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Original Article

The polymorphism and whole-genome DNA methylation level of DNMT1 gene in acute leukemia children

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Abstract: Acute leukemia (AL) is the most popular malignant tumor in children. Currently little has been known about the relationship between childhood AL pathogenesis and whole-genome methylation level. As the polymorphism of DNA methyltransferase (DNMT) gene can affect DNA methylation level, we investigated the whole genome methylation level in childhood AL patients. Meanwhile, the relationship between DNMT1 gene polymorphism and susceptibility of childhood AL was also been investigated. A case-control study was performed recruiting childhood AL patients and age-matched healthy children (N=168 each). PCR-ligase detective response (PCR-LDR) typing method was used to study the genotype distribution of human DNMT1 gene at rs2228611 and rs10854076 loci. Pyrophosphate sequencing was used to measure LINE-1 methylation level. Allele frequency of two loci fits Hardy-Weinberg equilibrium (P>0.05). Significant difference of genotype and allele frequency existed at locus rs10854076 but not locus rs2228611 between patients and healthy people. Allele C was found to be a risk factor for AL. A significant difference of LINE-1 methylation level existed between the two groups, as AL patients had lower methylation level (P<0.05). Specifically, LINE-1 methylation level at locus rs10854076 but not at rs2228611 had significant difference between patients and controls. Polymorphism of DNMT1 gene at locus rs10854076 is related with children AL susceptibility, possibly via affecting LINE-1 methylation level.

Keywords: DNA methylation, DNA methyltransferase 1, acute leukemia, LINE-1

Introduction

Leukemia is one malignant tumor in hematological system, and is the most frequent cancer in children. In China, there are about 15,000 newly diagnosed cases, with increased incidence. Among all childhood leukemia patients, there are more than 90% acute leukemia (AL), which includes acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML). ALL is the most common sub-type in children, as it has four folds increased incidence compared with AML [1]. Recently, as the advancement of medicine, remission rate of childhood AL, especially ALL, is rapid increasing. The overall survival rate of ALL has reached almost 80% [2], but still with 25%~30% of recurrence [3]. Therefore further studies on the pathogenesis of leukemia are of critical importance for the disease diagnosis, treatment and prognostic prediction.

DNA methylation is one frequent epigenetic regulation, and is catalyzed by DNA methyltransferase (DNMT) using S-adenosine methionine (SAM) as the methyl donor. Various studies revealed the close relationship between abnormal DNA methylation and tumor occurrence/progression, suggesting its potency as biomarker for tumors [4]. Within tumors, it is frequently to found hyper-methylation of specific tumor suppressor genes and hypo-methylation of whole genome, which induces instability of the chromosomes [5]. Hyper-methylation of DNA inactivates tumor suppressor genes, leading to tumor occurrence. Some studies found the close relation between childhood AL occurrence and alternation of DNA methylation level [6], such as hyper-methylation of DAPK, PTEN and P73 genes, accompanied with hypo-methylation of P16 gene. The relationship between whole-genome methylation level and childhood AL, however, has not been comprehensively

Table 1. The basic information and clinical features in AL patients and control cases

Characteristics	Control group	AL patients group	*P*-values
Case, number	168	168	-
Age, years (mean ± SD)	5.02±2.83	4.89±2.91	0.078
Sex			0.104
Female (n)	67	73	
Male (n)	101	95	
Diagnosis			0.112
De Novo (n)	55	0	
Secondary (n)	113	0	
WBC, $\times 10^9$/l (median, range)	28.73±1.45	8.83±0.67	0.014

studied. This study aimed to investigate the replacement of whole genome, LINE-1 gene, whose methylation level was measured [7, 8], in an attempt to illustrate the relationship between whole-genome methylation level and childhood AL pathogenesis, and to provide further evidences for pathogenesis.

Mammalian DNMTs which induces DNA methylation mainly include DNMT1, DNMT3a and DNMT3b. Among those DNMT1 is responsible for maintaining DNA methylation level during DNA replication and repair, while DNMT3a and DNMT3b are responsible for *de novo* DNA methylation. DNA methylation level can be affected by DNMTs function, as studies showed that deficiency of DNMT1 led to hypo-methylation of whole genome [9, 10]. Moreover, some studies found that DNA methylation level could be affected by polymorphism of DNMTs. For example, LINE-1 methylation level is correlated with DNMT3a polymorphism at locus rs7581217 [11]. Occurrence of childhood AL is also known to be closely related with DNA methylation level [12], Polymorphism of DNMTs genes is also related with tumor susceptibility such as throat cancer or pulmonary carcinoma [13, 14]. It is therefore reasonable to hypothesize that DNMTs gene polymorphism might be potentially related with susceptibility of childhood AL. Previous study found that polymorphism of DNMT1 gene at locus rs2228611 and locus rs10894076 is related with LINE-1 methylation level [15]. Polymorphism at locus rs2228611 was also correlated with occurrence of breast cancer [16], ovarian cancer [17]. This study thus focused on loci rs2228611 and rs10894076, whose relationship with childhood AL occurrence was investigated. Meanwhile the effect of such polymorphism on LINE-1 methylation was also identified, to provide evidences for locating susceptible factors for childhood AL.

Materials and methods

Research objects

A total of 168 AL children who were diagnosed in PLA Army General Hospital from January 2011 to December 2015 were recruited as the patients group (101 males and 67 females, aging from 1 to 13 years, average age =5.02±2.83 years). Another cohort of 168 healthy children with age-/sex-match were recruited from clinics (95 males and 73 females, aging from 1 to 12 years, average age =4.89±2.91 years). No significant differences existed regarding age or sex between these two groups. The basic information and the clinical characteristics were listed in **Table 1**. No recruited children had history of tumors or inheritable diseases. All patients were Han Ethic people living in Guangzhou. 5 ml fasted venous blood samples were collected from all participants. This study has been proved by the medicine ethical committee, and has obtained written consents from all guardians of children.

DNA extraction and bisulfite modification

Total DNA was extracted from peripheral blood samples using genomic DNA extraction kit (Tiangen Biochem, China) following instructions of test kit. 1 μg DNA was modified and purified using EpiTectBisufite kit (Qiagen, US). Genomic DNA processed by M.SssI methyltransferase was used as positive control (Universal methylation, UM).

LINE-1 methylation level detection

Using modified DNA as the template, PyroMarkPCR kit (Qiagen, US) was used to amplify PCR fragments. Amplification products were identified for length and specificity using agarose gel electrophoresis. One of PCR product strands was labeled with biotin. After mixing with streptomycin-magnetic beads, labeled and unlabeled strands were denatured and separate by vacuum processing. Single stranded DNA was mixed with LINE-1 sequencing primer for sequencing in PyroMark Q96 ID System

Table 2. Test of Hardy-Weinberg equilibrium of DNMT1 gene

SNP	Allele	Genotype			Allele		χ^2	*P*
		AA N (%)	AB N (%)	BB N (%)	A N (%)	B N (%)		
rs2228611	A/G	9 (5.4)	75 (44.6)	84 (50.0)	93 (27.7)	243 (72.3)	2.23	0.14
rs10854076	C/G	23 (13.7)	74 (44.0)	71 (42.3)	120 (35.7)	216 (64.3)	0.30	0.60

Table 3. Genotype and allele frequency distribution at loci rs2228611 of DNMT1 gene

Group	N	Genotype					Allele			
		GG	AG	AA	χ^2	*P*	G	A	χ^2	*P*
Control	168	84 (50%)	75 (44.6%)	9 (5.4%)	1.55	0.46	243	93	1.21	0.27
AL	168	76 (45.2%)	78 (46.4%)	14 (8.4%)			230	106		

Table 4. Genotype and allele frequency distribution at loci rs10854076 of DNMT1 gene

Group	N	Genotype					Allele			
		GG	CG	CC	χ^2	*P*	G	C	χ^2	*P*
Control	168	71 (42.3%)	74 (44%)	23 (13.7%)	8.61	0.01	216	120	7.97	<0.01
AL	168	56 (33.3%)	68 (40.5%)	44 (26.2%)			180	156		

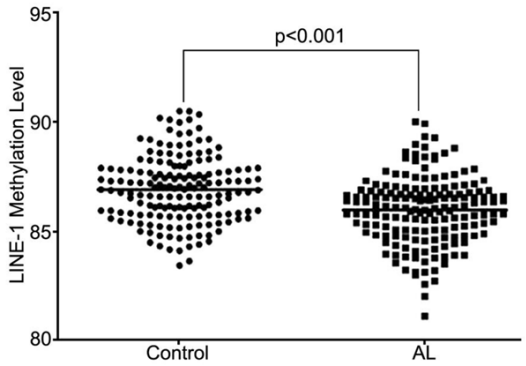

Figure 1. LINE-1 methylation level of control and AL group.

(Qiagen, US). Each sample was tested in triplicates, in parallel with negative and positive controls.

Genotyping by PCR-LDR method

PCR-LDR approach was employed for polymorphism test. Target DNA fragments were firstly amplified *in vitro* using multiple PCR targeting SNP loci fragment, using primers designed and synthesized by Jierui Bio Eng (China). Multi-PCR conditions were: 95°C pre-denature for 5 min, followed by 11 cycles each containing 94°C denature for 15 s, 60°C annealing (with 0.5°C decrease at each cycle), and 72°C elongation for 30 s; followed by 24 cycles (94°C denature for 15 s, 54°C annealing for 15 s, followed by 72°C elongation for 30 s), and ended with 72°C elongation for 5 min. NEB Taq DNA ligase system was employed in a 10 μl multiple ligase detection reaction (LDR) system, under the following conditions: 94°C 30 s, 56°C 3 min in 30 cycles. 1 μl LDR products were sequenced. Data analysis and genotyping were performed using peak_scanner_software_v1.0.

Statistical analysis

SPSS19.0 software was used to analyze all data. Chi-square test was used to test if allele frequency fitted Hardy-Weinberg equilibrium, and to analyze the genotype distribution between the two groups. The comparison of LINE-1 methylation level was performed by student t-test. Analysis of variance (ANOVA) was use to compare LINE-1 methylation level among different genotypes. Covariant analysis was used to rule out possible confounding factors for multi-group comparison. A statistical significance was defined when P<0.05.

Results

Hardy-Weinberg equilibrium of DNMT1 gene at loci rs2228611 and rs10854076

Using chi-square, all alleles at the two SNP loci in control children fitted Hardy-Weinberg equi-

Table 5. LINE-1 methylation level across different genotypes at SNP locus

SNP loci	AL group			Control group		
LINE-1	F	P	LINE-1	F	P	
rs2228611						
GG	85.99±1.59	0.16	0.85	86.95±1.50	0.07	0.93
GA	86.03±1.56			86.89±1.52		
AA	85.77±1.36			86.79±1.60		
rs10854076						
GG	87.35±1.09	63.66	<0.001	88.24±1.03	135.64	<0.001
CG	85.64±1.23[a]			86.21±0.86[a]		
CC	84.80±1.18[a,b]			85.13±0.82[a,b]		

Note: [a], P<0.05 compared to GG carriers; [b], P<0.05 compared to CG carriers.

librium regarding their frequency distribution (P>0.05), indicating no significant difference between control and experimental group, with population representatives (**Table 2**).

Significant differences for the different genotypes of loci rs2228611 and rs10854076

Gene distribution frequency of DNMT1 gene at loci rs2228611 and rs10854076 between two groups was shown in **Tables 3** and **4**. Results showed no significant differences of GG, AG or AA genotype at locus rs2228611 between AL and control group (χ^2=1.55, P=0.46). No significant difference existed for allele frequency of G or A (χ^2=1.55, P=0.27), indicating no correlation between polymorphism at loci rs2228611 and AL susceptibility.

Analysis of polymorphism loci rs10854076 revealed significant differences of GG, CG and CC genotypes between AL and control group (χ^2=8.61, P=0.01) (**Table 4**), as CC genotype had a higher frequency in AL group than control group. The frequency distribution of alleles showed higher C allele frequency in AL group compared with control group (χ^2=76.97, P< 0.01), suggesting that polymorphism at loci rs10854076 was correlated with AL susceptibility. Further statistical analysis showed that allele C was a risk factor for AL pathogenesis (OR=1.56, 95% CI, 1.15~2.13).

The LINE-1 methylation levels were lower in AL group compared with control group

LINE-1 methylation level was shown in **Figure 1**. Methylation levels of LINE-1 were 86.92±1.50% and 85.99±1.55% for control and AL group, respectively. Student t-test was used to compare the difference of LINE-1 methylation level between two groups. Results showed significantly lowered LINE-1 methylation level in AL group than that in control group (t=5.59, P< 0.001).

DNMT1 gene polymorphism correlated with the LINE-1 methylation level

ANOVA was employed to compare the difference of LINE-1 methylation level between different genotypes at SNP loci. Results showed the correlation between LINE-1 methylation level and DNMT1 polymorphism at rs10854076 loci. As shown in **Table 5** and **Figure 3**, significant difference of LINE-1 methylation level existed between three genotypes at this loci in two groups (P<0.001). Paired comparison also showed significantly lower LINE-1 methylation level in CC or CG genotype carriers compared with GG carriers (P<0.05). Covariant analysis was also employed to exclude possible confounding factors by sex or age. Results showed significant difference of LINE-1 methylation level between different genotypes of rs10854976 loci of two groups (P<0.05). No significant difference, however, was observed at rs2228611 loci between different genotypes. Therefore LINE-1 methylation level was not correlated with polymorphism at rs2228611 loci (**Table 5**; **Figure 2**).

The LINE-1 methylation and SNP are independent prognostic factor for AL

The median possibility of AL were 1.398 (95% CI: 1.075-1.763) and 1.459 (95% CI: 1.167-1.836) for rs2228611 and rs10854076, respectively (**Table 6**). The median possibility of AL were 2.065 (95% CI: 1.675-3.026) and 2.148 (95% CI: 1.703-3.235) for rs2228611 and rs10854076, respectively (**Table 6**). This prognostic impact of the LINE-1 methylation and SNP remained statistically significant and are the risk factors for the AL (**Table 6**).

Discussion

The alternation of whole-genome methylation level induces instability of chromosome [5],

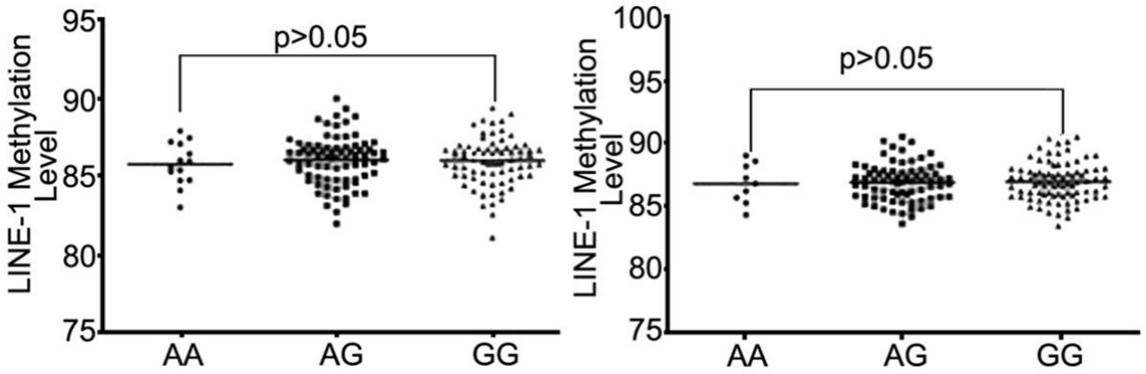

Figure 2. LINE-1 methylation level at rs2228611 loci among different genotypes in AL (left) and control (right) group.

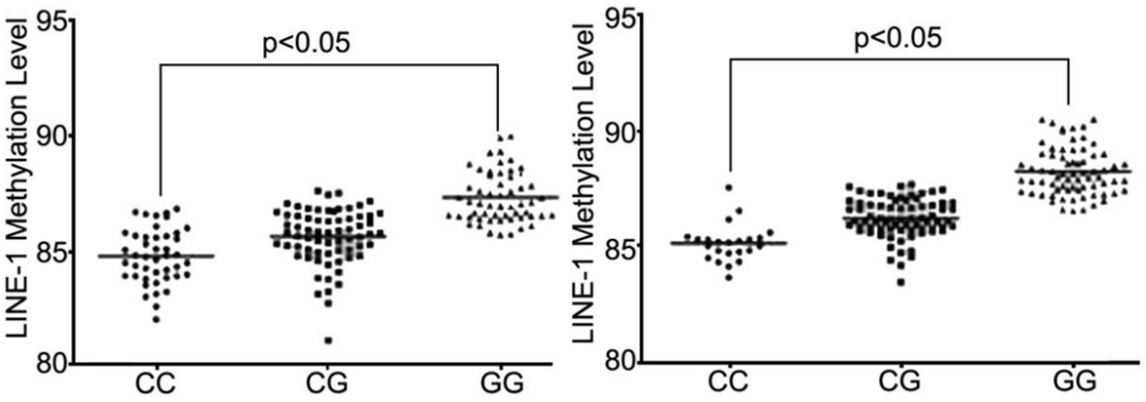

Figure 3. LINE-1 methylation level at rs10854076 loci among different genotypes in AL (left) and control (right) group.

Table 6. Multivariate logistic regression analysis in the AL patients using Cox Regression analysis of the SNP and methylation

Vaviable	Possibility of AL			
	P-value	HR	95% CI for HR	
			Lower	Upper
LINE-1 methylation				
rs2228611	<0.01	1.398	1.075	1.763
rs10854076	<0.01	1.459	1.167	1.836
SNP				
rs2228611	<0.01	2.065	1.675	3.026
rs10854076	<0.01	2.148	1.703	3.235

leading to various diseases. Previous studies showed significant hypo-methylation of whole genome during tumor pathogenesis, and had significant correlation with cancer prognosis [18, 19]. Little has been known, however, regarding the relationship between childhood AL and whole genome methylation level. We thus performed a case-control study to investigate childhood AL pathogenesis from the view of whole genome methylation.

LINE-1 methylation level was studied by pyrophosphate sequencing method. Results showed significantly lower LINE-1 methylation level in AL group compared with control ones, suggesting potential correlation between altered LINE-1 methylation level and childhood AL pathogenesis. A similar study performed by Bujko et al, however, made contrast conclusion as they found significantly higher LINE-1 methylation level in AL children from Poland [20]. Kroeger et al did not found alternation of LINE-1 methylation level from 30 AML patients from Houston, Texas [21]. Moreover, no significant change of LINE-1 methylation has been found in a cell study performed by Negrotto [22]. Such inconsistency might be due to variations in samples size, plus different ethnic groups enrolled. The definitive conclusion thus requires larger cohort clinical studies.

As polymorphism of DNMTs might affect methylation level of LINE-1, we investigated the correlation between rs2228611 and rs10894076 loci of DNMT1 gene and susceptibility of children AL. Results showed the relationship between rs10854076 and childhood AL, as allele C was shown to be a risk factor for children AL. However, rs2228611 loci was not related with AL. This was the first time that the correlation between rs10854076 loci and childhood AL occurrence has been studied. Similar research has been performed by Luo et al, who found that rs11085721 loci, but not rs2228611, was responsible for the susceptibility of childhood ALL [23], which were consistent with our results.

We further analyzed the relationship between two single nucleotide polymorphism (SNP) loci of DNMT1 and LINE-1 methylation level. Results showed significant difference of LINE-1 methylation level at rs10854076 loci, but not rs2228611 loci, indicating that LINE-1 methylation level was affected by polymorphism at rs10854076 loci. Such loci is one susceptibility factor for childhood AL, possibly via affecting LINE-1 methylation level. Hossain et al indicated that these two SNP locus might regulate the correlation between urine cadmium and LINE-1 methylation level [14]. This study further demonstrated that polymorphism at rs10854076 loci but not rs2228611 loci might modulate such correlation via affecting LINE-1 methylation level. Whether rs10854076 loci affected DNMT function to further modulate methylation level remains unclear and requires further investigations.

Conclusion

Polymorphism at rs10854076 loci of DNMT1 gene is correlated with childhood AL susceptibility, possibly via affecting LINE-1 methylation level.

Disclosure of conflict of interest

None

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