ORIGINAL ARTICLE

Male Infertility

Novel methylation specific real-time PCR test for the diagnosis of Klinefelter syndrome

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The aim of this study was to design a molecular assay for the diagnosis of Klinefelter syndrome (KS), based on the detection of supernumerary X-chromosomes (X-chs). DNA was extracted from peripheral blood samples of twenty-six 47,XXY males; two 46,XY/47,XXY males; twenty-two 46,XY males; and 15 females; and deaminated. Methylation-specific quantitative polymerase chain reaction (MS-qPCR) was performed using primers for unmethylated and methylated copies of the X-ch inactive-specific transcript (XIST-U and XIST-M) gene. X-ch disomy was determined on the basis of XIST methylation status. Degree of mosaicism in the 46,XY/47,XXY males was compared with karyotype and fluorescent *in situ* hybridization (FISH) results. Data analysis was performed using the Roche® LightCycler software V. 3.5.3., including determination of crossing points (CPs) by fit-point analysis and melting curve analysis. X-ch disomy was detected in all female controls and KS patients; male controls expressed XIST-M only. CPs ranged from 29.5 to 32.5 (standard deviation (s.d.) 0.8) for XIST-U and from 29 to 31 (s.d. 0.6) for XIST-M. Limit of detection of mosaicism was 1%. Based on XIST-U/XIST-M ratios for the two 47,XXY/46,XY patients, the calculated degree of mosaicism (1.8% and 17.8%) was comparable to FISH results (2.3% and 15%, respectively). Turnaround time from DNA deamination to final data analysis was under 9 h. We conclude that MS-qPCR is a sensitive, specific and rapid test for the detection of X-ch disomy, with applicability for the screening and diagnosis of KS, even in the setting of low grade 47,XXY/46,XY mosaicism.

INTRODUCTION

Klinefelter syndrome (KS) is the most common numerical chromosomal abnormality in men, affecting approximately one in 500 live births.[1] However, due to phenotypic variation amongst affected individuals and lack of a dedicated screening program, KS is appreciably underdiagnosed. It is estimated that only 25% of men with KS are diagnosed during their lifetime, with fewer than 10% being diagnosed before puberty.[2] As a result, the majority of affected patients are not eligible for early therapeutic interventions such as developmental therapy, hormonal therapy and fertility preservation.[3]

Currently available tests for the diagnosis of KS include conventional cytogenetics and fluorescent *in situ* hybridization (FISH), which are expensive, as well as labor- and time-intensive. Although Barr body cytology has been proposed as a more economical alternative, the reported 82% test sensitivity limits its utility as a diagnostic test.[4] The diagnosis of low grade mosaicism poses an additional clinical challenge, as the limit of detection of many of the available tests is often not low enough to consistently identify mosaicism.

We have previously demonstrated the use of methylation-specific polymerase chain reaction (MS-PCR) for the diagnosis of KS.[5] This test is based on the principle of dosage compensation in the setting of X-chromosome (X-ch) polysomy. Accumulating evidence suggests that supernumerary X-chs in men with KS undergo inactivation, by a mechanism similar to that seen in normal 46,XX females. Inactivation

of the X-ch is initiated within the X-ch inactivation center, by activation of the X-ch inactive-specific transcript (XIST) promoter.[6] XIST, in turn, binds to its specific site on the X-ch to turn off gene transcription.[7-9] Werler *et al.*[8] first discussed the use of XIST expression to study X-ch inactivation in the mouse model, and postulated that this finding could be translated to humans. In normal human males, XIST is methylated and, therefore, transcriptionally inactive. In females, one copy of XIST is methylated (inactive), while the other is unmethylated (active). Like females, 47,XXY men have one methylated (inactive) and one unmethylated (active) copy of XIST.

Differences in XIST methylation patterns can be detected between 46,XY and 47,XXY men using MS-PCR and gel electrophoresis.[5] Although this approach allows for rapid detection of the presence of an additional X-ch, it has several limitations. Firstly, it does not allow for quantitative analysis of X-ch disomy. Secondly, the ability of this technique to detect low-grade chromosomal mosaicism is unknown. And thirdly, the need for gel electrophoresis following MS-PCR adds to the time and resources required to carry out the test.

In contrast, quantitative PCR (qPCR) allows for both detection and quantification of DNA replication, and is amenable to automated data analysis. Compared to conventional PCR, qPCR requires a substantially lower amount of DNA as substrate, thereby limiting the blood sample volume required for the test. With these potential advantages in mind, the aim of this study was to design a sensitive, specific and rapid laboratory test for the diagnosis of KS, including

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low grade mosaicism, based on the detection of X-ch disomy and inactivation of the supernumerary X-ch by qPCR.

MATERIALS AND METHODS

Samples for this study were obtained from an existing blood sample repository, established and maintained with approval from the Institutional Review Board of Weill Cornell Medical College. All patients contributing to this repository provided informed consent for the explicit use of their blood samples for DNA extraction and genetic analysis. Electronic medical records of patients who had samples stored within the repository were reviewed. Twenty-two 46,XY males (male controls); fifteen 46,XX females (female controls); 26 non-mosaic 47,XXY males; and two 46,XY/47,XXY mosaic males were identified for inclusion into this study, based on the availability of confirmed karyotype analysis and availability of peripheral blood samples.

DNA extraction

DNA was extracted from 100 μl (less than 10 drops) of peripheral blood samples of all 65 study patients using the Promega Wizard Genomic DNA Purification Kit (Promega Inc, Madison, WI, USA), according to manufacturer's instructions. Following extraction, DNA concentration and purity was checked by spectrophotometry. All DNA samples were stored at −80°C.

DNA deamination

DNA was deaminated using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. Briefly, 0.5 μg of DNA was processed in 20 μl volume of water for deamination. Carrier RNA was not used. Deaminated DNA was eluted in 30 μl of elution buffer and stored at −80°C. Bisulfite conversion modifies unmethylated cytosine to uracil, disrupting the double-stranded structural configuration of DNA. Thus, spectrophotometry is unable to provide accurate determination of DNA concentration following deamination. As a result, subsequent steps in our experiments relied on DNA volume rather than DNA concentration.

MS real-time PCR

qPCR amplification was performed on the Roche 480 LightCycler platform (Roche, Basel, Switzerland), using primers for unmethylated and methylated copies of the X-ch inactive-specific transcript gene (XIST-U and XIST-M, respectively), and PerfeCta SYBR Green PCR master mix (Quanta BioSciences, Gaithersburg, MD, USA). Primers were selected based on previous experiments[5] and are listed in **Table 1**. All reactions were run in a volume of 20 μl on 96-well plates. The reaction mixture included 5 μl of deaminated DNA, 10 μl of master mix and two sets of primers for XIST-U and XIST-M at a final concentration of 0.4 μmol l^{-1} each. All samples were run in duplicate. Water was used as a negative control. In terms of qPCR settings, initial denaturation was performed at 95°C for 5 min, followed by amplification at 95°C for 10 s, 61°C for 20 s, 72°C for 25 s for 45 cycles. Melting curve analysis was carried out using a standard program, at 95°C for 5 s, followed by cooling to 65°C for 1 min, followed by heating to 97°C, with eight acquisitions per degree increase.

Intra-assay variation

To evaluate test repeatability, DNA samples from one female; one 46,XY male; and two 47,XXY males, chosen at random, were run in quintuplicate on the same 96-well plate. Water was used as a negative control.

Inter-assay variation

To evaluate test reproducibility, DNA samples from two females; two 46,XY males; and two 47,XXY males were run together, in triplicate, on a 96-well plate. This experiment was repeated on three different days. Water was used as a negative control.

Standard curves

Serial 10-fold dilutions of deaminated DNA from a randomly selected 46,XX patient were performed. qPCR was then performed, as described above, for each dilution of DNA. Each reaction was run in quintuplicate. Standard curves were generated based on the results, and were applied for quantitative data analysis.

Limit of detection

To determine the lowest percentage of XX:X mosaicism detectable by qPCR, DNA from one randomly selected 46,XX female and one randomly selected 46,XY male was mixed to achieve XX:X concentration ratios of 100:0, 99:1, 95:5, 90:10, 75:25, 50:50, 25:75, 10:90, 5:95, 1:99 and 0:100. Identical volumes of the prepared mixtures were subsequently deaminated and qPCR was performed as described above. Each reaction was run in triplicate.

Data analysis

Data analysis for qPCR reactions was performed using the Roche LightCycler software V 3.5.3., and included determination of crossing points (CPs) by fit-point analysis and melting curve analysis. Results for intra- and inter-assay variation were assigned by an automated second-derivative algorithm. Standard curves were applied for quantification of mosaicism.

Karyotype and FISH analysis

Low-grade 46,XY/47,XXY mosaicism was present in two study patients (fraternal twins) and was confirmed by karyotype and FISH analysis at our institution. Standard protocols were used for karyotype analysis; 20 metaphase cells from Twin 1 and 50 metaphase cells from Twin 2 were evaluated. FISH was performed using spectrum orange labeled CEP X and spectrum green labeled LSI Yq12 probes (Abbot Molecular Inc, Des Plaines, IL, USA). Following overnight cohybridization, cells were washed, counterstained with diamidino-2-phenylindole and imaged using Leica Microsystems Cytovision (Buffalo Grove, IL, USA). Two hundred interphase nuclei from Twin 1 and 500 interphase nuclei plus 50 metaphase cells from Twin 2 were evaluated. The degree of mosaicism in the two 47,XXY/46,XY study patients was compared with karyotype and FISH results.

Table 1: Primers used for methylation specific real-time PCR

Primer	Sequence	Final concentration (μmol l^{-1})	Position	Gene bank No.
XIST-U-L	5'-AAAAGTGGTTGTTATTTTAGATTTGTT-3'	0.4	19238–19260	U80460
XIST-U-R	5'-CTACCTCCCAATACAACAATCACAC-3'	0.4	19435–19411	U80460
XIST-M-L	5'-AATTAAAGTAGGTATTCGCGGTTTCG-3'	0.4	19049–19024	U80460
XIST-M-R	5'-TTTTTCCTTAACCCATCGAAATATCG-3'	0.4	18834–18809	U80460

XIST-M-L: X-chromosome inactive-specific transcript methylated left primer; XIST-M-R: XIST methylated right primer; XIST-U-L: XIST unmethylated left primer; XIST-U-R: XIST unmethylated right primer

RESULTS

Sensitivity and specificity

X-ch disomy, based on XIST-U and XIST-M expression, was detected in all 15 females and all 28 KS patients (100% sensitivity). In contrast, the 22 male controls expressed XIST-M only (100% specificity). Amplification curves demonstrated a narrow range of CPs for XIST-U (29.5–32.5, standard deviation (s.d.) 0.8) and XIST-M (29–31, s.d. 0.6) (**Figure 1**).

Assay repeatability and reproducibility

Assay repeatability was confirmed by evaluating intra-assay variation. X-ch disomy was detected in the female control and both KS patients, while the male control expressed XIST-M only. The mean value and s.d. of CPs for the intra-assay variation are summarized in **Table 2**. Assay reproducibility was confirmed by evaluating inter-assay variation. X-ch disomy was detected in the two female controls and two KS patients, while the two male controls expressed XIST-U only. The mean value and s.d. of CPs for the intra-assay variation are summarized in **Table 3**. The assay was noted to be both repeatable and reproducible, based on the narrow range of CPs and low s.d.

Limit of detection

X-ch disomy was detectable in samples with XX:X concentration ratios of 1:99 (**Figure 2**). Based on these results, the level of mosaicism using our assay was 1%. Uniformity of melting peaks on melting curve analysis confirmed specific amplification of a single PCR product in this experiment.

Comparison of qPCR with conventional cytogenetics and FISH

The two mosaic KS patients had undergone cytogenetic evaluation prior to referral to our institution. However, karyotype analysis performed in one of two independent laboratories had failed to detect mosaicism in one of the twins. At our institution, karyotype analysis of the two

mosaic KS patients showed 20% mosaicism in Twin 1, but was unable to confirm the low level of X-ch disomy in Twin 2 (**Figure 3**). FISH analysis demonstrated 15% mosaicism in Twin 1 and 2.3% mosaicism in Twin 2 (**Figure 3**). Based on XIST-U/XIST-M ratios for the two 47,XXY/46,XY patients, the calculated degree of mosaicism (17.8% and 1.8%) was comparable to the results of FISH analysis (15% and 2.3%, respectively).

Turnaround time

Turnaround time from DNA deamination to final data analysis was under 9 h.

DISCUSSION

Our results demonstrate that MS-qPCR can be used for the diagnosis of KS, including low-grade mosaicism, with high sensitivity and specificity. qPCR technology allows for the use of very small quantities of patient DNA, compared to other available diagnostic tests. Automated data analysis facilitates the objective interpretation of test results. Furthermore, with a turnaround time of less than 9 h, this assay is considerably more efficient than previously described methods. Other currently available tests for the diagnosis of KS, such as karyotype analysis, FISH, and Barr body analysis are expensive, labor-intensive and have varying sensitivity and specificity, particularly in the setting of low levels of mosaicism. The use of a molecular methodology based quantitative assay, as described in this manuscript, has the potential to obviate many of these limitations.

Table 2: Results of intra-assay variation

Sample	Mean CP±s.d.	
	XIST-U	*XIST-M*
46,XX	36.0±0.3	31.1±0.2
46,XY	NA	28.1±0.1
47,XXY	32.0±0.3	28.1±0.2
47,XXY	32.8±0.4	28.7±0.2
Water (negative control)	NA	NA

CP: crossing point; NA: not applicable; s.d.: standard deviation; XIST-M: X-chromosome inactive-specific transcript methylated; XIST-U: X-chromosome inactive-specific transcript unmethylated
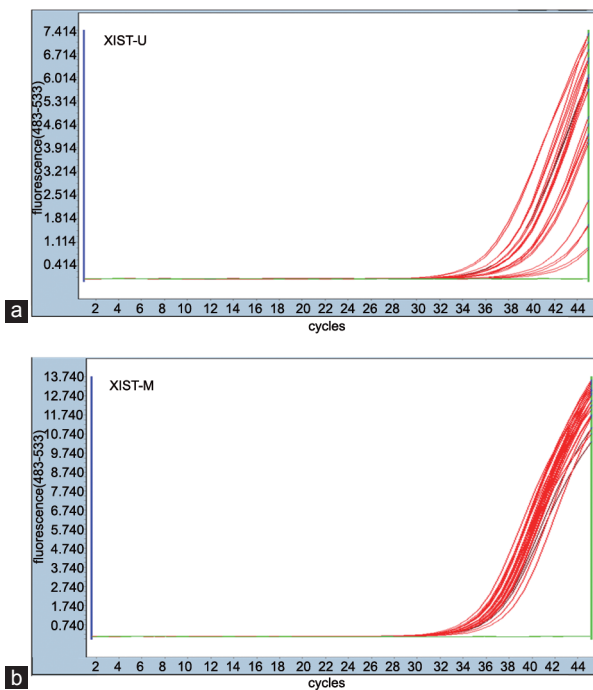

Figure 1: Determination of crossing points (CPs) for (**a**) XIST-U and (**b**) XIST-M. XIST-M: X-chromosome inactive-specific transcript methylated; XIST-U: X-chromosome inactive-specific transcript unmethylated.
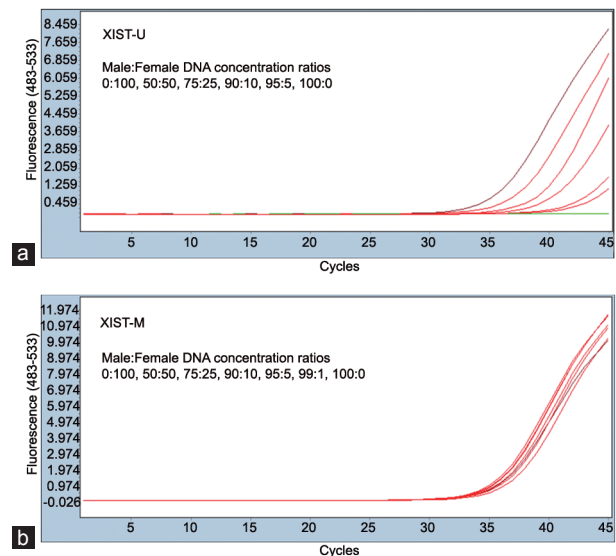

Figure 2: Limits of detection for (**a**) XIST-U and (**b**) XIST-M. XIST-M: X-chromosome inactive-specifi c transcript methylated; XIST-U: X-chromosome inactive-specifi c transcript unmethylated.

Table 3: Results of inter-assay variation (Data are presented as mean CP±s.d.)

Sample						
	Day 1 XIST-U	Day 2 XIST-U	Day 3 XIST-U	Day 1 XIST-M	Day 2 XIST-M	Day 3 XIST-M
46,XX	36.4±0.2	36.8±0.1	36.5±0.1	35.3±0.1	34.8±0.2	35.0±0.3
46,XX	36.8±0.1	36.3±0.2	36.7±0.1	35.2±0.03	35.0±0.02	34.7±0.1
46,XY	NA	NA	NA	32.4±0.1	32.4±0.2	32.2±0.03
46,XY	NA	NA	NA	32.6±0.1	32.4±0.2	32.1±0.2
47,XXY	33.8±0.1	33.3±0.1	33.3±0.3	31.5±0.1	31.9±0.02	31.0±0.2
47,XXY	37.1±0.03	36.7±0.2	36.7±0.1	35.0±0.1	34.7±0.01	34.6±0.1
Water (negative control)	NA	NA	NA	NA	NA	NA

CP: crossing point; NA: not applicable; XIST-M: X-chromosome inactive-specific transcript methylated; XIST-U: X-chromosome inactive-specific transcript unmethylated
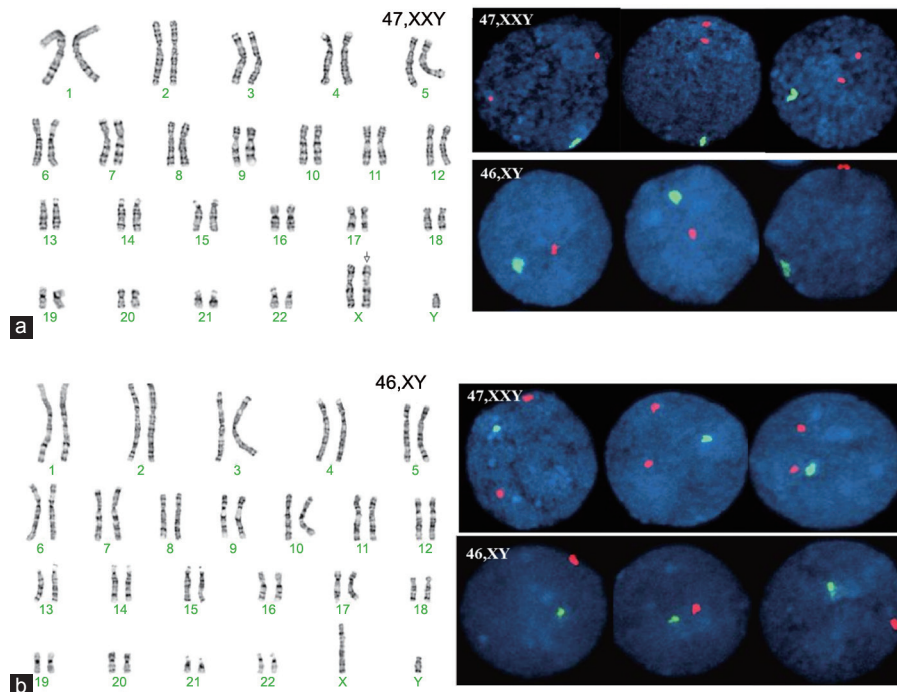

Figure 3: Results of karyotype and FISH analysis in mosaic KS patients. (**a**) Twin 1 conventional cytogenetic analysis showed a 47,XXY chromosome complement in 20% of cells analyzed and a 46,XY karyotype in the remaining 80%. Interphase FISH detected XXY signals in 15% of cells analyzed and XY signals in the remaining 85%. (**b**) Twin 2 conventional cytogenetic analysis showed a 46,XY karyotype in all evaluated cells. However, interphase FISH analysis detected XXY signals in 2.2% of analyzed cells and XY signals in the remaining 97.8%. FISH: fluorescent *in situ* hybridization; KS: Klinefelter syndrome.

MS-PCR was introduced by Herman and colleagues[10] in 1996, as an efficient and cost-effective test to analyze the methylation status of CpG dinucleotides in CpG islands. Since then, MS-PCR has been widely used to study promoter methylation in cancer states,[11,12] identify gene polymorphisms leading to conditions like Fragile X syndrome,[13] diagnose disorders of genomic imprinting such as Prader–Willi and Angelman syndromes,[14] and evaluate X-ch inactivation.[15] We have previously reported the use of MS-PCR for the diagnosis of KS, based on inactivation patterns of the FMR1 and XIST genes.[5] qPCR, however, represents a significant improvement upon standard PCR as a diagnostic test because it is faster, eliminates post-PCR data analysis steps such as gel electrophoresis or hybridization, and allows for higher throughput of clinical samples. Additionally, the use of a closed system minimizes the chance of workspace contamination during amplicon transfer, thereby decreasing false-positive results.

The amount of DNA required for this assay to be performed in duplicate was easily extracted from 100 μl of peripheral blood samples

in all cases. Coffee *et al.*[13] have described DNA extraction from dried blood spots following newborn heel sticks, for the diagnosis of Fragile X syndrome using qPCR for methylation analysis. It is conceivable that a similar approach could also be employed for the diagnosis of KS. However, given that KS is not a life-threatening condition warranting an immediate diagnosis after birth, this approach was not specifically explored by us in this initial study.

A high index of suspicion is needed to prompt genetic testing for KS, especially in the setting of low grade 46,XY/47XXY mosaicism. As noted in this study, conventional karyotype analysis was unable to confirm the presence of low-grade mosaicism in one of two patients. The sensitivity and specificity of FISH compared to karyotype analysis has been explored by several studies, but one technique has not been shown to be consistently superior to the other. Nevertheless, some authors have advocated the use of FISH to conform the presence or absence of mosaicism when clinical suspicion is high and karyotype analysis demonstrates <10% mosaicism.[16] While this approach would

have been sufficient for the diagnosis of KS in our two mosaic KS patients, the associated costs and time investment, would have been substantial. We report a very low level of detail (1%) associated with this qPCR assay, which is beneficial for the diagnosis of low grade mosaicism. Although our study population only included two low grade mosaic 46,XY/47,XXY males, our experimental data clearly indicates that our test is able to reliably diagnose low grade mosaicism.

The limited number of mosaic and non-mosaic KS patients, as well as normal male and female controls included in the study, may be perceived as a limitation of this analysis. Nevertheless, these preliminary results are promising with respect to the potential utility of a MS-qPCR assay for the diagnosis of KS. Validation of this test on a larger patient sample is required and planned. Secondly, patient DNA was extracted from peripheral blood samples for all individuals included in this study. It is important to remember that peripheral blood-based assays may not necessarily reflect tissue-specific chromosomal constitution, especially in cases of mosaicism. Theoretically, DNA extracted from testicular tissue could be used for this test following the same protocol. We did not specifically investigate this possibility. Lastly, we did not specifically perform cost-analysis for this assay. However, although the initial cost of a qPCR platform may be substantial, long-term costs associated with carrying out this assay would largely be attributed to reagents and basic lab supplies, especially if 96-well PCR plates are used to simultaneously analyze multiple patient samples.

The benefit of systematic screening for KS has been debated in the literature. A growing body of evidence suggests that early therapeutic interventions in boys with KS can have a beneficial impact on their physical, academic and social development, as well as their overall health.[17-19] However, there is concern that systematic screening for KS may be expensive, difficult to perform and challenging to interpret in the setting of genetic mosaicism. Therefore, the availability of a sensitive, specific and rapid test for the postnatal diagnosis of KS, including patients with low grade mosaicism, may lead to early diagnosis and intervention, thereby considerably improving the quality of life of affected individuals.

CONCLUSIONS

Methylation specific qPCR is a sensitive, specific and rapid test for the detection of X chromosome disomy, with applicability for the screening and diagnosis of KS, even in the setting of low degree of 47,XXY/46,XY mosaicism.

AUTHOR CONTRIBUTIONS

A Mehta participated in study design, data acquisition, analysis and interpretation and was responsible for authoring the manuscript. A Mielnik participated in data acquisition, analysis and interpretation, and contributed to manuscript review. PNS participated in data interpretation and manuscript review. DAP conceived the study and study design, participated in data interpretation and contributed to manuscript review. All authors gave approved the final version of the manuscript for publication.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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