jetter, 2006, Adv Comput Math 25, 323-344). The numerical kinetics modeling method has been used to model various kinetics systems (Onischenko et al., 2020, Cell 183, 1785-1800.e26; Thornburg et al., 2022, Cell 185, 345-360). The aforementioned information has been added in the Materials and Methods of the revised manuscript (Line 619-620, Page 23; Line 638-639, Page 24; Line 650-651, Page 24).

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Thank you for the submission of your revised manuscript to EMBO reports. After a careful check of your response to the referees, we have decided to proceed with publication after a few editorial requests have been addressed:

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1) We noticed that you have listed 4 co-first authors. In such cases, we generally ask for a justification of co-authorship and equal contribution.
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Taking these guidelines into account, we would welcome to acknowledge Huicheng Shi in the Acknowledgement section instead of full authorship.

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Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

Sep 6th, 2022

Dear Editor,

Thank you for the editorial comments on our manuscript (EMBOR-2022-55671V3) entitled "*Neighboring mutation-mediated enhancement of dengue virus infectivity and spread*". We are very pleased that the editors are in favor of publishing this manuscript in *EMBO reports.* We have taken all the editorial comments into consideration, and believe that the textual correction and modified presentation have improved the manuscript.

We now provide point-by-point responses to the editorial comments.

#1. *Your manuscript will be published in our Reports section. The character limit for Reports is currently 25,000 (+/- 2,000) characters, excluding references, materials and methods. Your article has currently 30,000 characters. If it is possible to shorten the text somewhat, please do so.*
Answer: We have revised the manuscript to meet the requirement of character limit. The revised manuscript has 26,766 characters.

#2. *We noticed that you have listed 4 co-first authors. In such cases, we generally ask for a justification of co-authorship and equal contribution.*
Answer: Lu Chen performed the mutants screening, animal experiments and mechanistic studies. Xianwen Zhang constructed the infectious clones and mutants of DENV1-4. Xuan Guo performed the mathematical modeling. Wenyu Peng performed the purification of proteins and contributed to the animal experiments and mechanistic studies. All four co-first authors contributed to the conception and design of the work.

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- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Taking these guidelines into account, we would welcome to acknowledge Huicheng Shi in the Acknowledgement section instead of full authorship.
Answer: Sorry we missed his additional contribution. Huicheng Shi also helped to answer the reviewers' questions and provided helpful suggestions for the mathematical modeling section. And all authors agreed to list Huicheng Shi as an additional author.

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Answer: We have revised the reference list according to the EMBO reports standard.

#6. - Appendix: as per our editorial policies we request that all methods must be part of the main manuscript text. In this case, the Appendix Supplementary text 1 might remain in the Appendix but can the Supplementary text 2 be incorporated in the methods section?
Answer: The Supplementary text 2 has been incorporated in the methods section (Line 631-646, Page23-24).

#7. -Work with Dengue Virus: Please add a biosafety statement in the materials and methods section and please identify the committee that approved the work with dengue virus and the approval #.

Answer: The biosafety statement has been added in the materials and methods section (Line 664-668, Page 24).

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Answer: We have provided a short summary, 3 bullet points, and a synopsis image along with the revised manuscript.

Prof. Gong Cheng
Tsinghua University
School of Medicine
Rm 4301, Biotech Building
Beijing 100084
China

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures/Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: 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.	Yes	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	