RESEARCH ARTICLE

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Leukocyte Telomere Length Shortening, hTERT Genetic Polymorphisms and Risk of Childhood Acute Lymphoblastic Leukemia

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Abstract

Background: Telomeres are involved in chromosomal stability, cellular immortality and tumorigenesis. Human telomerase reverse transcriptase (TERT) is essential for the maintenance of telomere DNA length. Recently, a variable tandem-repeats polymorphism, MNS16A, located in the downstream region of the TERT gene, was reported to have an effect on TERT expression and telomerase activity. Previous studies have linked both relative telomere length (RTL) and TERT variants with cancer. Therefore, we evaluated associations between RTL, TERT gene polymorphisms (hTERT, rs2735940 C/T and MNS16A Ins/Del) and risk of childhood acute lymphoblastic leukemia (ALL) in an Iranian population. **Methods:** RTL was determined by a multiplex quantitative PCR-based method, and variants of the hTERT, rs2735940 C/T and MNS16A Ins/Del, were genotyped by amplification refractory mutation system PCR (ARMS-PCR), and PCR, respectively. **Results:** Our results indicated that RTL was shorter in ALL patients (1.53±0.12) compared to the control group (2.04±0.19) (P=0.029). However, no associations between hTERT gene variants or haplotypes and the risk of childhood ALL were observed (P>0.05). Also hTERT polymorphisms were not associated with RTL or patient clinicopathological characteristics, including age (P=0.304), sex (P=0.061) organomegally (P=0.212) CSF involvement (P=0.966) or response to treatment (P=0.58). **Conclusions:** We found that telomere attrition may be related to the pathogenesis of childhood ALL, irrespective to TERT variants.

Keywords: Telomere length- TERT genetic polymorphisms- childhood acute lymphoblastic leukemia

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Introduction

Telomeres are repetitive DNA sequences shielding the ends of chromosomes in eukaryotic cells, and contribute to maintenance of chromosomal integrity and genome stability (Bellon and Nicot, 2008). Because of the end replication problem, telomeres progressively shorten with repeated cell division, and may result in telomere malfunction and tumorigenesis (Sheng et al., 2012; Dos Santos et al., 2015). During tumorigenesis, telomere length (TL) usually go through progressive attrition and telomerase activity (TA) is restored (Ghaffari et al., 2008; Savage and Bertuch, 2010). Short telomeres have been reported to increase the risk of several cancers including esophageal squamous cell carcinoma (ESCC) (Zheng et al., 2009), head and neck cancer (Zhu et al., 2016), non-Hodgkin lymphoma (Lan et al., 2009), acute myeloid leukemia (AML) (Hartmann et al., 2005), or chronic lymphocytic leukemia (CLL) (Bechter et al., 1998). In CLL, telomeric dysfunction has been proposed as an emerging prognostic factor as TL was associated with high-risk genomic aberrations and poor outcome (Dos Santos et al., 2015).

The telomerase complex includes human telomerase reverse transcriptase (hTERT), the shelterin complex and the RNA component of the telomerase (TERC) that adds telomere DNA repeats onto the 3' ends of linear chromosomes. Telomerase activity is regulated by the human TERT gene, which encodes the catalytic subunit of telomerase (Hoxha et al., 2014). The hTERT gene is located on chromosome 5p15.33 (gene ID: 7015), and strictly is regulated by the transcriptional activity of the promoter region. Inherited genetic variations in TERT gene have been suggested to affect TL (Matsubara et al., 2006). For instance, the -1327C>T (rs2735940) is a single nucleotide polymorphism (SNP) located in the promoter region of hTERT is a T/C transition 1327-bp upstream of the transcription start site and affects its transcriptional activity (Matsubara et al., 2006). This genetic variation

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has been associated with the risk of various types of cancer including ALL (Sheng et al., 2013) and epithelial cancer (Iizuka et al., 2013), and it has also been shown to affect telomere length (Matsubara et al., 2006). Sheng et al., (2013) reported that TERT -1327C>T TT genotype and T allele were associated with higher risk of childhood ALL in a Chinese population. Besides, this SNP has been suggested as an independent factor influencing the risk of various epithelial malignancies in elderly Japanese (Iizuka et al., 2013).

Another genetic variation in the downstream region of the hTERT gene locus (5p15.33) is MNS16A, which is a polymorphism tandem repeat functional minisatellite (Baird, 2010). MNSA16A polymorphism status is classified either as short or long, with short alleles being associated with increased hTERT mRNA (Wang et al., 2010). HTERT MNS16A status has been associated with risk for lung cancer, nasopharyngeal carcinoma, colorectal cancer and malignant gliomas (Zagouri et al., 2012).

Given the importance of telomere length to cancer biology, we hypothesized that these two TERT SNPs -1327C/T and MNS16A Ins/Del, and telomere length are associated with risk of pediatric ALL. To test this hypothesis, we genotyped these SNPs near TERT in childhood patients with ALL in an Iranian population and further measured relative telomere length (RTL) in our case-control study.

Materials and Methods

Patients

This study recruited a total of 215 subjects comprised of 98 children diagnosed with ALL (57 male and 41 female; mean age at diagnosis=6.23 years) and 117 age and sex matched children with no history of cancer of any type as the control group) (58 male and 59 female; mean age at diagnosis=5.77 years) from a south-east Iranian population. Table 1 shows clinical and biological characteristics of ALL patients such as age, sex, hemoglobin (Hb), white blood cell (WBC) and platelet count at diagnosis, and the status of organomegally, lymphadenopathy (LAP), cerebrospinal fluid (CSF), response to treatment, immunophenotype as well as chromosomal abnormalities. Our study included remission samples with the MRD level of 0.01. Ethical approvals for recruitment were taken from local Ethics Committee of Zahedan University of Medical Sciences, and informed consent was obtained from all patients and healthy subjects in agreement with our institutional guidelines. None of the patients received chemotherapy or radiotherapy prior to blood collection.

Telomere length measurement

Peripheral blood lymphocytes were collected in EDTA-containing tubes from patients and healthy controls and DNA samples were isolated by salting out method as described previously (Hashemi et al., 2012; Hasani et al., 2014; Hashemi et al., 2014b). Relative telomere length was measured by the multiplex quantitative real-time PCR-based assay described by Cawthon with minor modifications (Cawthon, 2009). In the present study, modifications to the original protocol included new sets of primers for endogenous single copy gene control albumin of albu and albd that were taken from the study of Zhu et al., (2015). The average RTL was estimated from the ratio of the telomere (T) repeat copy number to a single gene copy number (Alb gene; S), expressed as the T/S ratio for each sample using standard curves. The DNA quantity standards were serial dilutions of a reference DNA sample (a mixture of several unknown children's DNAs). All samples for both the telomeres and single-copy gene reactions were performed in triplicate.

Primer sequences were shown in the supplementary online resource Table S1. In the multiplex qPCR, we combined the telomere primer pair telg and telc (final concentrations 900nM each) with the albumin primer pair albu and albd. QPCR was performed in a final volume of 25 µl using 12.5 µl of 1X SYBRGreen Master Mix (Genetbio, South Korea), 100 nmol/L of primers, 10mM Tris–HCl pH8.3, 50mM KCl, 1mM DTT and 20 ng of DNA from patients and controls. The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at 88°C with signal acquisition. The 74°C reads indicates the Ct values for the amplification of the telomere template; the 88°C reads provided the Ct values for the amplification of the alb template.

We also determined the lower, middle and upper quartiles of RTL distribution (Q1, Q2, Q3 and Q4) based on the RTL distribution in control subjects.

Genotyping of hTERT gene polymorphisms

We genotyped hTERT rs2735940 C/T polymorphism by the amplification refractory mutation system PCR (ARMS-PCR) method (Hashemi et al., 2014a). The PCR cycling condition was an initial denaturation at 95°C for 5 min followed by 30 cycles of 30 s at 95°C, annealing temperature for 23 s at 60°C, and 30 s at 72°C, with a final extension of 72°C for 10 min. The product sizes were 197 bp for the C or T allele and 574 bp for the internal control. The presence of a 197-bp allele-specific band, in conjunction with a 574 bp control band, was considered to be positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered to be evidence for the absence of an allele.

PCR was used to genotype the MNS16A variable number of tandem repeat polymorphisms with the primer set, as we previously reported (Hashemi et al., 2014a). The amplification procedure consisted of an initial denaturing step for 5 min at 95°C followed by 30 cycles for 30 s at 95°C, 20 s at 67.5°C, and 17 s at 72°C, as well as a final extension step for 10 min at 72°C. The PCR products were visualized on 3% agarose gel containing 0.5 µg/ml of ethidium bromide and genotypes were assigned as as the short (S*) allele, 243 bp, or long (L*) allele, 302 bp, thus defining the MNS16A genotypes as L*/L*, L*/S* and S*/S*.

Statistical analysis

All statistical analyses were performed using the SPSS software for Windows, version 15.0 (SPSS Inc, Chicago IL, USA). Data were expressed as mean ± SD. Comparison of RTL among groups was performed using the Mann-Whitney test and paired T-test. Groupwise comparison of the distribution of clinical and laboratory variables was executed with the Student t test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). P-values below 0.05 were considered statistically significant. For genetic comparisons, differences in allele, genotype and haplotype frequencies were evaluated by the χ^2 test using SNPStats software (Sole et al., 2006).

Results

Association of hTERT -1327C/T and hTERT MNS16A Ins/Del polymorphisms with ALL

Our study revealed that none of hTERT -1327C/T and hTERT MNS16A Ins/Del polymorphisms were associated with the risk of ALL in our population as demonstrated in Table 2 (P>0.05). Although the hTERT -1327C/T heterozygote CT vs CC genotype in codominant model was more prevalent in ALL patients than in healthy subjects (47% vs 40%), the difference was not statistically significant (OR=1.57; 95%CI=0.79-3.10; P=0.190). Similar results obtained using other genetic models including codominant homozygote (P=0.332), dominant (P=0.213), recessive (P=0.877) and allele comparison (P=0.322). With respect to hTERT MNS16A Ins/Del polymorphism, no significant difference was found between case and controls regarding distribution of hTERT MNS16A allele and genotypes using multiple genetic models, e.g. codominant heterozygote (P=0.172), codominant homozygote (0.356), dominant (P=0.153), recessive (P=0.636) and allele comparison (P=0.144).

Table 1. Clinical Characteristics of Childhood Acute Lymphoblastic Leukemia Patients

Characteristics	Cases n (%)
Age at Diagnosis, year	
Mean	6.23
Range	2-18
Sex	
Male	58 (59.2)
Female	40 (40.8)
WBC (*10^3)	34.1
HB	7.5
PLT (*10^3)	59.7
Organomegally	
Positive	80 (82.5)
Negative	17 (17.5)
LAP	
Positive	55 (56.7)
negative	42 (43.3)
CSF Involvement	
Positive	6 (6.1)
negative	92 (93.9)
Response to treatment	
Yes	80 (82.5)
Metastatic	17 (17.5)
Immunophenotype	
B-cell lineage	89 (90.8)
T-cell lineage	9 (9.2)
Chromosomal abnormalities	
Negative	94 (95.9)
Philadelphia chromosome	4 (4.1)

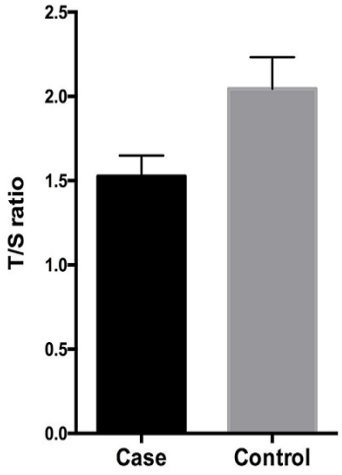

Figure 1. Comparison of Relative Telomere Length Distribution in Patients with Childhood ALL and Controls. Telomere length described as relative telomere copy to Single gene copy numbers (T/S ratio); P=0.029.

As shown in table 3, two -1327C/T and MNS16A INS/Del polymorphisms were not correlated with demographic and clinical characteristics of ALL patients. The ALL patients with MNS16A SS or -1327TT genotypes had shorter telomeres than those with LL (0.88 vs 1.62) or CC (1.35 vs 1.71), respectively, but the differences were not significant (P>0.05). Although the mean RTL was shorter in ALL patients with -1327TT genotype vs CC (1.35 vs 1.71) as well as in patients with SS vs LL (1.46 vs 1.75), the differences were not significant (P>0.05).

Table 4 indicates the haplotype analysis of -1327C/T and MNS16A INS/Del polymorphisms in ALL patients and controls. We found that the C/S haplotype (-1327/MNS16A) was more frequent in ALL patients than in controls (0.208 vs 0.126) but the difference was not statistically significant (OR=1.74; 95% CI=1.00-3.66; P=0.050).

Association of RTL and childhood ALL risk

In our series, the analysis of RTL showed a significant telomere shortening in ALL patients (Mean ± SEM=1.53±0.12) compared to the control group (Mean ± SEM=2.04±0.19) (p=0.029) (Figure 1). Then we assessed the distribution of different RTL quartiles in cases and controls, and we found a significant difference between two groups with respect to RTL quartiles distribution (P-trend=0.001). In case group, low quartiles

Table 2. Genotypic and Allelic Frequencies of hTERT rs2735940 C/T and hTERT MNS16A Ins/Del Polymorphisms in ALL Patients and Cancer Free Subjects

Genetic Model	Genotypes	ALLn (%)	Control n (%)	OR (95% CI)	P-value
hTERT rs2735940 C/T					
Codominant	CC	21 (23.3)	37 (31.6)	Ref.	-
	CT	42 (46.7)	47 (40.2)	1.57 (0.79-3.10)	0.190
	TT	27 (30.0)	33 (28.2)	1.44 (0.69-3.02)	0.332
Dominant	CC	21 (23.3)	37 (31.6)	Ref.	-
	CT+TT	69 (76.7)	80 (68.4)	1.51 (0.81-2.84)	0.213
Recessive	CC+CT	63 (70.0)	84 (77.8)	Ref.	-
	TT	27 (30.0)	33 (28.2)	1.09 (0.59-1.99)	0.877
Allele	C	84 (46.6)	121 (51.7)	Ref.	-
	T	96 (53.4)	113 (48.3)	0.82 (0.55-1.21)	0.322
MNS16A Ins/Del					
Codominant	LL	44 (50.0)	69 (60.5)	Ref.	-
	LS	34 (38.6)	35 (30.7)	1.52 (0.83-2.78)	0.172
	SS	10 (11.4)	10 (8.8)	1.57 (0.60-4.07)	0.356
Dominant	LL	44 (50.0)	69 (60.5)	Ref.	-
	LS+SS	44 (50.0)	45 (39.5)	1.53 (0.87-2.68)	0.153
		21 (28.0)			
Recessive	LL+LS	78 (88.6)	104 (91.2)	-	-
	SS	10 (11.4)	10 (8.8)	1.33 (0.53-3.36)	0.636
Allele	L	122 (69)	173 (76)	Ref.	-
	S	54 (31)	55 (24)	1.39 (0.90-2.16)	0.144

Table 3. Association among hTERT Polymorphisms, Telomere Length and Clinicopathological Characteristics of ALL Patients

Variables	MNS16A Ins/Del			P-value	hTERT rs2735940 C/T			P-value	Telomere length (T/S)
	LL	LS	SS		CC	CT	TT		
Telomere Length									
(T/S)	1.62	1.34	0.88	0.173	1.37	1.37	1.40	0.508	-
Age at Diagnosis									
(Mean, year)	6.65	5.65	6.48	0.381	3.02	4.44	3.64	0.315	P=0.304
Sex				0.763				0.399	P=0.061
Male	56	38	10		31	42	35		1.31
Female	57	31	10		27	47	25		1.78
Organomegally				0.086				0.947	P=0.212
Positive	38	29	6		17	35	23		1.41
Negative	5	5	4		3	7	4		1.03
LAP				0.196				0.216	P=0.032
Positive	29	18	4		14	21	18		1.56
Negative	14	16	6		6	21	9		1.07
CSF. involvement				0.227				0.305	P=0.966
Positive	5	1	0		2	1	3		1.38
Negative	39	33	10		19	41	24		1.36
WBC (*10^3)	33.1	33.5	33.8	0.856	21.7	42.7	30.6	0.270	P=0.914
HB (*10^3)	7.8	7.6	7.6	0.980	7.5	7.3	8.2	0.324	P=0.245
PLT(*10^3)	65.3	56.3	58.7	0.856	64.2	53.2	71.1	0.415	P=0.086
Response to treatment				0.761				0.385	P=0.58
Responsive	35	29	9		17	34	24		1.47
Metastatic	8	5	1		4	8	2		0.89

Table 4. Haplotype Analysis of Two hTERT Polymorphisms

SNP1 rs2735940	SNP2 MNS16A	ALL n (%)	Control n (%)	OR (95% CI)	P-value
C	L	0.367	0.401	Ref	-
T	L	0.326	0.357	1.01 (0.65 - 1.58)	0.96
C	S	0.208	0.126	1.74 (1.00 – 3.66)	0.05
T	S	0.100	0.117	1.16 (0.53 - 2.56)	0.71

Table 5. Association of Different Telomere Quartiles with Biological and Clinical Parameters of the Patients

Characteristic	Q1	Q2	Q3	Q4	P-trend
	0.317≤	>0.317 - ≤1.568	>1.568- ≤2.987	>2.987	
n (cases/controls)	10/25	55/25	18/26	6/25	**0.001**
Percent (cases/controls)	11/25	62/24	20/26	7/25	
Age at Diagnosis (Mean, year)	6.25	6.34	5.71	4.43	0.764
Sex					0.139
Male/Female	6/4	38/17	8/1	2/4	
Organomegally					0.726
Positive/Negative	8/2	44/11	14/4	5	
LAP					0.145
Positive/Negative	6/4	26/29	10/8	5	
CSF. involvement					0.202
Positive/Negative	2/8	2/53	1/17	1/5	
WBC (*10^3)	38.80	30.36	33.54	43.55	0.941
HB (*10^3)	6.91	8.08	7.25	5.54	0.153
PLT (*10^3)	97.30	56.15	47.73	49.68	0.059
Response to treatment					0.061
Responsive	6	43	18	5	
Metastatic	4	12	0	1	
Immunophenotype					0.552
Pre-B cell	9	51	15	6	
T cell	1	4	3	0	
Chromosomal aberrations					0.727
Negative	10	54	17	6	
Philadelphia positive	0	1	1	0	
MNS16A Ins/Del					0.652
LL/LS/SS	17/11/2004	33/31/8	22/12/2005		
hTERT rs2735940 C/T					0.365
CC/CT/TT	12/11/2010	18/31/25	11/18/9	10/16/4	

Bold number is indicative of significant p-value

(Q1 and Q2) were more frequent than those in controls, but in control group, high quartiles (Q3 and Q4) were more common indicating that shorter telomere length is a risk factor for childhood ALL (Table 5). We found no significant relationship between RTL quartiles and biological and clinical parameters of the patients.

Discussion

The present study demonstrated that telomere attrition occurred more frequently in ALL patients than in controls (T/S ratio: 1.36 vs 1.90). However, hTERT gene polymorphisms were not associated with the risk of ALL, and no relationship between these polymorphisms and RTL or clinical characteristics of ALL patients was found.

Previous literature demonstrated that short telomeres contributed to increased risk of cancer at several sites, including head and neck cancer (Zhu et al., 2016), non-Hodgkin lymphoma, a type of malignant hemopathy Lan et al., (2009) or CLL Bechter et al., (1998). In a meta-analysis, Liu et al., (2011) indicated that shorter telomeres were significantly associated with cancer risk compared with longer telomeres. In the stratified analysis by tumor type, the significant association was observed in subgroups of bladder, lung cancer, cancers in the digestive system and the urogenitals system. Additionally, Wu et al., (2003) reported reduced TL in multiple myeloma (MM) patients compared with the TL

in plasma cells (PCs) of healthy donors. They suggested that the telomere attrition observed in ALL patients might be caused by a rapid and extensive cell proliferation and population doubling that occurs before telomerase activation. In CLL, telomeric dysfunction has been proposed as an emerging prognostic factor as TL was associated with high-risk genomic aberrations and poor outcome (Dos Santos et al., 2015). The study of Borssen et al., (2011) indicated that telomeres were shorter at ALL diagnosis compared to at cessation of therapy, suggesting that the leukemia blasts had shorter telomeres than normal hematopoietic stem cells from which the bone marrow cells at remission originated. This is in line with previous data focusing on AML Hartmann et al., (2005) and CML Palma et al., (2013), demonstrating that telomeres in leukemic blasts compared to in normal blast and progenitor cells (Drummond et al., 2007). A possible explanation for this finding is a difference in the proliferative history (i.e., the number of cell cycle rounds) of leukemic vs normal blasts and progenitors.

Dissimilar to our results, long telomere length was found in various cancers. Sheng et al., (2012) indicated that extreme telomere length may be a potential predictor for future risk of ALL, and TERC rs16847897 may contribute to the development of childhood ALL supporting the results of Ojha et al., (2016) on CLL. In their study, Ojha et al., (2016) collected genome-wide data from two groups of patients with CLL and two control populations. They observed that CLL patients were predisposed to longer LTL than controls in both case-control sets. One explanation for the conflicting reports could be that telomere length is controlled by both genetic and environmental factors, such as race, smoking, inflammation and oxidative stress (Epel et al., 2004). Differences in gene – gene and gene – environment interactions from different genetic backgrounds may also play a role in the discrepancy.

The association between the -1327C>T polymorphism and cancer susceptibility has been controversial. Our study indicated that none of hTERT -1327C>T and hTERT MNS16A Ins/Del polymorphisms affected the risk of ALL. However, Iizuka T., et al (Iizuka et al., 2013) presented evidence that the -1327C>T polymorphism in the hTERT promoter-1327 T allele was associated with a lower risk of epithelial malignancy, particularly prostate (latent), colorectal and lung cancers in the elderly Japanese population. However, Sheng et al., (2013) reported that TERT -1327 TT genotype and T allele were associated with higher risk of childhood ALL in a Chinese population . A possible explanation for opposing effects of the -1327 T on cancer risk could be the difference in the genetic background or linkage disequilibrium. It has been shown that carriers of -1327 T allele had significantly longer telomeres than those of -1327 T non-carriers (Matsubara et al., 2006), but our study indicated no correlation between TL and -1327T/C polymorphism.

With respect to MNS16A polymorphism, the meta-analysis of Chen et al,. (2013) suggested that this polymorphism was associated with cancer risk. In the subgroup analysis stratified by ethnicity, a significantly increased cancer risk was suggested for Caucasians but not for Asians in all genetic models, which supports our findings.

Optimal telomere length is a balance of cell proliferation, senescence and control. Telomere shortening to a critical length results in loss of telomere protection, leading to chromosomal instability and loss of organismal viability, which could reflect a constitutional or acquire tendency toward leukemogenesis. Hartmann et al., (2005) have confirmed that telomere shortening was significantly more pronounced in adult patients with leukemia with cytogenetic alterations as compared to patients with normal karyotypes. On the other hand, maintaining long telomeres for longer periods may facilitate tumorigenesis by predisposing cells to delayed senescence, with greater risk of opportunity for acquiring genetic abnormalities and higher risk of transformation (Sheng et al., 2012). Hence a tendency to have extreme, shorter or longer telomeres could each contribute to carcinogenesis.

In childhood ALL patients, the Philadelphia chromosome translocation is rare, with a frequency of less than 5%. However, it is classified as a high or very high risk, and only 20-30% of Philadelphia chromosome-positive (Ph+) children with ALL are cured with chemotherapy alone. In our population, only 4.1 percent of patients (4 out of 98) were positive for Philadelphia chromosome.

In conclusion, our study provided evidence regarding the contribution of telomere shortening in pediatric ALL development, with no correlation with hTERT genetic variants. Further studies are warranted to confirm our findings.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Conflict of Interest

All authors declare that there is no conflict of interest.

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