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Original Research

Investigation of association between CD40 current gene variants (rs4810485, rs1883832 and rs3765459) and serum CD154 protein levels in Iranian migraineurs

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Abstract: Migraine is a chronic neurological disease described by recurrent moderate to severe headaches often in association with neuro-inflammation. As cytokines are affect the immune response and migraine exacerbation, the current study aimed to investigate the possible associations between CD40 polymorphisms and level of soluble CD154 protein with migraine. In a prospective case-control study, we studied blood samples of 190 patients with migraine (migraineurs) and 200 healthy controls (HCs) from southeast Iran. Genotyping for the CD40 (rs4810485-intron, rs1883832-5′-UTR, and rs3765459-intron) gene variants were executed using PCR-RFLP and soluble CD154 protein levels were measured via ELISA method. Among CD40 gene variants, rs1883832 (TC genotype) was significantly associated with migraine (P = 0.007, OR = 2.326, 95% CI = 1.258-4.303). No significant associations observed between the rs4810485 and rs3765459 SNPs with migraine. The most frequent genotypes for CD40 were GG in rs4810485 (51.5%) and rs3765459 (62.1%) as well as TC in rs1883832 (53.7%). There was no statistically relationship between these gene variants and different subclasses of migraine. Concentration of soluble CD40L among patients with rs1883832 (TC genotype) were significantly (P = 0.027, OR = 0.417, CI = 0.192-0.906) higher in compared to healthy controls. Our findings showed that in CD40 rs1883832, TC genotype may have a role in migraine susceptibility. Therefore, it suggested that in addition to other factors, CD40 rs1883832 (TC genotype) genetic variation may also play a critical role in the etiology of migraine.

Key words: CD40; CD154; Inflammation; Migraine; Polymorphisms.

Introduction

Migraine is a painful and severe headache that accompanying with sensory warning and is a public health problem with great impact on both the society and patient (1). Since about half of migraineurs do not pursue medical attention besides lacking of social, economic or ethnic distribution, it is difficult to determine exact prevalence of disease in the community (2). It seems that about 15 to 16 percent of women and 5 to 9 percent of men are affected with migraine and its pervasiveness is highest among the ages of 30-49 worldwide (3). Migraine etiology is complex, involving both multiple genetic and environmental factors, but scientists propose three different mechanisms for its pathophysiology including: cardiovascular, neurological and neuro-inflammatory impairments (4, 5). The two major subclasses with different neurological symptoms of migraine are common migraine (without aura) and classic migraine with aura (6). According to the theory of neuro-inflammation, ions and inflammatory agents release in meninges sensory fibers nerve endings and stimulates pain receptors in these area (7). In addition, the inflammatory conditions cause changes in serum levels of immune mediators in migraine patients but diverse results have been reported on the mechanisms involved (8-12). The interaction between immune cells is regulated by several mechanisms, including cytokines, which play a crucial role in physiological and pathological processes such as, Immunity, inflammation and pain (13). Widely, cytokines and their receptors are present in the central nervous system (CNS) and have been proposed as important inflammation mediators in neuro-vascular system and likely to be involved in pain threshold modulation (14-20). The CD40 surface molecule is a 277-amino-acid glycoprotein expressed on B lymphocytes, and other cells occasionally present this antigen. The CD40 expression could be trigger with stimulation of CD154 (CD40L ligand) which is synthesized by CD4 positive cells or by NK cells, monocytes and lymphocytes B in case of inflammation (21). Soluble CD154 is a ligand of glycoprotein IIb-IIIa receptor that has inflammatory property including expression of adhesive molecule, chemokines and metalloproteinases (22). To understand the possible role of CD40/CD154 interaction and soluble CD154, in migraine headaches in the leading research we analyzed its important polymorphisms in migraineurs with two different subclasses of disease and results compared with healthy controls.

Materials and Methods

Patients and samples

The study approved by the ethics committee of Za-

hedan University of medical sciences and conducted using clinical samples from migraine patients (N = 190, age: 13 to 66 years, mean = 31.72) who were treated at the Department of Neurology, Ali-ebn Abitaleb Hospital, Zahedan, Iran, from August 2013 to February 2014. Healthy controls (HCs) without any inflammatory, neurological diseases, migraine headache and specific systemic disease (N = 200, age: 15 to 75 years, mean = 35.1) from volunteer blood donors were selected at the same time. Diagnoses of migraine was made according to standardized criteria (23). Patients were excluded if they had history of any inflammatory diseases or received any kind of anti-inflammatory medicines for past one month. Patients adjusted in two definite common (without aura, N = 112, 76 female and 36 male, age mean = 31.0) classic (with aura, N = 78, 56 female and 22 male, age mean = 32.5) subtypes of migraine. All patients were informed of the study and participated voluntarily and written consents were taken (23).

Blood collection, serum and DNA extraction

Whole peripheral blood (10 mL) samples were taken from all subjects and collected in separator tubes (contain EDTA, 0.5 M) and centrifuged for 15 min at 150 *g* at 20 °C and then serum stored at -20 °C in sterile plastic tubes for DNA extraction. Genomic DNA was extracted from the serum of 195 subjects with migraine headaches and 200 HCs using the DNA extraction kit (DIAtom DNA Prep., GORDIZ, Moscow, Russia) according to the manufacturer's instruction. DNA quality extracts were analyzed by electrophoresis. DNA concentration measured using NanoDrop device and concentration of 60 ng/µl as well as ratio of 260/280 nm between 1.7-1.9 were acceptable (24). For measurement of soluble CD154 venous blood samples were centrifuged within 15 min at 3,000 rpm for 10 min, and the supernatant were transferred into polypropylene tubes at -80 °C until the assays were performed (25). Soluble CD154 levels were measured by ELISA kit (Abcam, London, UK) according to the manufacturer's instruction.

CD40 PCR analysis

PCR amplifications for CD40 target sequences were performed in a final volume of 20 µl containing, 10 µl master mix (TAKARA, Tokyo, Japan), 0.7 µl (10 pmol) of each primer, 2 µL template DNA, and 6.6 µl DNase-free water was used (26). For CD40 single nucleotide polymorphisms (SNPs) rs4810485 (located in the intron 1 of the gene), rs1883832 (located in the Kozak consensus sequence of the 5′-UTR) and rs3765459 (located in the intron 8 of the gene) the amplification was performed with an initial denaturation step at 95 °C for 5 minutes; followed by 35 cycles at 94 °C for 30 s, 58 °C for 35 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min. We should mention that rs4810485 is in high linkage disequilibrium with rs1883832 (r2 = 0.95). The PCR product was checked for size and purity by 3% agarose gel electrophoresis. The locus of genes and primer information were indicated in Table 1.

CD40 RFLP analysis

CD40 single nucleotide polymorphisms (SNPs) rs4810485, rs1883832 and rs3765459 were analyzed through polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method (27). Final volume of 20 µL including 2 µL of 10×Buffer, 0.5 µL of related restriction enzyme, 7 µL of PCR product, 10.5 µL of double distilled water were used for all amplification products, overnight at 37 °C and 10 µL digestion product was loaded for electrophoresis. The restriction enzymes and sizes of the fragments as well as electrophoresis map were indicated in Table 1 and Figure 1 respectively.

Sample size and power estimation

Case-control analysis of SNPs have traditionally been carried out in the context of binary phenotypes. An effective sample size (SS) can be defined as the minimum number of samples that achieves suitable statistical power (SP). Statistical power is the possibility to reject a null hypothesis (H0) while the alternative hypothesis (HA) is true. The SP of 80% is used generally to avoid false negative associations and to determine a cost-effectiveness of SS (28). In this study, we calculate SP and SS according to the 0.09 disease prevalence in our region (29), 1.053 case-to-control ratio in our study, complete linkage disequilibrium (LD, D′=1), and 0.05

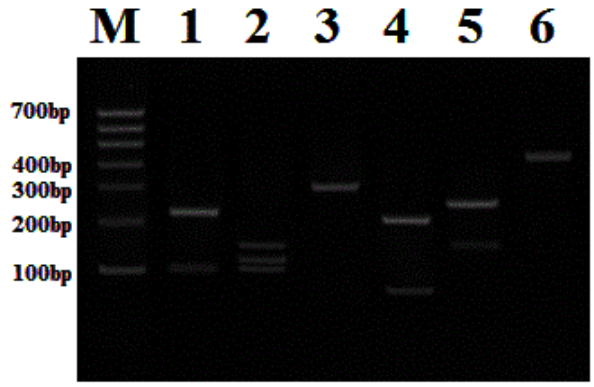

Figure 1. Genotypes of three CD40 SNPs. M: marker; rs4810485 T>G polymorphism: 1, TT genotype; 2, GG genotype; rs1883832 T>C polymorphism: 3, TT genotype; 4, CC genotype; rs3765459 A>G polymorphism: 5, AA genotype; 6, GG genotype.

Table1. Primer sequences and restriction enzymes used for detection of CD40 gene polymorphisms.

Locus (gene)	Reference SNP ID	Sequence (5`-3`)	Digestion pieces (RE)
20q13.1 (CD40)	rs4810485	F: TTAGGAGACCAGAGTTCT R: AAAGCTGTGGGACCAAAGCA	TT: 259+102 (MspI) GG: 148+111+102 (MspI)
20q13.1 (CD40)	rs1883832	F: TACACAGCAAGATGCGTCCCT R: AACAACTCACAGCGGTCAGCAA	TT: 291 (NcoI) CC: 229, 62 (NcoI)
20q13.1 (CD40)	rs3765459	F: ATGCTCCTTCCATCCAGA R: TCGTCGGGAAAATTGATCTCCT	AA: 263, 158 (HpyCH4III) GG: 421 (HpyCH4III)

F: Forward, **R:** Reverse, **RE:** Restriction enzyme.

type I error rate (α) under various conditions such as genetic (i.e., allelic, dominant, and recessive), marker allele frequencies (MAFs), and each single SNP markers ($P \leq 0.05$) via power for genetic association analyses (PGA) software (30).

Statistical analysis

SPSS version 22.0 (SPSS, Chicago) and SNPStats version 1.14.0 were used for all the statistical analyses. The association between CD40 genotypes and their relationship with soluble CD154 or migraine subtypes were estimated using the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. The Hardy-Weinberg equilibrium (HWE) was used with the X2 test for any of the SNPs under consideration. The significance level was set at $P \leq 0.05$ for all the tests.

Results

Our study had overall more than 80% power to detect the association between CD40 SNPs and the risk of migraine if OR=1.5 under a dominant model (Table 2).

Association of CD40 SNP (rs 4810485T/G) with migraine and sCD40L

The G/G, G/T and T/T genotypes were found in 57%, 37% and 6% in HCs, in comparison with 51.5%, 43.2% and 5.3% in migraineurs, respectively (Table 2). The allele frequency of (G/T) were 75% (G), 25% (T) in HCs and 73% (G), 27% (T) in migraineurs, respectively (Table 2). Distributions of CD40 polymorphisms in rs4810485G/T were not significantly different between patients and controls for GT (*OR* = 1.289, *P* = 0.396), and TT (*OR* = 0.969, *P* = 0.961) genotypes and also G (*OR* = 0.884, *P* = 0.591) and T (*OR* = 1.130, *P* = 0.596) alleles (Table 2). Similarly, there were no association between migraine subtypes (classic and common) and this CD40 SNPs in studied population (Table 3). On the other hand, concentration of sCD40L among patients with different rs4810485T/G SNPs have not shown any statistically significant changes compare to control group (Table 4). Moreover, there were no association between sCD40L and CD40 rs4010585T/G genotypes in different subclasses of migraine (Table 5).

Association of CD40 SNP (rs1883832T/C) with migraine and sCD40L

The C/C, T/C and T/T genotypes were found in 52%, 38% and 10% in HCs, in comparison to 31.6%, 53.7% and 14.7% in migraineurs, respectively (Table 2). The allele frequency of CD40 rs1883832T/C were 71% (C), 29% (T) in HCs and 59% (C), 41% (T) in migraineurs, respectively (Table 2). There were significant associations between TC (OR = 2.326, *P* = 0.007) genotype and also C (*OR* = 3.440, *P* = 0.000) and T (*OR* = 2.290, *P* = 0.000) alleles of CD40 rs1883832T/C SNP and migraine (Table 2). Moreover, there were significant association between migraine subtypes (classic and common) and TC+TT genotypes in studied population (Table 3). On the other hand, concentration of sCD40L among the patients with CD40 rs1883832 T/C genotype were significantly (OR = 0.417, *P* = 0.027) higher in comparison to control group (Table 4). Moreover, there were no association between sCD40L and CD40 rs1883832T/C genotypes in different subclasses of migraine (Table 5).

Association of CD40 SNP (rs3765459A/G) with migraine and sCD40L

The G/G, A/G and A/A genotypes were found in 49%, 41% and 10% in HCs, in comparison to 62.1%, 30.5% and 7.4% in migraineurs, respectively (Table 2). The allele frequency of CD40 rs3765459A/G (A/G) were 69.5% (G), 30.5% (A) in HCs and 77% (G), 23% (A) in migraineurs (Table 2). Distributions of CD40 polymorphisms in rs3765459A/G were not significantly

Table 2. Genotype and allelic frequencies of CD40 SNPs in patients and control subjects.

Reference SNP-ID (statistical power %)	Genotypes/alleles	Patient number (%)	Control number (%)	·OR (95% CI)	*P*-value
	GG	98 (51.5%)	114 (57%)	1.00	-
	TG	82 (43.2%)	74 (37%)	1.289 (0.717-2.316)	0.396
rs4810485	TT	10 (5.3%)	12 (6%)	0.969 (0.279-3.372)	0.961
(87%)	TG+TT	92 (48.5%)	86 (43%)	1.244 (0.708-2.188)	0.448
	G	139 (73%)	150 (75%)	0.884 (0.561-1.393)	0.591
	T	51 (27%)	50 (25%)	1.130 (0.717-1.781)	0.596
	CC	60 (31.6%)	104 (52%)	1.00	-
	TC	102 (53.7%)	76 (38%)	2.326 (1.258-4.303)	**0.007****
rs1883832	TT	28 (14.7%)	20 (10%)	2.427 (0.96-6.136)	0.061
(79%)	TC+TT	130 (68.4%)	96 (48%)	2.347 (1.309-4.209)	**0.004****
	C	112 (59%)	142 (71%)	3.440 (2.259-5.236)	**0.000*****
	T	78 (41%)	58 (29%)	2.290 (0.190-0.442)	**0.000*****
	GG	118 (62.1%)	98 (49%)	1.00	-
	AG	58 (30.5%)	82 (41%)	0.587 (0.320-1.079)	0.086
rs3765459	AA	14 (7.4%)	20 (10%)	0.581 (0.206-1.641)	0.306
(87%)	AG+AA	72 (37.9%)	102 (51%)	0.586 (0.331-1.037)	0.06
	G	146 (77%)	139 (69.5%)	1.500 (0.951-2.362)	0.079
	A	44 (23%)	61 (30.5%)	0.66 (0.423-1.049)	0.08

**p<0.01, ***p<0.001-significant p-value.

Table 3. Genotype and allelic frequencies of CD40 SNPs in different subclasses of migraine.

Reference SNP-ID	Genotypes/alleles	Common number (%)	Classic number (%)	˙OR (95% CI)	*P*-value
rs4810485	GG	72 (64%)	34 (43%)	1.00	-
	TG	40 (36%)	37 (48%)	2.009 (0.855-4.722)	0.110
	TT	0	7 (9%)	1.682 (0.046-0.786)	0.999
	TG+TT	40 (36%)	44 (57%)	2.381 (1.026-5.524)	**0.04****
	G	92 (82%)	52 (67%)	0.832 (0.399-1.733)	0.240
	T	20 (18%)	26 (33%)	1.201 (0.577-2.500)	0.623
rs1883832	CC	31 (28%)	31 (40%)	1.00	-
	TC	63 (56%)	41 (52%)	0.763 (0.302-1.928)	0.567
	TT	18 (16%)	11 (14%)	0.772 (0.212-2.813)	0.695
	TC+TT	81 (72%)	51 (66%)	0.765 (0.314-1.863)	0.555
	C	63 (56%)	47 (60%)	0.869 (0.484-1.561)	0.638
	T	49 (44%)	31 (40%)	1.150 (0.640-2.066)	0.638
rs3765459	GG	67 (59.5%)	50 (64%)	1.00	-
	AG	43 (38%)	20 (25%)	1.181 (0.558-2.498)	0.664
	AA	3 (2.5%)	9 (11%)	3.833 (0.433-33.93)	0.258
	AG+AA	45 (40.5%)	28 (36%)	0.799 (0.345-1.850)	0.600
	G	66 (59%)	51 (66%)	0.753 (0.406-1.396)	0.367
	A	46 (41%)	27 (34%)	1.328 (0.716-2.462)	0.367

***p*<0.01-significant *p*-value.

Table 4. Association of sCD40L with CD40 genotypes in patients and control subjects.

Reference SNP-ID	Genotypes	Controls Mean±SD (ng/ml)	Patients Mean±SD (ng/ml)	˙OR (95% CI)	*P*-value
rs4810485	GG	11.17±2.13	13.19 ±1.84	1.00	-
	TG	11.45±1.95	13.15±2.01	0.634(0.226-1.781)	0.387
	TT	9.51±2.91	11.90±1.92	0.497(0.174-1.423)	0.193
rs1883832	CC	11.09±2.11	13.04±1.63	1.00	-
	TC	11.17±2.30	12.92±2.04	0.417(0.192-0.906)	**0.027***
	TT	11.58±1.81	13.89±1.97	1.308(0.616-2.777)	0.484
rs3765459	GG	10.87±2.12	13.11±1.86	1.00	-
	AG	11.34±2.20	13.38±1.98	2.192(0.955-5.028)	0.064
	AA	11.92±1.96	11.91±2.02	0.822(0.348-1.942)	0.655

**p*<0.05-significant *p*-value.

Table 5. Association of sCD40L with CD40 genotypes in different subclasses of migraine.

Reference SNP-ID	Genotypes	Common number Mean±SD (ng/ml)	Classic number Mean±SD (ng/ml)	˙OR (95% CI)	*P*-value
rs4810485	GG	13.11±2.01	13.26±1.66	1.00	-
	TG	13.32±2.07	13.07±1.99	0	0.999
	TT	-	11.90±1.92	0	0.999
rs1883832	CC	12.71±1.54	13.24±1.67	1.00	-
	TC	13.01±2.13	12.85±1.99	1.125(0.397-3.190)	0.824
	TT	14.71±1.80	13.29±1.92	0.811(0.321-2.050)	0.658
rs3765459	GG	12.97±2.01	13.20±1.76	1.00	-
	AG	13.72±1.91	13.02±2.03	0.290(0.056-1.511)	0.142
	AA	10.32±0	12.17±2.07	0.205(0.038-1.118)	0.067

different in patients and controls for AG (*OR* = 0.587, *P* = 0.086), and AA (*OR* = 0.581, *P* = 0.306) genotypes and also G (*OR* = 1.500, *P* = 0.079) and A (*OR* = 0.66, *P* = 0.08) alleles (Table 2). Moreover, there are no significant association between migraine subtypes (classic and common) and these genotypes in studied population (Table 3). On the other hand, concentration of sCD40L among patients with different rs3765459A/G SNPs have not shown any statistically significant changes compare to healthy controls (Table 4). However, there were no association between sCD40L and CD40 rs3765459A/G genotypes in different subclasses of migraine (Table 5).

Discussion

Migraine is a chronic headache which is triggered by the changes in trigeminovascular system, but the pathophysiological mechanisms not well understood so far. Today, it is crystal clear that the vascular alterations are not limited to cranial vessels, and migraine is suggested to be a systemic vasculopathy (31). The vasculopathy of migraine is thought to reflect the endothelial dysfunction and impaired vascular reactivity. The activation of the platelets and the coagulation factors (32), the increased secretion of von Willebrand factor and tissue plasminogen activator from endothelium (33), the decrease in the circulating endothelial progenitor cells (34), which are all seen in migraine, supporting this theory. From increase in number of studies in the past decade, have been conducted that the risk of cardiovascular disease is increasing in migraineurs (35-38). Genetic association studies may point to the novel molecules that mediate migraine disorder and enabling its easier and more efficient management (23 and 39). Common genetic features, increased susceptibility, and/or vascular endothelial dysfunction may play a role in pathogenesis of migraine. Several studies utilized a candidate gene approach to elucidate genetic contribution to neuropathic pain phenotypes; however, the data is limited and inconsistent (40). The genetics of migraine is an interesting approach and its common or overlapping pathways involving the responsible genes may provide insight regarding the pathophysiological mechanisms that can explain their comorbidity with migraine (41 and 42). Cytokines and cytokine-inducible inflammatory molecules are small protein molecules secreted in response to immune stimuli and recent research has outlined important roles for cytokines in the migraine pathophysiology. Cytokines are involved in signaling that activates CNS glial cells. This activation is part of a poorly understood interaction between immune challenge and host that can lead to the development or facilitation of pathologic pain (43). CD40/CD154 pathway activation and a subsequent pro-inflammatory situation were reported in metabolic disorders such as obesity and atherosclerosis (44 and 45), diabetes mellitus (46) and hypertension (47). Whether migraine patients constitute a low- or high-risk group for cardiovascular disease is obscure, but high soluble CD154 levels in migraine patients support the presence of a vascular damage in migraine (45). In this study, we focused on type I TNF receptor CD40 (which is synthesized in inflammation by NK cells, monocytes and lymphocytes B) and its receptor CD154 in patients with migraine headaches to examine the hypothesis that say migraine headaches could be caused by an immune dysfunction (48). Guldiken et al have found neither significant differences in the soluble CD154, C-reactive protein (CRP) and prolactin levels in migraine patients with/without aura (49). Soluble CD40L has inflammatory property including expression of metalloproteinases, chemokines, cell adhesive molecule, and cytokines such as interleukin 1 (IL-1), IL-6, IL-8, IL-10 and tumor necrosis factor (TNF) from monocytes, dentritic cells, fibroblasts and epithelial cells. Matrix metalloproteinase 9, whose levels are found high during migraine attacks, degrades laminin, collagen type IV, a critical component of brain blood levels. TNF alpha, IL-6, IL1 beta and IL10 were found to be increased during migraine attacks (49). In the present study, since we did not measure the levels of these pro-inflammatory cytokines, it is not possible to conclude any association of the pro-inflammatory property of sCD40L with the inflammation in migraine. In Han Chinese population it has been suggested that TT genotypes of CD40 rs4810485 and rs1883832 genotypes may be predisposing genotypes for autoimmune diseases like Behçet's disease (50). In another study in Europeans population it has been confirmed that the CD40 rs4810485 G/T polymorphism is associated with susceptibility to rheumatoid arthritis and systemic lupus erythematosus (51). It has been reported that CD40 gene polymorphisms exert a genetic effect on IgE production in patients with asthma through translational regulation of CD40 expression on B cells (52). Buck and colleagues have been found neither significant differences between patients with multiple sclerosis and SNP of the CD40 (C/T21) gene variant (53). In another study it has been demonstrated that CD40 (rs1883832, rs4810485, and rs1535045)/CD154 (rs3092952, and rs3092920) SNPs has not any role in the susceptibility to systemic sclerosis (54). Here we have shown that among CD40/CD154 gene variants, CD40 rs1883832 is associated with susceptibility to migraine in Iranian population. As it has been shown that CD40/CD154 interaction, on the surface of activated T cells initiates a variety of signals in B cells including the activation of MAP kinases and NF-kB (55), it has been hypothesized that this above polymorphism may affect cellular immune responses and neuro-inflammation. In accordance with our result, other supporting study also claimed that the rs1883832 T allele is protective in Graves' disease but elevate risk of disease in Crohn's disease and multiple sclerosis (56). It has been confirmed that, inflammatory chemokines and cytokines such as interleukin 1 (IL-1), IL-6, IL-8, IL-10 and tumor necrosis factor (TNF) from monocytes, dendritic cells, fibroblasts and epithelial cells have a predominant role in the progress of cute CNS inflammation via induction of microgliosis and astrogliosis in the brain (57). Thus, our results for the first time provide evidence that improving our understanding toward how migraine have been related to CD40/CD154 gene variation and indicates that CD40/CD154 signaling could be a potential target for future development of migraine-specific preventive therapies. The data presented here must be viewed with caution due to the small number of patients enrolled and so these results should be taken as preliminary investigation of its kind. Similar studies recruiting larger sample sizes and conduct other ethnic groups and mixed-race studies on Iranian population may contribute to confirming our findings.

Conflict of interest

All the authors declare that they do not have financial disclosure or conflicts of interest.

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