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RESEARCH

Genetic polymorphism of the thrombospondin-related apical merozoite protein (TRAMP) of *Plasmodium knowlesi* in Malaysia

Yee Ling Ng[1] · Yee Ling Lau[1] · Mohd Hafizi Abdul Hamid[2] · Jenarun Jelip[2] · Choo Huck Ooi[3] · Rose Nani Mudin[4] · Joel Judson Jaimin[5] · Mun Yik Fong[1]

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Abstract

Plasmodium knowlesi is a simian malaria parasite that causes significant zoonotic infections in Southeast Asia, particularly in Malaysia. The *Plasmodium* thrombospondin-related apical merozoite protein (TRAMP) plays an essential role in the invasion of the parasite into its host erythrocyte. The present study investigated the genetic polymorphism and natural selection of the full length *PkTRAMP* from *P. knowlesi* clinical isolates from Malaysia. Blood samples ($n=40$) were collected from *P. knowlesi* malaria patients from Peninsular Malaysia and Malaysian Borneo. The *PkTRAMP* gene was amplified using PCR, followed by cloning into a plasmid vector and sequenced. Results showed that the nucleotide diversity of *PkTRAMP* was low (π: 0.009). *Z*-test results indicated negative (purifying) selection of *PkTRAMP*. The alignment of the deduced amino acid sequences of PkTRAMP of Peninsular Malaysia and Malaysian Borneo revealed 38 dimorphic sites. A total of 27 haplotypes were identified from the amino acid sequence alignment. Haplotype analysis revealed that there was no clustering of PkTRAMP from Peninsular Malaysia and Malaysian Borneo.

Keywords *Plasmodium knowlesi* · Thrombospondin-related apical merozoite protein · Genetic polymorphism · Haplotypes

Introduction

The World Health Organization (WHO) reported approximately 224 million malaria cases and 627,000 malaria death cases globally in 2020 (WHO 2021). *Plasmodium knowlesi* is endemic in Southeast Asia with the majority cases being reported from Malaysia (Luchavez et al. 2008; Ng et al. 2008; Van den Eede et al. 2009; Chin et al. 2020). Malaysia is vulnerable to transmission of malaria due to the tropical climate and its location in the humid equatorial region. In Malaysia, most of the cases of human *P. knowlesi* malaria have been reported in Malaysian Borneo. In the period 2013–2017, 77.1% of malaria cases in Malaysian Borneo were *P. knowlesi* malaria. Furthermore, the average malaria death rate in Malaysian Borneo is higher than in Peninsular Malaysia (Hussin et al. 2020). The reasons for the disparity in case numbers and severity between Peninsular Malaysia and Malaysian Borneo are unknown. However, genetically distinct haplotypes of some *P. knowlesi* genetic markers were detected in these two regions (Peninsular Malaysia and Malaysian Borneo) such as Duffy binding protein (*PkDBPαII*), cytochrome c oxidase subunit I (*PkCOXI*), type A small subunit ribosomal 18S RNA (*PkA-type 18S rRNA*), and apical membrane antigen-1 (*PkAMA-1*) (Fong et al. 2015; Yusof et al. 2016; Ng et al. 2021).

P. knowlesi malaria is a potentially life-threatening disease due to the parasite's much shorter (i.e., 24 h) asexual erythrocytic stage development than other medically important malaria parasites (Knowles and Gupta 1932; Cox-Singh et al. 2008). The erythrocytic cycle of the parasite is responsible for the manifestation of

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✉ Mun Yik Fong
fongmy@um.edu.my

[1] Department of Parasitology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia

[2] Vector Borne Disease Sector, Disease Control Division, Ministry of Health Malaysia, Putrajaya, Malaysia

[3] Sarawak Health Department, Ministry of Health Malaysia, Kuching, Sarawak, Malaysia

[4] Sabah Health Department, Ministry of Health Malaysia, Kota Kinabalu, Sabah, Malaysia

[5] Public Health Laboratory Kota Kinabalu, Ministry of Health Malaysia, Kota Kinabalu, Sabah, Malaysia

symptoms suffered by malaria patients. The invasion of erythrocytes by *Plasmodium* merozoites involves multiple steps which includes initial attachment, followed by apical reorientation of the merozoite. A tight junction is subsequently formed, and the junction moves to the posterior of erythrocytes powered by actin-myosin motor. This is followed by the entry of the merozoite into a parasitophorous vacuole in the erythrocyte (Cowman and Crabb 2006).

The *P. knowlesi* thrombospondin-related apical merozoite protein (PkTRAMP) is a 360-amino acid invasion-related protein that is part of a protein family containing the thrombospondin structural homology repeat (TSR) domain (Thompson et al. 2004). These TSR-containing proteins are known to play a crucial role in cell adhesion and cell interactions during cell migration (Adams and Tucker 2000). The essential role of TRAMP in merozoite invasion and the parasite blood-stage development has been shown in previous studies, where TRAMP specific-induced antibodies have been shown to inhibit parasite invasion in vitro (Uchime et al. 2012; Siddiqui et al. 2013). However, the genetic diversity of TRAMP is yet to be fully investigated.

P. knowlesi is presently the main cause of human malaria infection in Malaysia, and there are higher case numbers and malaria mortality rate reported in Malaysian Borneo compared with Peninsular Malaysia. The aim of the present study, therefore, is to conduct a comparative analysis on the genetic polymorphism and natural selection of *PkTRAMP* in Malaysian *P. knowlesi* isolates. This is the first study on *TRAMP* sequences from *P. knowlesi* malaria clinical samples, and the findings will be beneficial in understanding the level of polymorphism in PkTRAMP for future functional and vaccine development studies.

Materials and methods

Human blood samples and DNA extraction

Blood samples from *P. knowlesi* malaria patients were collected from hospitals in the Peninsular Malaysia ($n = 20$) states of Johor, Pahang, Kedah, Johor, Kelantan, Negeri Sembilan, Perak, Terengganu, Selangor, and Federal Territory of Kuala Lumpur, as well as the Malaysian Borneo ($n = 20$) states of Sabah and Sarawak. Most of the blood samples for this study were obtained in 2019–2020. The presence of *P. knowlesi* in the samples was screened by microscopic examination of thick and thin blood smears and nested polymerase chain reaction (PCR) based on the *Plasmodium* 18S rRNA locus (Snounou et al. 1993; Imwong et al. 2009). *Plasmodium* DNA was extracted from the blood samples using the QIAGEN Blood and Tissue Kit (QIAGEN, Hilden, Germany). One hundred µl of blood was used for DNA extraction, and 50 µl of EB Buffer was used for DNA elution. The DNA was stored at -20 °C until use.

Polymerase chain reaction (PCR) amplification of PkTRAMP gene

PCR of the *PkTRAMP* gene was carried out using forward primer PkTRAMPfull-F: 5'-GGATCCATGCGGAGC TTCACCTTCATA-3' and reverse primer PkTRAMPfull-R: 5'-GGATCCTTAATCGTACATAAATCATCCAGC CAC-3'. Approximately 0.5 µg of genomic DNA was used in a final amplification volume of 25 µl containing 2 mM MgCl2, 0.2 mM of dNTPs, 0.25 µM of forward and reverse primers, 1 unit of GoTaq® DNA polymerase, and 1X GoTaq® buffer (Promega, Madison, Wisconsin, USA). Cycling conditions for PCR were 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 100 s and a final extension at 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Eugene, USA). PCR products were ligated into the pGEM-T® TA cloning vector (Promega, Madison, Wisconsin, USA), followed by transformation into *Escherichia coli* TOP10F' competent cells using heat-shock method. The transformation colonies were screened for the presence of *PkTRAMP* DNA fragment. Positive recombinant plasmids were extracted and then sent to a commercial laboratory (Apical Scientific Sdn. Bhd., Malaysia) for DNA sequencing. For each isolate, three recombinant plasmid clones were sequenced to ensure that the *PkTRAMP* sequence obtained was consistent.

Genetic diversity analysis of PkTRAMP gene

DnaSP ver 6 (Rozas et al. 2017) was used to perform polymorphism analysis on the ~ 1020 bp sequences ($n = 40$). Information such as nucleotide diversity (π) and haplotype diversity (Hd) was calculated. The natural selection of *PkTRAMP* was determined by the *Z*-test ($P < 0.05$) in MEGA7 software based on Nei and Gojobori's method with Jukes and Cantor correction (Nei and Gojobori 1986). Multiple nucleotide and deduced amino acid sequence alignments of generated *PkTRAMP* sequences including a reference sequence (*P. knowlesi* strain H, GenBank Accession Number XM_002262219) were performed using BioEdit sequence alignment editor ver 7.2.0. The haplotype network of PkTRAMP amino acid sequences was constructed using NETWORK program ver 4.6.1 (Bandelt et al. 1999).

Results

All newly generated *PkTRAMP* sequences ($n = 40$) from Peninsular Malaysia and Malaysian Borneo were deposited in the GenBank database under Accession Numbers ON892606-ON892645. The genetic diversity and natural selection pressure index of the full length *PkTRAMP* for whole Malaysia, Peninsular Malaysia, and Malaysian Borneo are presented in Table 1.

The overall nucleotide diversity of *PkTRAMP* from Malaysia was low ($\pi = 0.009$). The nucleotide diversity of *PkTRAMP* from Peninsular Malaysia and Malaysian Borneo was very similar ($\pi = 0.008$ and $\pi = 0.007$, respectively). Meanwhile, the overall haplotype diversity of *PkTRAMP* from Malaysia was high (Hd = 0.999). The haplotype diversity of *PkTRAMP* from Peninsular Malaysia and Malaysian Borneo was high (Hd = 1.000 and Hd = 0.995, respectively).

A sliding window plot with a window length of 100 bp and a step size of 25 bp was used to determine nucleotide diversity along the entire *PkTRAMP* sequence. The nucleotide diversity of *PkTRAMP* from Peninsular Malaysia and Malaysian Borneo ranged from 0.000 to 0.027 (Fig. 1a) and from 0.001 to 0.016 (Fig. 1b), respectively. For *PkTRAMP* from Peninsular Malaysia, the most conserved regions ($\pi = 0.000$) were within nucleotide positions 551–650, whereas the highest peak diversity ($\pi = 0.027$) was within nucleotide positions 826–925. In contrast, the same nucleotide position (826–925) was the most conserved region ($\pi = 0.001$) for *PkTRAMP* from Malaysian Borneo, whereas the highest peak diversity ($\pi = 0.016$) was within nucleotide positions 201–300. The *Z*-test revealed purifying (negative) selection on the *PkTRAMP* across Malaysia, Peninsular Malaysia, and Malaysian Borneo (dN < dS, $P = 0.000$).

The *PkTRAMP* sequences (including strain H) were translated into amino acid sequences for analysis of haplotype polymorphism (Fig. 2). In the analysis, the PkTRAMP amino acid sequence of strain H was used as reference. Close examination identified 38 polymorphic sites, and all 38 were dimorphic. Overall, the amino acid sequences could be categorised into 27 haplotypes

Table 1 Estimates of genetic diversity and natural selection of the full length of *PkTRAMP* (1020 bp) from Malaysian *P. knowlesi* malaria patients

Location	Nucleotide diversity ($\pi \pm$ SD)	Haplotype diversity (Hd ± SD)	*Z*-test *P* values	
			Positive selection $dN > dS$	Negative selection $dN < dS$
Malaysia	0.009 ± 0.001	0.999 ± 0.006	1.000	0.000*
Peninsular Malaysia	0.008 ± 0.001	1.000 ± 0.016	1.000	0.000*
Malaysian Borneo	0.007 ± 0.001	0.995 ± 0.018	1.000	0.000*

π nucleotide diversity, *Hd* haplotype diversity, *SD* standard deviation, *d*N nonsynonymous mutation rate, *d*S synonymous mutation rate; *significance at $P < 0.05$

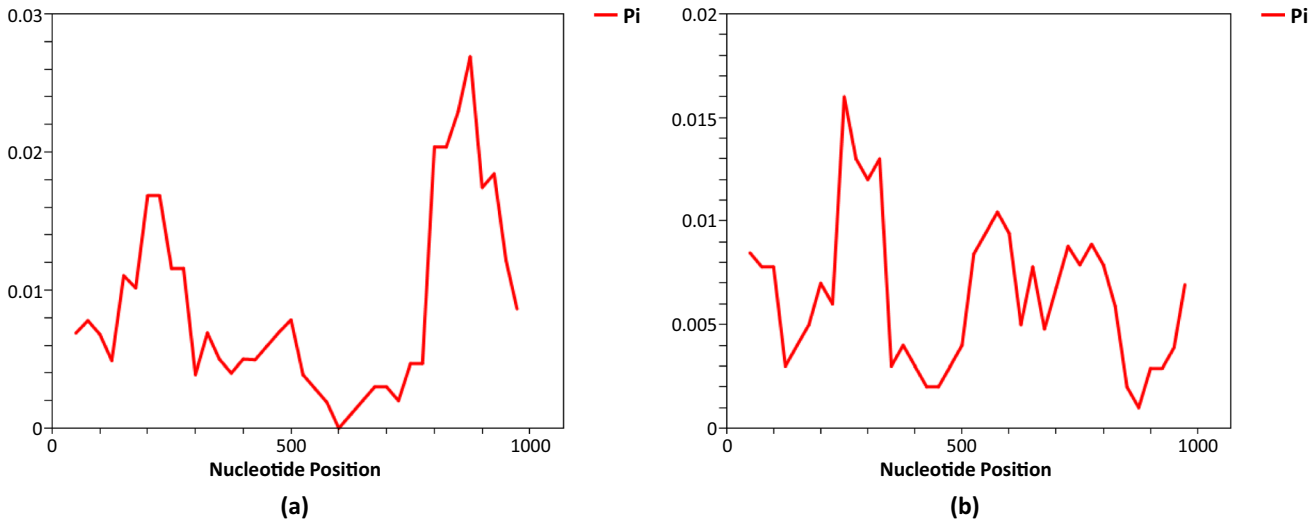

Fig. 1 Sliding window plot of the nucleotide diversity (π) along the *PkTRAMP*, generated with a window length of 100 bp and step size of 25 bp. **a** Nucleotide polymorphism in the *PkTRAMP* from Peninsular Malaysia. **b** Nucleotide polymorphism in the *PkTRAMP* from Malaysian Borneo

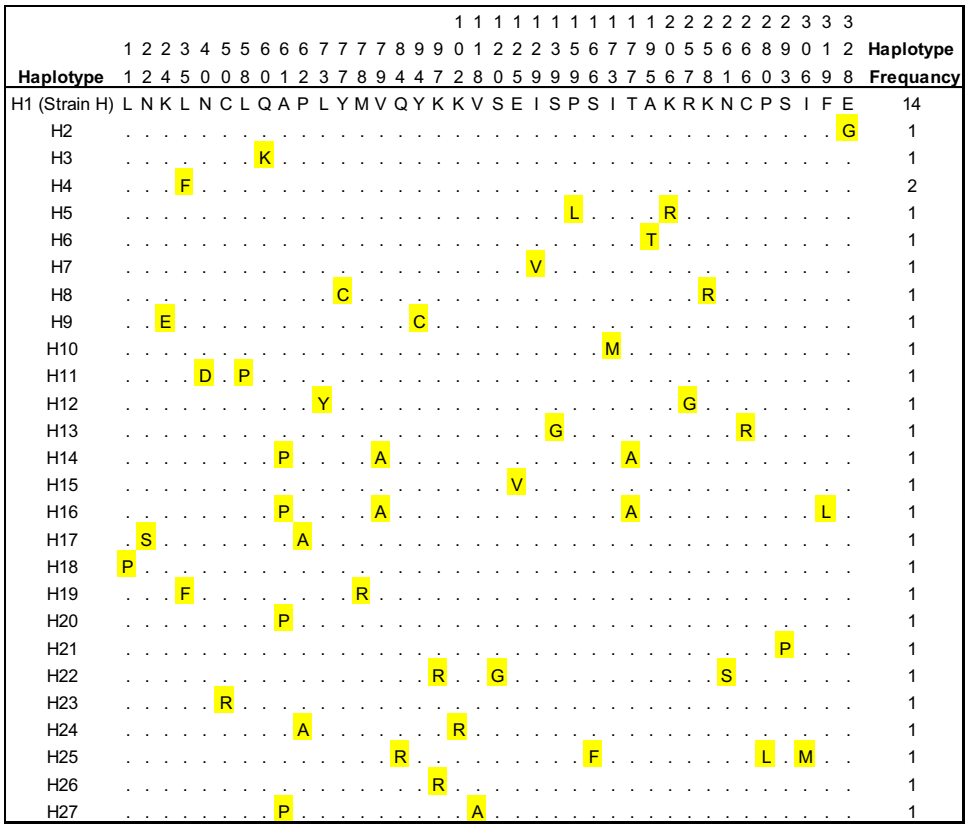

Fig. 2 Amino acid sequence polymorphism in PkTRAMP from Peninsular Malaysia and Malaysian Borneo. Polymorphic amino acid residues are listed for each haplotype. Amino acid residues identical to those of the reference sequence [strain H (haplotype 1)] are marked by dots. Dimorphic amino acid positions are marked in yellow shading. Haplotype frequency for each haplotype is listed in the right panel

(H1-H27). Haplotype H1 had the highest frequency ($n = 14$), thus being the most predominant in the population. Haplotype network analysis (Fig. 3) of the PkTRAMP did not reveal any specific separation between Peninsular Malaysia and Malaysian Borneo. The largest node was H1, which contained members from both Peninsular Malaysia and Malaysian Borneo. All other haplotypes basically originated from H1.

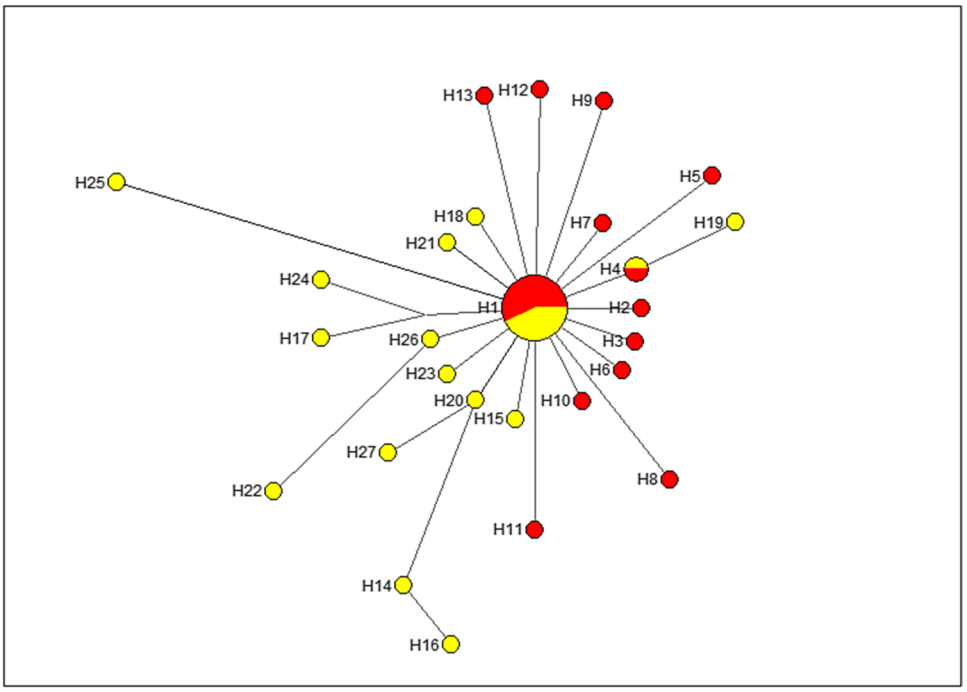

Fig. 3 Network analysis of 27 haplotypes of PkTRAMP. Red nodes indicate Malaysian Borneo haplotype members, and yellow nodes indicate Peninsular Malaysia haplotypes. The size of each node reflects the number of isolates in each haplotype

Discussion

In the current study, the nucleotide diversity of *PkTRAMP* ($\pi = 0.009$) was found to be lower than that of other *P. knowlesi* genes such as merozoite surface protein 7D (*MSP7D*) ($\pi = 0.052$) (Ahmed and Quan 2019), merozoite surface protein 3 (*MSP3*) ($\pi = 0.046$) (De Silva et al. 2017), and circumsporozoite protein (*csp*) ($\pi = 0.020$) (Chong et al. 2020), but almost similar to other low polymorphism genes such as *AMA-1* ($\pi = 0.006$) (Ng et al. 2021) and merozoite surface protein 4 (*MSP4*) ($\pi = 0.007$) (Ahmed et al. 2019). The haplotype diversity of *PkTRAMP* (Hd = 0.999) was similar to other *P. knowlesi* genes such as *AMA-1* (Hd = 1.000) (Ng et al. 2021) and *MSP3* (Hd = 0.999) (De Silva et al. 2017).

The identification of high haplotype diversity but low nucleotide diversity in *PkTRAMP* sequences was similar to those of other *P. knowlesi* genes such as *AMA-1* (Ng et al. 2021), rhoptry-associated protein 1 (*RAP-1*) (Rawa et al. 2016), and *MSP4* (Ahmed et al. 2019). This may suggest that the population may have undergone a recent expansion (Grant and Bowen 1998). Although haplotype diversity was high, the low nucleotide diversity values indicate minor variations between haplotypes. This is supported by the haplotype network analysis, which revealed mostly single or minor differences between haplotypes (Fig. 3).

The haplotype network revealed no distinct geographical separation of PkTRAMP between Peninsular Malaysia and Malaysian Borneo. This is similar to *P. knowlesi* proteins such as the PkCSP (Chong et al. 2020) and PkMSP7D (Ahmed and Quan 2019). However, some *P. knowlesi* genetic markers such as PkDBPαII (Fong et al. 2015), PkAMA-1 (Ng et al. 2021), PkCOXI, and PkA-type 18S rRNA (Yusof et al. 2016) showed distinct geographical separation.

The *Z*-test for natural selection on *PkTRAMP* indicated that *PkTRAMP* was under negative (purifying) selection, which is similar to other *P. knowlesi* genes (Fong et al. 2015; Rawa et al. 2016; Ahmed et al. 2019; Ng et al. 2021). This could possibly be due to functional constraints of the proteins that limit diversity since these genes have been shown to play important roles in the parasite's invasion into host erythrocyte. Another possible explanation could be that the *P. knowlesi* samples collected in these studies originated from human infections. Previous studies have shown that *P. knowlesi* invasion into macaque and human erythrocytes differs. For example, the *P. knowlesi* Duffy binding protein (DBP) has been shown to be crucial for human erythrocyte invasion but not macaque erythrocyte invasion (Dankwa et al. 2016). Thus, in theory, there would be a greater selection pressure towards certain *P. knowlesi* proteins in the human host rather than the macaque host, resulting in an increased representation of these haplotypes in the parasite population infecting humans.

Conclusion

The present study revealed low polymorphism of *PkTRAMP* and an absence of geographical clustering of the protein. In addition, *PkTRAMP* was found to be undergoing negative selection. Future genetic studies involving larger number of samples and immuno-characterisation of PkTRAMP should be carried out to validate the protein as a vaccine candidate for *P. knowlesi* malaria.

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Author contribution Mun Yik Fong and Yee Ling Lau conceived the idea, design the study, and developed the methodology. Mohd Hafizi Abdul Hamid, Jenarun Jelip, Choo Huck Ooi, Rose Nani Mudin, and Joel Judson Jaimin were involved in the collection and screening of human blood samples for malaria infection. Yee Ling Ng performed the DNA sequencing of the PkTRAMP gene and molecular data analysis. Yee Ling Ng wrote the manuscript, and other authors reviewed and edited the manuscript.

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Data availability All datasets are presented in the manuscript. Nucleotide sequences generated in the study are deposited into GenBank.

Declarations

Ethics approval Ethical approval for the use of these blood samples was granted by the Medical Research Subcommittee of the Ministry of Health, Malaysia (NMRR-15–67223975).

Consent to participate Human blood samples used in this study were taken as part of routine (planned) blood sampling for national malaria screening. No additional blood was drawn. No patients were subjected to additional procedures or were required to follow the rules of behaviour. Therefore, regulations on medical research involving human subject do not apply.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interest.

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