

Long non-coding RNA PAX8-AS1 polymorphisms increase the risk of childhood acute lymphoblastic leukemia

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Abstract. The present case-control study was conducted on 110 children with acute lymphoblastic leukemia (ALL) and 120 healthy children to determine the impact of polymorphisms in paired-box gene 8 (PAX8) antisense RNA 1 (PAX8-AS1), namely rs4848320 C>T, rs6726151 T>G and rs1110839 G>T, on ALL risk. Genotyping was performed through the polymerase chain reaction-restriction fragment length polymorphism method. The findings indicated that the rs4848320 variant increased the risk of ALL in codominant [CT vs. CC: odds ratio (OR)=2.13, 95% confidence interval (CI)=1.16-3.90, P=0.014; and TT vs. CC: OR=2.21, 95% CI=1.03-4.74, P=0.041], dominant (CT+TT vs. CC: OR=2.15, 95% CI=1.22-3.81, P=0.009,) and allele (T vs. C: OR=1.55, 95% CI=1.07-2.25, P=0.024) inheritance models. The rs6726151 variant significantly increased the risk of ALL in codominant (GT vs. GG: OR=1.88, 95% CI=1.08-3.27, P=0.036) and overdominant (GT vs. GG+TT: OR=2.08, 95% CI=1.23-3.53, P=0.008) inheritance models. No significant relationship was identified between the rs1110839 G>T variant and disease risk/protection in childhood ALL. In conclusion, the findings of the present study indicated that rs4848320 and rs6726151 polymorphisms of PAX8-AS1 may be a risk factor for the development of childhood ALL. Further studies with larger sample sizes and different ethnicities are now required to confirm these findings.

Introduction

Acute lymphoblastic leukemia (ALL) is the most prevalent malignancy in children and constitutes approximately 75% of pediatric acute leukemias (1). While the etiology of ALL is not fully understood, previous reports have indicated that genetic factors serve a role in the development of childhood ALL (2-5).

Non-coding RNAs, comprising microRNAs and long non-coding RNAs (lncRNAs), do not encode protein sequences, yet are involved in various biological processes (6-8). In particular, lncRNAs, as transcripts of >200 nucleotides in length that lack protein-coding potential, regulate gene expression at various levels, including at the chromatin remodeling (9), transcription and post-transcriptional processing stages (10,11).

Paired-box gene 8 (PAX8) encodes a transcription factor required for cell growth and differentiation during embryonic development (12). Overexpression of PAX8 has been identified in various cancers (13-17). Though the precise role of PAX8 in cancer remains uncertain, it has been proposed that PAX8 contributes to the development and progression of specific cancers by maintaining tissue specific stem cells, by inhibiting terminal differentiation and apoptosis (18).

lncRNA PAX8 antisense RNA 1 (PAX8-AS1) is mapped to chromosome 2q13 in the upstream region of PAX8 (19). An expression quantitative trait loci (eQTL) is a locus containing a genetic variant that influences the expression level of a gene (20). PAX8-AS1, a potential regulator of PAX8, may contain polymorphisms that represent eQTLs for PAX8 (21). In particular, previous bioinformatics analyses have revealed that the polymorphisms rs4848320 C>T and rs1110839 G>T in PAX8-AS1 may be eQTLs for PAX8 (21). Furthermore, it has been suggested that rs4848320 and rs1110839 may affect the function or expression of PAX8-AS1, thereby influencing PAX8 expression (22,23). Few previous studies have evaluated the impact of PAX8-AS1 variants on cancer risk. Han *et al* (19) reported that rs4848320 and rs1110839 variants of PAX8-AS1 significantly decreased the risk of cervical cancer (19). Ma *et al* (24) identified that the two variants of PAX8-AS1 were significantly associated with the prognosis of hepatocellular carcinoma (HCC). However, to the best of

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our knowledge, no previous study has investigated the impact of PAX8-AS1 polymorphisms on childhood ALL. Based on the previous findings on PAX8-AS1 and cancer risk, it was hypothesized that polymorphisms of PAX8-AS1 may affect the risk of childhood ALL by disturbing the interaction between PAX8-AS1 and PAX8, to in turn influence PAX8 expression. PAX8 has been demonstrated to serve an important role in the pathogenesis of cancer by inhibiting cell differentiation and apoptosis (18). Therefore, the present study aimed to assess the possible association between the PAX8-AS1 polymorphisms rs4848320 C>T, rs1110839 G>T and rs6726151 T>G and the risk of childhood ALL in a Southeast Iranian population sample. In the analysis, the polymorphisms which have been implicated as potential risk factors for cancer were selected (19,24), while the rs6726151 T>G variant, with a minor allele frequency of 0.486 (25), was examined for the first time. The findings of the present study highlight the potential role of PAX8-AS1 variants in the pathogenesis of childhood ALL.

Materials and methods

Patients. A total of 230 subjects including 110 children diagnosed with ALL and 120 age- and sex-matched healthy children were enrolled in the present case-control study. The study design including the enrolled patients has been reported previously by our group (2,8). The local Ethics Committee of Zahedan University of Medical Sciences (Zahedan, Iran) approved the project (approval no. IR.ZAUMS.REC.1395.270) and informed consent was obtained from the parents of all participants. Extraction of genomic DNA from whole blood was performed using the salting out method as described previously (26).

Genotyping. Polymorphism genotyping was performed through the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primer sequences and restriction enzymes are summarized in Table I. The primers were produced by Bioneer Corp., (Daejeon, Korea). Into a 0.20 ml PCR reaction tube, 1 μ l genomic DNA (~100 ng/ml), 1 μ l (10 μ M each) forward and reverse primers, 10 μ l 2X Prime Taq Premix, all from Genet Bio, Inc., (Daejeon, Korea), and 7 μ l ddH₂O were added. The PCR conditions were as follows: Preheating for 6 min at 95°C; 30 cycles of 95°C for 30 sec, 64°C for rs1110839 and rs4848320 for 30 sec or 62°C for rs6726151 for 30 sec, and 72°C for 30 sec; followed by a final extension step for 5 min at 72°C. Subsequently, 10 μ l of amplified product was digested with the appropriate restriction enzyme (New England BioLabs, Inc., Ipswich, MA, USA), resolved on 2.5% agarose gel containing 0.5 μ g/ml ethidium bromide, observed under a UV transilluminator (DigiDoc H101; UVP, LLC, Upland, CA, USA) and photographed. For quality control, 15% randomly selected samples were re-genotyped and the outcome revealed 100% concordance.

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM, Corp., Armonk, NY, USA). The categorical and continuous data were analyzed using χ^2 and t-test, respectively. Individual single nucleotide polymorphism (SNP) associations with childhood ALL risk were assessed using unconditional logistic regression analyses, in which odds ratios (ORs) and 95% confidence

intervals (CIs) were determined for codominant, dominant, recessive, overdominant and allele inheritance models. $P < 0.05$ was considered to indicate a statistically significant difference. Haplotype and linkage disequilibrium analyses were conducted using SNPStats (<https://www.snpstats.net/snpstats>) (27) and Haploview 4.2 software both from Broad Institute (Cambridge, MA, USA) (28), respectively. Linkage disequilibrium between the PAX8-AS1 polymorphisms was estimated through calculation of D' (correlation coefficient between pairs of loci) and r^2 (square of the correlation coefficient between two indicator variables) with Haploview 4.2.

Results

Patient characteristics. The demographic characteristics of the patients considered in the present study are reported previously (2,8).

Genotyping of the variants. Variants were genotyped by PCR-RFLP. When genotyping the rs4848320 variant, digestion of the PCR product (222 bp) yielded a fragment of 195 bp (and presumed 27 bp fragment not visible on agarose gel) for the C allele, and remained undigested for the T allele (Fig. 1). Regarding the rs1110839 variant, the T allele remained undigested (270 bp), while the G allele was digested and produced a fragment of 244 bp (and presumed 26 bp fragment not visible on agarose gel; Fig. 2). For rs6726151, the T allele was digested and produced 211 and 160 bp fragments while the G allele was undigested (371 bp; Fig. 3). The lengths of all fragments following restriction digestion are summarized in Table I.

Association between the variants and childhood ALL risk. The genotype and allele distributions of PAX8-AS1 polymorphisms in pediatric patients with ALL and healthy controls are presented in Table II. The findings suggested that the rs4848320 variant was associated with risk of ALL in codominant (CT vs. CC: OR=2.13, 95% CI=1.16-3.90, $P=0.014$; and TT vs. CC: OR=2.21, 95% CI=1.03-4.74, $P=0.041$), dominant (CT+TT vs. CC: OR=2.15, 95% CI=1.22-3.81, $P=0.009$) and allele (T vs. C: OR=1.55, 95% CI=1.07-2.25, $P=0.024$), inheritance models. For the rs6726151 variant, the findings indicated that this variant significantly increased the risk of ALL in codominant (GT vs. GG: OR=1.88, 95% CI=1.08-3.27, $P=0.036$) and overdominant (GT vs. GG+TT: OR=2.08, 95% CI=1.23-3.53, $P=0.008$) inheritance models. No significant association was observed between the rs1110839 G>T variant and disease risk/protection in childhood ALL.

Results of the haplotype analysis of the three variants are presented in Table III. The findings did not support an association between haplotype and risk of childhood ALL. Associations between the PAX8-AS1 polymorphisms and patient clinical characteristics were also estimated. As depicted in Table IV, a significant association between rs4848320 and sex was observed [$\chi^2=8.45$, degrees of freedom (df)=2, $P=0.015$]. Notably, the CT genotype significantly decreased the risk of ALL in females (OR=0.32, 95% CI=0.13-0.84, $P=0.0355$; data not shown). For rs6726151, the findings indicated that this variant was associated with organomegaly ($\chi^2=8.21$, df=2, $P=0.017$) and lymphadenopathy ($\chi^2=11.48$, df=2, $P=0.003$;

Table I. Primers and restriction enzymes used in the detection of paired-box gene 8 antisense RNA 1 polymorphisms.

Polymorphism	Sequence, 5'-3'	Restriction enzyme	Product size, bp
rs4848320 C>T	F: CTGCTTAGCATGTGCTTGGTGATG R: GAAACACTGAGAACTAAGAGAAGCCTGCA	<i>Pst</i> I	T allele: 222; C allele: 195+27
rs1110839 G>T	F: TCATCTCCCCAGGAGAGGTCCTCAGC R: ACAGTCCGGTTGGAGACTG C	<i>Hha</i> I	T allele: 270; G allele: 244+26
rs6726151 T>G	F: CCCAAAGACCAGCACACA R: AGACCCACCATTTCATAACA	<i>Mbo</i> I	G allele: 371; T allele: 211+160

F, forward; R, reverse.

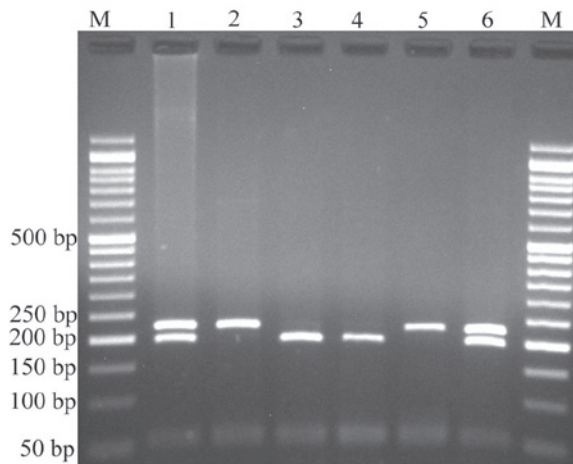


Figure 1. Electrophoresis of rs4848320 C>T polymorphism fragments. The C allele was digested by *Pst*I and produced a 195 bp fragment and presumed 27 bp fragment not visible on agarose gel, while the T allele remained undigested (222 bp). M: DNA marker; lanes 1 and 6: CT; lanes 2 and 5: TT; lanes 3 and 4: CC.

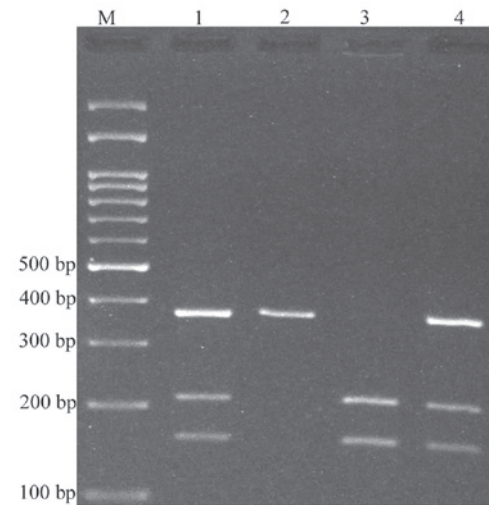


Figure 3. Electrophoresis of rs6726151 T>G polymorphism fragments. The T allele was digested by *Mbo*I and produced 211 and 160 bp fragments, while the G allele remained undigested (371 bp). M: DNA marker; lanes 1 and 4: GT; lane 2: GG; lane 3: TT.

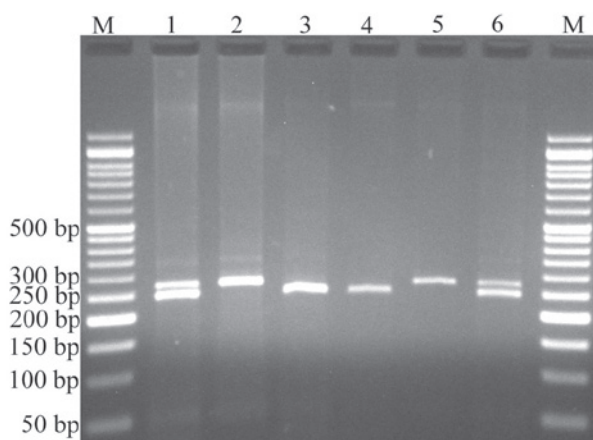


Figure 2. Electrophoresis of rs1110839 G>T polymorphism fragments. The G allele was digested by *Hha*I and produced 244 bp fragment and presumed 26 bp fragment not visible on agarose gel, while the T allele remained undigested (270 bp). M: DNA marker; lanes 1 and 6: GT; lanes 2 and 5: TT; lanes 3 and 4: GG.

Table IV). No significant associations were identified between rs1110839 and patient clinical characteristics.

Furthermore, linkage disequilibrium was observed between rs4848320 and rs1110839 (D' =0.2242, r^2 =0.0455); rs4848320 and rs6726151 (D' =0.5268, r^2 =0.1117); and rs1110839 and rs6726151 (D' =0.2279, r^2 =0.0189; Fig. 4).

Discussion

In the present study, the possible association between PAX8-AS1 polymorphisms and risk of childhood ALL in a Southeast Iranian population was investigated. The findings indicated that the rs4848320 and rs6726151 variants of PAX8-AS1 significantly increased the risk of developing childhood ALL, while there was no association between rs1110839 and disease risk/protection. By contrast, the haplotype analysis did not identify a significant association of any of the variants with risk of childhood ALL. However, stratification of the variants according to the clinical characteristics of patients indicated that the rs4848320 variant was associated with sex while the rs6726151 variant was associated with organomegaly and lymphadenopathy.

Previous results have indicated that non-coding transcripts in the human genome serve crucial and diverse biological roles (29). The findings of macromolecular interactions have

Table II. Association of paired-box gene 8 antisense RNA 1 polymorphisms and risk of acute lymphoblastic leukemia.

Polymorphism	Cases, n (%)	Controls, n (%)	OR (95% CI)	P-value
rs4848320				
Codominant				
CC	26 (23.6)	48 (40.0)	1.00	-
CT	60 (54.6)	52 (43.3)	2.13 (1.16-3.90)	0.014
TT	24 (21.8)	20 (16.7)	2.21 (1.03-4.74)	0.041
Dominant				
CC	26 (23.6)	48 (40.0)	1.00	-
CT+TT	84 (76.4)	72 (60.0)	2.15 (1.22-3.81)	0.009
Recessive				
CC+CT	86 (78.2)	100 (83.3)	1.00	-
TT	24 (21.8)	20 (16.7)	1.39 (0.72-2.70)	0.322
Overdominant				
CC+TT	50 (45.4)	68 (56.7)	1.00	-
CT	60 (54.6)	52 (43.3)	1.57 (0.93-2.64)	0.090
Allele				
C	112 (50.9)	148 (61.7)	1.00	-
T	108 (49.1)	92 (38.3)	1.55 (1.07-2.25)	0.024
rs1110839				
Codominant				
TT	43 (39.1)	54 (45.0)	1.00	-
TG	43 (39.1)	34 (28.3)	1.59 (0.87-2.90)	0.132
GG	24 (21.8)	32 (26.7)	0.94 (0.48-1.83)	0.860
Dominant				
TT	43 (39.1)	54 (45.0)	1.00	-
TG+GG	67 (60.9)	66 (55.0)	1.27 (0.75-2.16)	0.365
Recessive				
TT+TG	86 (78.2)	88 (73.3)	1.00	-
GG	24 (21.8)	32 (26.7)	0.77 (0.42-1.41)	0.393
Overdominant				
TT+GG	67 (60.9)	86 (71.7)	1.00	-
TG	43 (39.1)	34 (28.3)	1.62 (0.93-2.82)	0.085
Allele				
T	129 (58.6)	142 (59.2)	1.00	-
G	91 (60.4)	98 (40.8)	1.02 (0.70-1.48)	0.924
rs6726151				
Codominant				
GG	40 (36.3)	56 (46.6)	1.00	-
GT	63 (57.3)	47 (39.2)	1.88 (1.08-3.27)	0.036
TT	7 (6.4)	17 (14.2)	0.58 (0.22-1.52)	0.576
Dominant				
GG	40 (36.4)	56 (46.6)	1.00	-
GT+TT	70 (63.7)	64 (53.4)	1.53 (0.90-2.60)	0.141
Recessive				
GG+GT	103 (93.6)	103 (85.8)	1.00	-
TT	7 (6.4)	17 (14.2)	0.41 (0.16-1.04)	0.082
Overdominant				
GG+TT	47 (42.7)	73 (60.8)	1.00	-
GT	63 (57.3)	47 (39.2)	2.08 (1.23-3.53)	0.008
Allele				
G	143 (65.0)	159 (72.3)	1.00	-
T	77 (35.0)	81 (27.7)	1.06 (0.72-1.55)	0.844

OR, odds ratio; CI, confidence interval.

Table III. Association of paired-box gene 8 antisense RNA 1 haplotypes and risk of acute lymphoblastic leukemia.

Polymorphism			Cases, frequency	Controls, frequency	OR (95% CI)	P-value
rs4848320	rs1110839	rs6726151				
C	T	T	0.2025	0.2216	1.00 [ref.]	-
T	G	G	0.2010	0.1680	1.32 (0.71-2.47)	0.390
T	T	G	0.2177	0.1472	1.63 (0.83-3.19)	0.160
C	T	G	0.1402	0.2042	0.77 (0.38-1.58)	0.480
C	G	G	0.0910	0.1431	0.68 (0.33-1.42)	0.310
C	G	T	0.0754	0.0478	1.87 (0.55-6.43)	0.320
T	G	T	0.0463	0.0495	1.09 (0.38-3.11)	0.880
T	T	T	0.0259	0.0186	1.35 (0.20-9.20)	0.760

OR, odds ratio; CI, confidence interval.

revealed that tissue-specific lncRNAs form base-pairing interactions with numerous mRNAs associated with tissue-differentiation, indicating that tissue specificity is an critical factor in controlling human lncRNA-mRNA interactions (30). LncRNAs have tissue-specific expression and serve an important role in the human transcriptome by regulating normal tissue differentiation as well as cancer development (30). Notably, a number of previous studies have implicated a role of lncRNA dysregulation, of transcripts including leukemia-induced non-coding activator RNA, B-ALL-associated long RNA (BALR)-6 and -2, NOTCH1-associated lncRNA in T-ALL and CCDC26, in tumorigenicity in leukemias (31-34). SNPs may significantly influence gene expression and function. Altered expression of lncRNAs in various cancers indicates the potential tumor suppressor or oncogenic functions of the lncRNAs (35-39). Recently, an association between the rs2147578 polymorphism of lnc-LAMC2-1 and risk of childhood ALL has been demonstrated (6).

There is limited information on the impact of PAX8-AS1 polymorphisms on cancer risk (19,24). Han *et al* (19) demonstrated that PAX8-AS1 rs4848320 and rs1110839 polymorphisms decreased the risk of cervical cancer, while Ma *et al* (24) reported that rs4848320 and rs1110839 were associated with prognosis of HCC in a Chinese population. It has been proposed that the PAX genes act as oncogenes, and that PAX overexpression facilitates malignant development through effects on apoptotic resistance, tumor cell proliferation and migration, and repression of terminal differentiation (40). As PAX8-AS1 is a potential regulator of PAX8, polymorphisms in the PAX8-AS1 may affect its function and alter the expression of PAX8.

There are a number of limitations to the present study. First, a relatively small sample size was used. Second, there was a lack of data regarding the response of patients to treatment; therefore, it was not possible to analyze the association between the variants and response to treatment.

In conclusion, the present results suggested that PAX8-AS1 polymorphisms significantly increased the risk of childhood ALL in a Southeast Iranian population. As this, to the best of

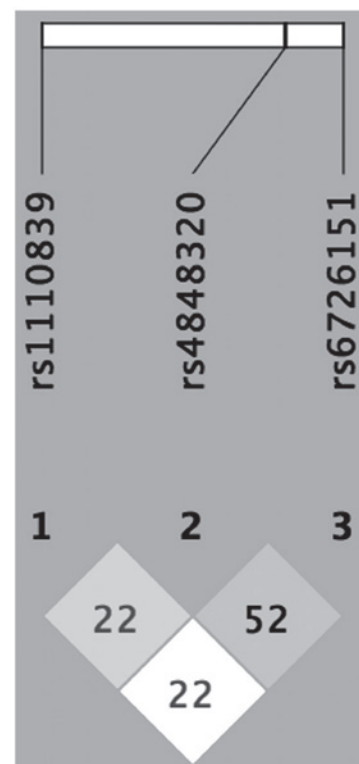


Figure 4. Haplotype linkage disequilibrium graph of the three polymorphisms analyzed in long non-coding paired-box gene 8 antisense RNA 1. Pairwise linkage disequilibrium coefficients (D' x100) are indicated in each cell linkage.

our knowledge, was the first study to examine the association of polymorphisms in PAX8-AS1 with risk of childhood ALL, future studies with larger sample sizes and different ethnicities are required to confirm the findings.

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Table IV. Association of paired-box gene 8 antisense RNA 1 polymorphisms with demographic and clinical features of patients.

Factor	rs4848320				rs1110839				rs6726151			
	CC	CT	TT	P-value	TT	TG	GG	P-value	GG	GT	TT	P-value
Sex, n												
Male	19	28	18	0.015	28	24	13	0.583	22	36	7	0.073
Female	7	32	6		15	19	11		18	27	0	
Age at diagnosis, years	5.2±3.1	6.5±3.9	5.7±4.5	0.355	5.9±3.7	5.5±3.9	7.2±4.1	0.231	5.6±4.0	6.2±3.9	6.3±3.8	0.757
WBC, x10 ⁶ /ml, mean ± SD	31.3±49.6	40.8±55.3	40.9±46.8	0.715	33.8±42.9	44.0±66.2	37.5±36.4	0.656	42.1±50.4	39.5±54.9	10.1±15.5	0.318
Hemoglobin, g/dl, mean ± SD	7.2±2.1	7.0±2.3	7.8±2.3	0.362	7.1±2.3	7.1±2.4	7.6±2.0	0.607	7.6±2.3	7.9±2.2	8.0±1.6	0.172
Platelet, x10 ⁶ /ml, mean ± SD	71.9±64.0	52.3±45.4	49.1±38.0	0.173	54.4±44.0	60.8±61.1	56.2±49.4	0.874	61.6±50.6	54.3±50.5	42.9±430.0	0.587
Organomegaly				0.152				0.163				0.017
Positive	24	52	24		39	37	24		40	55	5	
Negative	2	8	0		4	6	0		0	8	2	
Lymphadenopathy				0.105				0.649				0.003
Positive	14	44	19		31	28	18		31	45	1	
Negative	12	16	5		12	15	6		9	18	6	
Cerebrospinal fluid involvement				0.536				0.498				0.669
Positive	1	5	3		5	2	2		4	5	0	
Negative	25	55	21		38	41	22		36	58	7	

Emboldened values indicate statistical significance (P<0.05). WBC, white blood cell; SD, standard deviation.

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