ORIGINAL ARTICLE

Loss of PPM1F expression predicts tumour recurrence and is negatively regulated by miR-590-3p in gastric cancer

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Abstract

Objectives: MicroRNAs (miRNAs) as small non-coding RNA molecules act by negatively regulating their target genes. Recent studies have shown that protein phosphatase Mg²⁺/Mn²⁺-dependent 1F (PPM1F) plays a critical role in cancer metastasis. But, the regulation mechanisms of PPM1F by miRNAs in gastric cancer (GC) remain undefined.

Methods: The correlation of PPM1F or miR-590-3p (miR-590) expression with clinicopathological features and prognosis of the patients with GC was analysed by TCGA RNA-sequencing data. The miRNAs that target PPM1F gene were identified by bioinformatics and Spearman correlation analysis, and the binding site between miR-590 and PPM1F 3'UTR was confirmed by dual luciferase assay. MTT and Transwell assays were conducted to evaluate the effects of miR-590 or (and) PPM1F on cell proliferation and invasion.

Results: We found that PPM1F expression was downregulated in GC tissues and cell lines and was correlated with tumour recurrence in patients with GC. The decreased expression of PPM1F was attributed to the dysregulation of miR-590 expression rather than its genetic or epigenetic alterations. Overexpression of miR-590 promoted cell proliferation and invasion capability of GC cells, while knockdown of miR-590 reversed these effects. Moreover, PPM1F was validated as a direct target of miR-590 and counteracted the tumour-promoting effects caused by miR-590. The expression of miR-590 presented the negative correlation with PPM1F expression and acted as an independent prognostic factor for tumour recurrence in patients with GC.

Conclusion: PPM1F may function as a suppressive factor and is negatively regulated by miR-590 in GC.

1 | INTRODUCTION

Despite the decreased incidence of gastric cancer (GC), it remains the fourth gastrointestinal malignant tumour and the third leading cause of cancer-related deaths worldwide.¹ *Helicobacter pylori* (HP) infection acts a pivotal role in the occurrence and initial development of GC, and due to not timely and effective eradication of this bacterial infection, GC is still the second most common cancer in East Asia,² and presents at an advanced stage owing to its invasiveness. Thus, to

uncover the molecular mechanisms of GC, tumorigenesis may provide insights into developing effective anti-cancer strategies for GC.

Protein phosphatase Mg²⁺/Mn²⁺-dependent 1F (PPM1F), a Ser/Thr protein phosphatase that belongs to PPM family has been reported to exhibit cellular functions by repressing filament association³ and affecting cell polarity and centrosome placement.⁴ Moreover, PPM1F (also known as POPX2) promotes cancer cell motility and invasiveness^{5,6} through the regulation of the GSK/ β -catenin⁷ and MAPK1/3 pathways,⁸ and accelerates smoking-caused breast cancer by inactivating p53 signals, indicating a prognostic target for cancer prevention and therapy.⁹

Jing Zhang, Ming Jin and Xiaoyu Chen contributed equally to this work.

MicroRNAs (miRNAs) as small non-coding RNAs with 21-23 nucleotides act via negatively regulating gene expression by binding with the 3' UTR of cognate mRNA targets.¹⁰ Accumulating evidence shows that dysregulation of miRNAs is involved in multiple cellular processes of GC including cell proliferation, migration and metastasis,¹¹⁻¹³ of which miR-149 and miR-200c inhibit tumour metastasis in hepatocellular carcinoma (HCC) and breast cancer by targeting PPM1F^{14,15} and uncover the regulation link between PPM1F and miRNAs in cancer.

However, in the present study, TCGA data and tissue microarray analysis indicated that PPM1F expression was downregulated in GC and correlated with tumour recurrence of the patients. Taken account into no significant alterations in genetic and epigenetic dysregulation for PPM1F in GC, miR-590-3p was further identified to display an oncogenic role by targeting PPM1F and acted as an independent prognostic factor for tumour recurrence of patients with GC.

2 | MATERIALS AND METHODS

2.1 | Clinical data

The clinicopathological and prognostic data for 415 cases of GC patients and 33 adjacent normal tissues as well as the relative expression levels of PPM1F and miRNAs (has-miR-590-3p, has-miR-186-5p, has-miR-200b, has-miR-200c and has-miR-429) were downloaded from The Cancer Genome Atlas 2015 RNA-sequencing database (<https://genome-cancer.ucsc.edu>). The protocols used in our study were approved by the Ethics Committee of Shanghai Sixth People's Hospital. The clinicopathological characteristics of 308 GC patients for PPM1F expression were summarized in Table S1.

2.2 | Immunohistochemistry (IHC) analysis

The tissue microarray of GC was purchased from the Outdo Biotech Co, LTD (HStmA030PG03, Shanghai, PR, China), and it included 15 GC samples and corresponding 15 adjacent normal tissues. The expression or cellular localization of PPM1F in GC tissue cells was determined by IHC analysis. The GC tissues were stained for anti-PPM1F (ab156222, Rabbit polyclonal antibody, Abcam, Cambridge, MA, USA) and its expression level was quantified as previously described.¹⁶

2.3 | Identification of miRNAs in cancer tissues

The miRNAs that target 3'UTR of PPM1F gene were identified in cancer tissues by using the StarBase v2.0 (<http://starbase.sysu.edu.cn>) under the strictly incorporated conditions with 2 prediction algorithms (TargetScan 7.1 and microRNA.org) and very high stringency (>5).

2.4 | Cell culture

GES-1, SGC-7901, MKN-45, MKN-28, HGC-27, MGC-803, AGS and BGC-823 cell lines were stored at Digestive Disease Laboratory

of Shanghai Sixth People's Hospital, and cultured in Dulbecco's Modified Eagle medium (DMEM) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin (HyClone) in a humidified atmosphere containing 5% CO₂ at 37°C.

2.5 | Quantitative real-time PCR (qRT-PCR)

Total RNA of AGS and MKN-28 cells was collected by using TRIzol and reverse transcription was conducted by using M-MLV and cDNA amplification by using the SYBR Green Master Mix kit (Takara, Otsu, Japan). In addition, total RNA for miRNAs was isolated by using a High Pure miRNA isolation kit (Roche) and RT-PCR using a TaqMan MicroRNA Reverse Transcription kit (Life Technologies). A miScript Primer Assay (QIAGEN) was used for the miR-193a and U6. Data were analysed using the comparative Ct method ($2^{-\Delta\Delta C_t}$). Three separate experiments were performed for each clone. The primers were listed in Table S2.

2.6 | Western blotting analysis

AGS and MKN-28 cells were harvested and their proteins were extracted by using lysis buffer. The primary antibody against PPM1F (ab156222, Rabbit polyclonal antibody, Abcam, Cambridge, MA, USA) was diluted at a ratio of 1:1000 and the second antibody Goat anti-Rabbit IgG H&L (HRP) (ab6721, Abcam, Cambridge, MA, USA) was diluted at a ratio of 1:10000 according to the instructions. Western blotting analysis was conducted as previously described.¹⁶

2.7 | Luciferase reporter assay

Luciferase reporter assay was performed as previously described.¹⁶

2.8 | Plasmid, siRNAs and miR-590 mimic and inhibitor

Plasmid-mediated pcDNA3.1-PPM1F vector, siRNA targeting PPM1F (si-PPM1F) vector, miR-590 mimic and inhibitor were purchased from Genesharma (Shanghai, PR, China) and the empty vector was used as a control. AGS and MKN-28 cells were planted in 6-well plates 24 hour prior to si-PPM1F, pcDNA3.1-PPM1F, miR-590 mimic or inhibitor transfection with 50%-70% confluence, and then were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture instructions.

2.9 | Cell migration assay

Cell migration was determined by wound healing. Briefly, the adherent AGS cells transfected with miR-590 mimic and PPM1F or MKN-28 cells transfected with miR-590 inhibitor and si-PPM1F in a 6-well plate were wounded with a standard 200 µL pipette tip to form a 1 mm wide strip across the well. After 48-hour

incubation, the wound closure was observed and photographed with a microscope.

2.10 | Cell viability, colony formation and transwell invasion assays

Cell viability, colony formation and transwell assays were performed as previously described.¹⁶

2.11 | Statistical analysis

Statistical analyses were conducted by SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism. Student's *t* test or chi-square test was used to evaluate the statistical significance for the comparisons of 2 groups. Pearson's correlation coefficient analysis was used to analyse the correlations of PPM1F with miRNAs. Overall survival (OS) was defined as the interval between the dates of surgery and death and OS and disease-free survival (DFS or recurrence)

curves were analysed with the Kaplan-Meier method and log-rank test. Univariate analysis and multivariate models were performed by using a Cox proportional hazards regression model. *P* < .05 was considered statistically significant.

3 | RESULTS

3.1 | Downregulation of PPM1F expression is correlated with tumour recurrence of GC patients

Distinct from the previous studies indicating that PPM1F is up-regulated in HCC and breast cancer,^{7,12,13} our results demonstrated that the expression level of PPM1F was markedly decreased in GC samples (*n* = 415) as well as in the pair-matched GC samples (*n* = 30) in comparison to the adjacent normal tissues (*n* = 33) by using The Cancer Genome Atlas (TCGA) sequencing data (Figure 1A). Except for the AGS cell line, PPM1F expression was also lowered in other GC cell lines compared with the GES-1 indicated by qRT-PCR

FIGURE 1 Loss of PPM1F expression was associated with tumour recurrence in patients with GC. (A) TCGA cohort analysis of the differentially expressed levels of PPM1F in the total GC samples as well as in the pair-matched tumour tissues. (B) qRT-PCR analysis of the expression level of PPM1F in GC cell lines and GES-1 cells. (C) IHC analysis of the expression level and cellular localization of PPM1F in GC tissue cells (×400). (D) The cut-off value, sensitivity and specificity of PPM1F was assessed in GC samples (*n* = 308). (E) The patients with GC were divided into PPM1F high expression group (*n* = 214) and low expression group (*n* = 94). (F) Kaplan-Meier analysis of the link of PPM1F high or low expression with the tumour recurrence of the patients with GC

TABLE 1 Cox regression analysis of PPM1F expression as a recurrence predictor

Variables	Univariate Cox regression analysis		Multivariate Cox regression analysis	
	RR (95% CI)	P value	RR (95% CI)	P value
Age (years)				
<60 vs ≥60	0.898 (0.545 to 1.480)	.674	NA	NA
Gender				
Male vs female	1.925 (1.096 to 3.384)	.023	1.981 (1.126 to 3.483)	.018
Pathological stage				
III/IV vs I/II	1.075 (0.663 to 1.743)	.769	NA	NA
T stage				
T3+T4 vs T1+T2	0.915 (0.542 to 1.544)	.739	NA	NA
N staging				
Positive vs negative	1.199 (0.709 to 2.027)	.499	NA	NA
M stage				
Positive vs negative	0.899 (0.361 to 2.238)	.819	NA	NA
PPM1F expression				
High vs low	0.465 (0.249 to 0.870)	.017	0.453 (0.242 to 0.847)	.013

NA, not analysed.

analysis (Figure 1B). IHC analysis showed that PPM1F was mainly localized in the cytoplasm and its positive rate was much lower in GC tissues than the adjacent normal tissues (53.33% vs 86.67%, $P = .021$) (Figure 1C). These findings suggested that loss of PPM1F expression was a frequent event in GC.

Moreover, according to the OS time, OS status and PPM1F expression level, we gained the cut-off value (1.21) of PPM1F (Figure 1D) in GC samples ($n = 308$), and divided the patients into 2 groups: PPM1F high expression and PPM1F low expression (Figure 1E). ROC curve and AUC were used to calculate the sensitivity and specificity of PPM1F expression, which demonstrated that the sensitivity, specificity and AUC of PPM1F were respectively 33.9%, 81.8% and 0.55% (Figure 1D), indicating that PPM1F expression might be a potential marker for GC patients. We further analysed the correlation of PPM1F expression with the clinicopathological characteristics and prognosis of GC patients and found that PPM1F expression was associated with the age ($P = .033$), but had no correlation with the other factors such as gender, pathological stage and TNM stage (each $P > .05$, Table S3). Kaplan-Meier analysis revealed that the patients with PPM1F low expression harboured higher tumour recurrence rate (Figure 1F), but had no significant difference in OS (Figure S1A) compared with those with PPM1F high expression. Besides, the patients of early stage (stage I+II) or late stage (stage III+IV) with PPM1F low expression displayed no difference in tumour recurrence compared with those with PPM1F high expression (Figure S1B). Multivariate analyses were further conducted by a Cox proportional hazard model, which showed that, PPM1F expression as well as gender was an independent prognostic factor for tumour recurrence of GC patients (Table 1).

3.2 | PPM1F displays a negative correlation with miR-590 expression in GC samples

To uncover the reason for PPM1F downregulation in GC, we evaluated the genetic or epigenetic dysregulation of PPM1F expression in GC ($n = 370$), which indicated that PPM1F had no significant alterations at the genetic (Figure S2A,B) and methylation levels (Figure S2C) and gene amplification, deletion, mutation, copy number deletion and methylation modification could not account for the downregulation of PPM1F in GC.

Additionally, it is known that miRNAs act by negatively regulating their target gene.¹⁰ To illustrate whether PPM1F expression is regulated by miRNAs, the prediction tools microRNA.org and TargetScan 7.1 were applied to identify the potential miRNAs that target 3'UTR of PPM1F gene, of which 5 miRNAs (miR-590-3p, miR-186-5p, miR-200b, miR-200c and miR-429) have the potential to bind to the 3'UTR of PPM1F (Figure 2A). Then, we assessed the expression levels of these 5 miRNAs in GC samples and found their expression levels were substantially increased in the pair-matched GC samples except for the miR-200c (Figure 2B) as well as in the total tumour samples compared with the normal tissues (Figure 2C). The spearman correlation analysis revealed the negative correlation of PPM1F expression with these 5 miRNAs in GC (Figure 2D1-5).

However, the high expression levels of miR-186, miR-429, miR-200b and miR-200c (Figure 2B,C) and the high recurrence tendency for miR-186 (Figure S3A) were inconsistent with the previously reported results, which indicated that these 4 miRNAs as tumour suppressors have low expression levels and low recurrence rate in GC.¹⁷⁻²¹ Moreover, our studies showed that the high expression levels of miR-429 and miR-200b were also contradictory with their low

FIGURE 2 PPM1F was identified to have negative correlation with miR-590 expression in GC. (A) Bioinformatic software identification of the 5 miRNAs that target 3'UTR of PPM1F in cancer tissues. (B and C) TCGA cohort analysis of the differentially expressed levels of these 5 miRNAs in the pair-matched GC samples as well as in the total tumour tissues. N: normal, T: tumour. (D1-5) Pearson correlation analysis of the correlation of PPM1F expression with these 5 miRNAs in GC samples

recurrence tendency in GC patients (Figure S3B,C). Thus, only miR-590 high expression (Figure 2B,C) was consistent with the previously reported results²² and was selected for further investigation.

3.3 | PPM1F was verified as a target of miR-590 in GC cells

Having demonstrated the negative correlation of PPM1F expression with miR-590 in GC samples (Figure 2D4), we further verified whether PPM1F is a target of miR-590 in GC. We first detected the expression levels of miR-590 in GC cell lines by qRT-PCR, indicating that miR-590 possessed higher expression level in MKN-28 cells and lower expression level in AGS cells compared with the GES-1 (Figure 3A). Spearman correlation analysis showed the negative correlation of miR-590 with PPM1F expression in GC cell lines (Figure 3B). Then, miR-590 mimic (100 μ M) or inhibitor (100 μ M) was respectively transfected into AGS cells with miR-590 low expression and MKN-28 cells with miR-590 high expression. After transfection for 48 hour, qRT-PCR and western blot analysis demonstrated that miR-590

overexpression substantially reduced the expression level of PPM1F in AGS cells, while miR-590 knockdown increased its expression in MKN-28 cells (Figure 3C,D). Furthermore, luciferase reporter system containing the wild-type (wt) and mutant (Mut) 3'UTR of PPM1F (Figure 3E) was co-treated with miR-590 mimic or inhibitor in AGS or MKN-28 cell line. The results showed that the luciferase activity of wt 3'UTR of PPM1F was decreased by miR-590 mimic in AGS cells, but increased by miR-590 inhibitor in MKN-28 cells (Figure 3F). However, the luciferase activity of Mut 3'UTR of PPM1F was not significantly influenced by miR-590 mimic or inhibitor in GC cells.

3.4 | PPM1F rescued the cell viability and colony formation induced by miR-590

To further investigate the effects of miR-590 on PPM1F expression in GC cells, we conducted the MTT and cell colony formation assays. First, the transfection efficiency of PPM1F overexpression in AGS cells or si-PPM1F in MKN-28 cells was determined by qRT-PCR (Figure 4A) and Western blotting analysis (Figure 4B).

FIGURE 3 PPM1F was validated as a target of miR-590 in GC cells. (A) qRT-PCR analysis of the expression level of miR-590 in GC cell lines and GES-1 cells. (B) Pearson correlation analysis of the correlation of PPM1F with miR-590 expression in GC cell lines. (C and D) qRT-PCR and western blot analysis of the expression levels of miR-590 and PPM1F after the transfection with miR-590 mimic in AGS cells or miR-590 inhibitor in MKN-28 cells. (E) Schematic representation of the binding sites of miR-590 with wt or Mut 3'UTR of PPM1F. (F) The luciferase activity of wt or Mut 3'UTR of PPM1F was evaluated after transfection with miR-590 mimic in AGS cells or miR-590 inhibitor in MKN28 cells. Data are the means $\pm$ SEM of 3 experiments. * $P < .05$, ** $P < .01$

Then, miR-590 mimic promoted cell viability and colony formation ability, and these effects were reversed by co-transfection with PPM1F overexpression vector in AGS cells (Figure 4C,E), but miR-590 inhibitor inhibited cell viability and colony formation and these effects were attenuated by co-transfection with si-PPM1F in MKN-28 cells (Figure 4D,F). These results suggested that miR-590 might promote proliferation and colony formation of GC cells by regulating PPM1F expression.

3.5 | PPM1F counteracted the cell migration and invasion caused by miR-590

We also performed the wound healing and transwell assays to observe the effects of miR-590 on PPM1F expression in GC cells. After transfection with miR-590 mimic or (and) PPM1F overexpression vector in AGS cells or miR-590 inhibitor or (and) si-PPM1F in MKN-28 cells for 48 h (Wound healing assay) or 24 hour (Transwell assay), the results showed that miR-590 mimic enhanced cell migration and invasion capacity, which was counteracted by PPM1F overexpression in AGS cells (Figure 5A,C), whereas miR-590 inhibitor suppressed cell migration and invasion ability and these effects were rescued by PPM1F knockdown in MKN-28 cells (Figure 4B,D). These results suggested that miR-590 might promote the migration and invasion of GC cells by regulating PPM1F expression.

3.6 | Increased expression of miR-590 was positively associated with tumour recurrence of GC patients

Having defined the increased expression of miR-590 (Figure 2B,C) and its negative correlation with PPM1F expression (Figure 2D4) in

GC samples, we further analysed the relationship between its expression with clinicopathological features and prognosis of the patients with GC. According to the OS time, OS status and miR-590 expression level, the cut-off value (3.763) of miR-590 (Figure 6A) in GC samples ($n = 315$) was determined, by which the patients were classified into 2 groups: miR-590 high expression and miR-590 low expression (Figure 6B). ROC curve was drawn to assess the sensitivity and specificity of miR-590 expression in GC, which indicated that the AUC, sensitivity and specificity of miR-590 were respectively 0.53, 19.7% and 91.6% (Figure 6A), suggesting that miR-590 expression might be a potential marker for GC patients. Furthermore, we found that miR-590 expression had no association with the age, gender, pathological stage and TNM stage (each $P > .05$, Table S4). Kaplan-Meier analysis referred that the patients with miR-590 high expression presented higher tumour recurrence rate, but had no significant difference in OS compared with those with miR-590 low expression (Figure 6C). Nevertheless, the patients of early stage (stage I+II) or late stage (stage III+IV) with miR-590 high expression exhibited no difference in tumour recurrence compared with those with miR-590 low expression (Figure S4). Multivariate analyses illustrated that, miR-590 expression as well as gender was an independent prognostic factor for tumour recurrence of GC patients (Table S5).

4 | DISCUSSION

Some studies have shown that PPM1F expression is upregulated in HCC and breast cancer^{9,14,15} and participates in tumour cell motility and invasion.⁵⁻⁹ But, the association between PPM1F expression and the clinicopathological characteristics and prognosis of these patients is not elucidated. In our study, inconsistent with the

FIGURE 4 Overexpression of miR-590 enhanced GC cell viability and colony formation by regulating PPM1F expression. (A) and (B) qRT-PCR and western blot analysis of the transfection efficiency of PPM1F in AGS cells or si-PPM1F in MKN-28 cells. (C) and (D) MTT assay analysis of the cell viability and (E and F) colony formation assay analysis of cell colony formation ability after transfection with miR-590 mimic or (and) PPM1F overexpression in AGS cells or miR-590 inhibitor or (and) si-PPM1F in MKN-28 cells. Data are the means $\pm$ SEM of 3 experiments. * $P < .05$; ** $P < .01$

FIGURE 5 Overexpression of miR-590 accelerated GC cell migration and invasion by regulating PPM1F expression. A and B, Wound healing analysis of the cell migration and (C, D) Transwell analysis of the cell invasion capability after transfection with miR-590 mimic or (and) PPM1F overexpression in AGS cells or miR-590 inhibitor or (and) si-PPM1F in MKN-28 cells. Data are the means $\pm$ SEM of 3 experiments. * $P < .05$; ** $P < .01$

FIGURE 6 Increased expression of miR-590 was positively correlated with tumour recurrence of the patients with GC. (A) The cut-off value, sensitivity and specificity of miR-590 were determined in GC samples ($n = 315$). (B) The patients with GC were classified into miR-590 high expression ($n = 34$) or low expression group ($n = 281$). (C) Kaplan-Meier analysis of the association of miR-590 high or low expression with the tumour recurrence of patients with GC

results,^{9,14,15} PPM1F expression was found downregulated and negatively correlated with the age and tumour recurrence of the patients with GC in a large sample size from TCGA data. Multivariate analysis revealed PPM1F as an independent prognostic factor for tumour recurrence of GC patients. Our results were sustained by another report indicating that PPM1F low expression is related to distant metastasis and poor survival of lung cancer.²³ These findings suggested that loss of PPM1F might be involved in the GC tumorigenesis.

To further reveal the reason for PPM1F downregulation in GC, we found no significant alterations in genetic and epigenetic dysregulation of PPM1F in GC. Then, we attempted to predict 5 miRNAs (miR-590, miR-186, miR-200b, miR-200c and miR-429) that could target PPM1F gene for explaining the downregulation of PPM1F

in GC, of which miR-590 had the strong relevance with PPM1F expression in GC samples. Luciferase assay showed the binding site of miR-590 with the wt 3'UTR of PPM1F and miR-590 mimic decreased PPM1F expression, while miR-590 inhibitor increased its expression in GC cells. Some other studies also show that PPM1F is negatively modulated by miR-149 and miR-200c in HCC and breast cancer.^{14,15} These results implied that the aberrant expression of PPM1F in GC might be caused by the dysregulation of miRNAs.

Functionally, contrary with previous reports,⁵⁻⁹ we found that PPM1F overexpression suppressed cell proliferation and invasion and counteracted the tumour-promoting role of miR-590, but PPM1F knockdown reversed these effects. Multivariate analysis also revealed miR-590 as an independent prognostic factor for tumour

recurrence of GC patients. Intriguingly, our findings for the functions of miR-590 in GC were also supported by some other studies in a variety of cancers, such as colorectal cancer (CRC),²⁴ cervical cancer²⁵ and glioblastoma,²⁶ but opposed by those in CRC^{27,28} and glioblastoma multiforme.²⁹ Thus, our above results showed that miR-590 might act as an oncogenic marker in GC by targeting PPM1F.

5 | CONCLUSIONS

Taken together, PPM1F expression was downregulated, but miR-590 was upregulated in GC samples and their expression levels were correlated with tumour recurrence of the patients with GC. miR-590 was further identified to exhibit an oncogenic role by targeting PPM1F and acted as an independent prognostic factor for tumour recurrence of GC patients.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was approved by the Hospital's Protection of Human Subjects Committee.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

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